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REVIEW

CHARACTERIZATION OF LIPID SUBSTANCES OF ROSE HIP SEEDS AS A POTENTIAL SOURCE OF FUNCTIONAL COMPONENTS: A REVIEW

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ABSTRACT

Functional foods receive the greatest attention for nutritional needs of specific consumers. The rose hip fruit, besides carotenoids and polyphenols, are also good sources of lipid substances (fatty acids, sterols and tocopherols), which can be used as functional foods instead of being discarded as waste. The aim of this review is to present an overview of the lipid characterization of rosehip seeds as affected also by the oil extraction procedure.
The rosehip seeds oil is proven to be rich in polyunsaturated fatty acid (PUFA), sterols and tocopherols, which provide specific biological activities (anti-inflammatory, anti-obesity, antioxidant, anti-diabetic activity). In particular, the oil content of rose hip seeds ranges from 5 to 18 % and is composed of unsaturated fatty acids such as linoleic acid (36-55 %) which is the most abundant one, linolenic (17-27 %) and oleic acid (15-22 %) respectively. As for the sterols, its content ranges around 5 g/kg constituting predominantly β-Sitosterol, whereas, the tocopherols amount to around 1 g/kg with γ-tocopherol being the most abundant.

Keywords: rosehip oil, oil extraction, fatty acids, unsaponifiable matter components

1. INTRODUCTION

Nowadays, the optimization of food production in sustainable way is a requirement with the rise in world population and the reduction of natural resources. As such, the use of abundant natural food sources could be a solution, since food stuffs provide not only nutrition but also exercise a great potential for health benefits (PATEL, 2015). Moreover, the global interest in functional foods increased owing to their promising response to numerous diseases. In accordance, numerous works have reported the potential health properties of plant based products that are rich sources of nutrients and phytochemicals as evidenced through their biological activities (TYLEWICZ *et al*., 2019; NADPAL *et al*., 2016; MOZZON *et al*., 2015; GUIMARÃES *et al*., 2013; DELIORMAN ORHAN *et al*., 2007; REIN *et al*., 2004). In recent times, food wastes are classified as valuable constituents with the emerging technologies ensuring the extraction of target compounds through the food chain (GALANAKIS, 2012).Among fruit and vegetable products, rose hips have gained considerable attention due to their highest content of bioactive compounds (ILYASOĞLUl, 2014).

Rose hips are included in the *Rosaceae* family, with the rose species being large shrubs or small trees that grow in various regions of the world. They are perennial woody plants, with more than 200 species and 18000 cultivars in the world, geographically distributed mainly in Europe, Asia and North America (PATEL, 2017). The rose hips constitute the aggregate fruit of the rose bush plants and are formed of a stretched, pulpy shell called "hypanthium" which encloses the real fruits known as "achenes" (WINTHER *et al*., 2016) (Fig. 1).

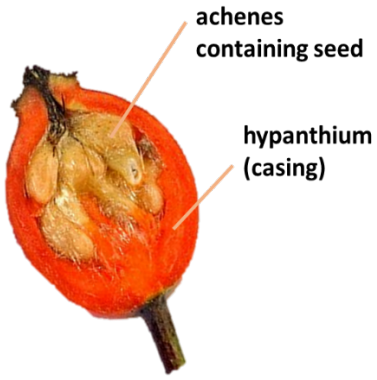

Figure 1. Anatomical scheme of rose hip.

The achenes represent the thin membranes close to the seeds of rose hip, which are 30-40 % of the overall weight of fruit. The fleshy part of the rose hips is usually utilized in production of different kind of food products (juice, jam, bakery products, candies etc.), while the seeds are discarded as waste. The nutritional characterization has proven that rose hips are rich in nutrients and are good source for dietary supplements, for direct use as functional food product or as food ingredients and additives such as natural colorants for industrial production process (PATEL, 2017; ROSU *et al*., 2011).

Rose hip seeds furthermore, have been evidenced to exhibit a series of biological activities such as gastrointestinal protection, diabetic (insulin mimicking), anti-aging, enhancing

immunity properties. These can be attributed to their rich chemical composition which include not only phenolic components, mainly flavonoids and proanthocynidins,(FASCELLA *et al*., 2019; KOCZKA *et al*., 2018), but also other nutrients such as fatty acid, terpenes, tocopherol, carotenoids, proteins, sugars and minerals (BHAVE *et al*., 2017; DEMIR *et al*., 2014; NADPAL *et al*., 2016). The expression of the studied biological activities and the functional compounds are strictly related to the harvest period, genotypes and species (BARROS *et al*., 2011; ÇELIK *et al*., 2010; GÜNEŞ *et al*., 2017). In addition, parts of plant such as fruits, seeds, nuts and sprouts could represent a rich sources of oil with valuable components, which usually have discarded as waste (PATEL, 2017).
In particular, the rose hip seeds acquire oil content from 5 to 18 % which includes varying amounts of unsaturated fatty acids such as linoleic acid (36-55 %), followed by linolenic and oleic acid respectively (17-27 % and 15-22 %). Due to their higher favourable polyunsaturated fatty acids and sterols, rose hip seeds oil represents a high value added compound that can be extracted from vegetable wastes and reused in food processing (SALGIN *et al*., 2016). The oil constituents were found to exhibit anti-inflammatory, anti-bacterial, antioxidant activity and potentiality in the cosmetic products production (NADPAL *et al*., 2016; REIN *et al*., 2004).
In consideration, the seed oil extraction methods are extremely important as well as their efficiency in order to preserve the bioactive compounds such as the unsaturated fatty acids, and also to obtain an economical oil yield. Generally, cold pressing is used in the production of seeds oil. However, many other technologies have been investigated such as Soxhlet, organic solvent extraction, ultrasound, microwave and supercritical CO_2 extraction with the latter exhibiting a higher solubility of the oils and short extraction times, thus minimizing the degradation of bioactive compounds due to thermal and oxygen exposure (DĄBROWSKA *et al*., 2019a; SALGIN *et al*., 2016).
The objective of this review is to summarize the state of the research, to underline the attention in the lipophilic components extracted from rose hip seeds oil for further use in the food process and pharmaceutical industries in order to enhance the health benefits.

1.1. Proximate and energy profile of Rose hip seed oil

The different nutritional profiles and assessed energy values of rose hip seeds are shown in Table 1.
The predominant constituent in the seed was the carbohydrate while the protein represented the lowest content. High carbohydrate content and low protein content avoids protein emulsification, which may limit the oil release.
The difference among the nutritional profiles of rosehip seed is mainly due to the different species and the growing conditions (temperature, rainfall and harvest stage) (İLYASOĞLUl, 2014; DU *et al*., 2017; CONCHA *et al*., 2006; ÖZCAN, 2002).

2. OIL EXTRACTION METHODS

Standard procedures for the oil extraction of plant tissues are cold pressing and solvent extraction.
The cold pressing method ensures the production of safe oil since neither heat nor chemical substances are used. On the contrary, this procedure results in low oil yield and hence economically inconvenient in case of rose hip seeds oil (20 % of oil). Whereas,

solvent extraction requires large amounts of solvent, long extraction times and high temperatures, which may provoke the destruction of sensitive bioactive compounds.

Table 1. Nutritional composition and energy value of the rosehip seeds.

References **Samples origin**	Ilyasoğlu (2014) Turkey *(Rosa canina)*	Du *et al.* (2017) China *(Rosa acicularis)*	Concha *et al.* (2006) Chile *(Rosa affinis rubiginosa)*	Özcan (2002) Turkey *(Rosa canina)*
		Parameters		
Moisture g/100g, dry weight	10.3 (g/100 g of fresh weight)	9.0	6.0	5.7-6.6
Carbohydrate g/100g, fresh weight	89.1	40.4 Crude fiber	56.0 Crude fiber	47.1-65.1 Crude fiber
Fat g/100g, dry weight	6.3	6.7	9.0	13.4-17.8
Ash g/100g, dry weight	1.6	3.9	2.0	1.2-2.1
Protein g/100g, dry weight	3.0	3.8	3.0	9.6-11.5
Energy value kcal/100g, dry weight	425.0	-	-	3797.0-5086.0 cal/g

Studies show that the extraction efficiency of *Rosa affinis rubiginosa* can be increased by enzymatic pre-treatment (up to 30 %) (CONCHA *et al*., 2006). Hydrolytic enzymes are useful tools to selectively depolymerize and break down the cell walls.
Among the innovative technologies ultrasound, microwave, subcritical and supercritical fluid extraction are used for the recovery of rose hip seeds oil fatty acids (JAHONGIR *et al*, 2019; SZENTMIHÁLYI *et al*., 2002). Microwave extraction at 40°C for 30 min, supercritical CO_2 extraction (35°C, 250 bar for 80 min.) and subcritical CO_2 + C_3H_8 (28°C, 100 bar for 35 min.) enhanced the oil extractability compared to Soxhlet and ultrasound methods.
Efficiency of supercritical fluid extraction (SFE) and the bioactive components extractability are ascribed to many factors such as temperature, pressure and flow rate (SALGIN *et al*., 2016; DEL VALLE *et al*., 2004). In contrast to the solvent extraction, SFE works at low temperature and short process times, thus reducing the thermal damages and degradation of oxygen sensitive compounds. Carbon dioxide (CO_2) is commonly used as solvent for SFE which is a non-toxic, inert, non-flammable, odourless and cheaper compound. Fatty acids in the oil are soluble in supercritical CO_2 at 40°C and up to 280 bar and subsequently increased solubilities can be reached with co-solvent addition (SALGIN *et al*., 2016; SZENTMIHÁLYI *et al*., 2002).

3. LIPOPHILIC COMPONENTS OF THE ROSEHIP SEEDS OIL

The oil extracted from rose hip seeds presents high level of bioactive compounds and for this reason it could be used for the enrichment of food products. In particular, the lipophilic part of oil is rich in polyunsaturated fatty acid (PUFA), sterols and tocopherols.

3.1. Fatty acid oil composition

The fatty acid composition of the different seed oils extracted from rose hips is affected to the different rose species, environmental factors, agronomic practices, cultivation area and oil extraction methods.

Table 2 provides an overview of the fatty acid composition of rosehip seed oil subjected to different oil extraction methods. The traditional method for oil extraction from plants is a cold pressing procedure (A). This extraction method employs neither the use of chemical solvents nor heat treatment, thus allows the production of natural and safe oils without compromising their quality. However, the cold pressing method produces low oil yield therefore economically inconvenient for raw materials such as rosehip seeds, which contain a low percentage of oil. In fact, CONCHA *et al*. (2006) obtained 30-40 % of oil from *Rosa affinis rubiginosa* by cold pressing procedure, however, the yield was increased to 72 % by the enzymatic pre-treatment (H) which allows the cell wall degradation enhancing the extractability of oil. Nevertheless, several authors reported similar results for what concerns the fatty acid composition of oil by using cold pressing with or without enzymatic pre-treatment. Indeed, the fatty acid composition of oils originating from *R. canina* and *R. rubiginosa* grown in Poland and Chile were characterized by high content of linoleic content (42.20-51.70 %) followed by α-linolenic (21.50-34.00 %) and oleic (12.36-18.42 %) rather than palmitic (3.33-4.97 %), stearic (0.11-3.00 %) and arachidonic (0.6-0.7 %) acids (CONCHA *et al*., 2006; DĄBROWSKA *et al*., 2019; GRAJZER *et al*., 2015; PRESCHA *et al*., 2014).

Soxhlet method (B) by using different solvents for extraction such as hexane (ÇELIK *et al*., 2010; DĄBROWSKA *et al*., 2019; FROMM *et al*., 2012; SALGIN *et al*., 2016; SZENTMIHÁLYI *et al*., 2002), petroleum (KAZAZ *et al*, 2009), methanol, chloroform (YILMAZ *et al*., 2011) and diethyl ether (ÖZCAN, 2002) had been widely used for rose seeds. The oil yield ranged from 2.75-12.90 % and varied strongly depending on the solvent type, variety and origin of the raw material. ÇELIK *et al*. (2010) reported seed oil composition of five different rose hip species growing in Turkey (*R. dumalis var. boissieri, R. pulverulenta, R. canina L., R. iberica and R. heckeliana subsp. vanheurckiana*). Among the studied species, soxhlet extraction yield was highest for *R. heckeliana subsp. vanheurckiana* (7.95 %) and the lowest for *R. canina L.* (4.97 %). Although in all reported rose species the main unsatured fatty acids were linoleic, α-linolenic and oleic acids, *R. heckeliana subsp. vanheurckiana* contained high quantitie of linoleic acid (51.06 %), while *R. iberica* oil was caractherized by high α-linolenic (23.83 %) and oleic acid (23.03 %) contents, respectively. In contrast to these values, other studies reported lower linoleic and α-linolenic acids that ranged from 35.94-36.7 and 14.3-21.15 %, respectively (FROMM *et al*., 2012; SZENTMIHÁLYI *et al*., 2002) These discrepancies in studiescan be attributed to the different climatic conditions, geological location and other agronomic factorswhich might have influenced the biosynthesis of fatty acids.

DĄBROWSKA *et al*., (2019) reported fatty acid oil profile, obtained from *Rosa canina* grown in Bulgaria, by Soxhlet with hexane as extraction solvent, and the oil was found to be rich in palmitic (17.80 %) and linoleic (52.60 %) acids and poor in α-linolenic (2.10 %) and oleic acids (1.60 %).

When petroleum was used as solvent, theoil content was respectively 7.15 and 2.75 % for *Rosa canina* and *Rosa damascena*. Despite the higher oil yields detected, only 48.84 % linoleic and 22.14 % oleic acids have been found in oil of *Rosa canina*, whereas, 54.18 % linoleic and 23.91 % oleic acids have been found in oil of *Rosa damascena,* respectively (KAZAZ *et al*., 2009). The specific fatty acid profile is also strongly dependent on the species of plant but

even on the growing conditions. In fact, ÖZCAN, 2002 found different percentage of fatty acids for rose seeds coming from different regions of Turkey.
Several studies proposed supercritical fluid extraction (SFE), as a solid-liquid process for extraction of seed oil as an alternative to the traditional methods. Contrary to the traditional solvent extraction, SFE works with low temperature and short process times, thus, allowing for reduced thermal and oxygen degradation of sensitive compounds. Moreover, SFE is widely recognized as a valuable technology due to the extraction efficiency for the use of the fluid (mostly carbon dioxide) which has a high density, a high diffusivity and low viscosity thus leading to rapid solute mass transfer (DASSOFF and LI, 2019).
SALGIN *et al*. (2016) investigated the influence of particle size (125-1000 μm), pressure (20-40 MPa), volumetric flow rate of fluid solvent (0.75-3.5 mL min^{-1}) and temperature (40-60°C) on the extraction yield and oil composition of *Rosa canina* by SFE. The oil yields obtained with seed particles lower than 500 μm were about 50 % higher compared to the bigger particle sizes (>1000 μm). This is probably due to the difficulty in extraction of smaller particles (around 30 μm) closer to the bigger fractions, which may limit the mass transfer inside the pores, causing less enhanced release of oil. Extraction rate of oils increased with decreasing particle sizes (18 % at 250 bar, 50°C and 3 mL min^{-1}) (MACHMUDAH *et al*., 2007). Separation process carried out with CO_2 (E) provoked the highest extraction yield (16.5 % oil) by the application of 30 MPa, 40°C, 0.75 mL min^{-1} and 355-500 m in extraction time of 150 min. However in the case where 5 % vol. ethanol (G) was used as solvent, the same amount of oil was extracted in about 90 min. Concerning these oils, no significant differences were found in the fatty acid profiles for the different SFE extraction processes, and were composed mainly by linoleic (48.3-49.0 %), α-linolenic (19.9-21.2 %) and oleic (19.5-20.7 %) acids (SALGIN *et al*., 2016).
SZENTMIHÁLYI *et al*. (2002) emphasized the oil yield extractability of *Rosa canina* hip seeds by SFE with CO_2+C_3H_8 (F) (28°C, 100 bar and 35 min.) and SFE with CO_2 (E) (35°C, 250 bar and 80 min.), respectively. The yields were determined to be around 6.68 and 5.72 % for (F) and (E) respectively, and were higher compared to that of Soxhlet, microwave and ultrasound water bath extraction (4.85, 5.26 and 3.25 %). Thus, determined an enhanced fatty acids percentage of linoleic acid from 8.18 to 18.81 % rather than oleic and α-linolenic acids, which reported similar amount compared to the other extraction method.
The effect of temperature, pressure and CO_2 flow rate on fatty acids content of rosehip seeds was investigated (MACHMUDAH *et al*., 2007). The linoleic acid (47.02-49.14 %) and α-linolenic (33.02-40.21 %) significantly increased with increasing temperatures (40, 60 and 80°C). Above 300 bar the linoleic acid content tends to be steady; this is probably due to the increased CO_2 density at higher pressure causing the enhancement of acid dissolution, while α-linolenic acid significantly increased with increasing pressure applied. However, palmitic and stearic acids were not affected by any of the considered parameters for SFE, rather they were not quantified at 150 bar.
Rosa woodsii provided a rose seed oil, extracted with Folch method (I), rich in linoleic (37.10 %), α-linolenic (30.75 %) and oleic (19.70 %) acids (ANWAR *et al*., 2008).

Table 2. Fatty acid composition of rose hip seed oils under different oil extraction methods.

References	Grajzer *et al.* (2015)	Presha *et al.* (2014)	Szentmihályi *et al.* (2002)	Çelik *et al.* (2010)	Salgin *et al.* (2016)	Du *et al.* (2017)	Yilmaz (2011)	Concha *et al.* (2006)
Origin of raw materials	Poland	Poland	Hungary	Turkey	Turkey	China	Turkey	Chile
Oil extraction system	A	A	B, C, D, E, F	B	B, E, G	C	B-H	A-H
N. of species	2	4	5	5	6		4	9
Fatty acid (%)								
C16:0	4.2-4.8	3.8	3.60-7.87	4.25-5.15	2.3-3.8	4	1.18-3.39	3.33-4.97
C16:1	-	-	-	0.22-0.89	-	-	0.50-1.88	-
C18:0	2.1-3.0	1.8	2.45-3.27	1.80-2.87	1.9-2.5	2.9	0.84-2.58	Traces 0.11-1.75
C18:1	14.7-16.3	14.6	l6.25-22.11	20.35-23.03	19.5-20.5	34.2	0.48-29.96	12.36-14.82
C18:2	44.4-51.7	44.1	35.94-54.75	41.l4-51.06	47.0-49.2	56.5	3.21-36.33	42.20-47.87
C18:3	21.5-31.8	34.0	20.29-26.48	19.66-23.83	19.9-22	1.7	0.22-0.31	26.41-31.09
C20:0	nd 0.7	0.6	-	0.94-1.29	0.8-1	-	0.65-0.79	-
C20:2	nd 0,4		-	-	-	-	-	-
Others	1.7-2.4	0.4	-	-	-	-	-	-
Oil content (%)	-	-	3.25-6.68	4.97-7.95	16.5	-	-	-
Σ SFA	7.1-8.0	6.5	-	-	-	-	-	4.63-5.08
Σ MUFA	15.2-16.4	15.2	-	-	-	-	-	12.36-14.82
Σ PUFA	73.3-76.3	78.4	-	-	-	-	-	73.29-76.21

A: cold pressing procedure; B: Soxhlet; C: ultrasound; D: microwave; E: SFE with CO_2; F: SFE with CO_2+C_3H_8; G: FSE with 5%vol. ethanol; H: enzymatic pretreatment; I: Folch procedure.

Table 2. Continues.

References	Kazaz *et al.* (2009)	Machmudah *et al.* (2007)	Dabrowska *et al.* (2019)	Özcan (2002)	Fromm *et al.* (2012)	Anwar *et al.* (2008)	Ilyasoğlu (2014)
Origin of raw materials	Turkey	France	Bulgary Germany Hungary Poland Turkey	Turkey	Germany	Canada	Turkey
Oil extraction system	B	E	A, B, E	B	B-H	I	E
N. of species	2	9	1	1	1	3	1
Fatty acid (%)							
C16:0	5.26-5.30	0-4.68	2.72-17.80	1.71-3.17	3.1	3.70	3.34
C16:1	-	-	0.04-2.60	0.24-1.01	0.6	0.57	-
C18:0	2.02-3.13	0-2.88	2.05-8.80	1.69-2.47	2.2	1.59	1.69
C18:1	22.14-23.91	-	13.17-52.60	14.71-18.42	18.8	19.70	19.50
C18:2	48.84-54.18	47.02-50.25	2.10-55.70	48.64-54.41	36.7	37.10	54.05
C18:3	15.09-20.65	33.02-40.21	1.60-31.80	16.42-18.41	14.3	30.75	19.37
C20:0	-	-	0.23-3.50	1.87-2.61	1.3	0.80	1.00
C20:2	-	-	-	-	-	0.10	-
Others	-	-	-	-	-	-	-
Oil content (%)	2.75-7.15	18	3.1-12.90	13.37-17.82	10	-	6.29
Σ SFA	-	-	-	-	7.1	8.20	-
Σ MUFA	-	-	-	-	20.1	21.73	-
Σ PUFA	-	-	-	-	51.0	68.10	-

A: cold pressing procedure; B: Soxhlet; C: ultrasound; D: microwave; E: SFE with CO_2 F: SFE with CO_2+C_2H_6; G: FSE with 5%vol. ethanol; H: enzymatic pretreatment; I: Folch procedure.

3.2. Sterols profile

The recent interest for enriched functional foods with plant sterols is due to their demonstrated reducing effects of cholesterol level well as anti-inflammatory and anticarcinogenic properties (ALVAREZ-SALA *et al*., 2018). In particular, among the several plant sterols, approved by European Commission, β-sitosterol, campesterol and stigmasterol are allowed to be used in a higher proportions than the sterol content commonly added as ingredients in functional foods (BARRIUSO *et al*., 2016).
The rosehip seed oil was characterized by higher sterol contents than economically available vegetable oils such as soybean and sunflower (< 5 g/kg) (AMAROWICZ and PEGG, 2019).
Characterization of phytosterols profile of rosehip seed oil is reported in Table 3.
GRAJZER *et al*. (2015) observed that the total content of sterols was high in both rosehip oils (obtained from two different manufacturers), which are respectively 5.891 and 6.485 g/kg compared to camellia (2.312 g/kg) and walnut (1.791 g/kg) oils. No difference in the β-Sitosterol and Δ^5–Avenasterol content of *Rosa canina* oil was found between cold pressing and Folch procedure (GRAJZER *et al*., 2015; ILYASOĞLU, 2014). However, Δ^7-Stigmasterol and clerosterol were quantified by the DGF official method (ILYASOĞLU, 2014)and not confirmed by GC-MS method, whereas up to 0.6 g/kg of cycloartenol was found (GRAJZER *et al*., 2015). Beside that ZLATANOV (1999) in *Rosa canina* oil from Bulgaria β-Sitosterol followed by Δ^5–Avenasterol (81.5 and 4.6 g/kg respectively), which were the main sterols, and were found twenty times more compared to the one obtained by ILYASOĞLU (2014) and GRAJZER *et al*. (2015). While ZLATANOV (1999) reported values of phytosterol, which are not comparable with the other data available from similar studies.

Table 3. Phytosterol composition of rose hip seed oils.

References	Zlatanov (1999)	Ilyasoğlu (2014)	Grajzer *et al*. (2015)	Turan *et al*. (2018)
		Phytosterols (g/kg)		
Brassicasterol	5.4	-	nd	1
Campesterol	1.8	0.233	0.192-0.205	43
Cholesterol	0.5	-	-	4
Clerosterol	-	0.014	-	-
Stigmasterol	3.5	0.189	0.077-0.060	
β-Sitosterol	81.5	5.44	4.753-5.297	780
Δ^5-Avenasterol	4.6	0.316	0.242-0.379	39
Δ^7-Stigmasterol	nd	0.414	nd	43
$\Delta^{7.25}$-Stigmasterol	1.8	-	-	-
Δ^7-Avenasterol	0.9	0.019	0.037-0.056	15
Cycloartenol	-	-	0.589-0.649	-

3.3. Tocopherol content

Tocopherols are important compounds of the unsaponifiable fraction, which are present as liposoluble phenols in vegetable oils. Different isomer forms could be found (α, β, γ, and δ) depending on the number and position of methyl groups in the phenolic ring (HERNANDEZ, 2015). They exhibit antioxidant properties and that ensure the oxidative stability of oils.
Table 4 provides a summary of different tocopherols composition of rosehip seed oils.
The cold-pressed oil of rosehip obtained from two different manufacturers were characterized respectively by 1.0 and 1.1 g/kg of total tocopherols content, which were higher compared to camellia and walnut oils (0.7 and 0.4 g/kg) (GRAJZER *et al*., 2015). These results were in agreement with the one obtained by FROMM *et al*. (2012) for *Rosa canina* oil extracted with Soxhlet procedure. However, the tocopherols amount was not comparable with the results obtained by ZLATANOV (1999). Besides, a high level of tocopherols is associated with high oil rich in PUFA
Rose woodsii oil obtained with Folch procedure was constituted by a high amount of α-tocopherol (0.4 g/kg) followed by δ-tocopherol and γ-tocopherol (0.09 and 0.002 g/kg) (ANWAR *et al*., 2008). Instead, in experiments with *Rosa canina* oils, the most abundant isomer form was γ-tocopherol ranging from 0.6 to 0.9 g/kg (GRAJZER *et al*., 2015; FROMM *et al*., 2012). In all rosehip oils, β-tocopherol was not detected (ANWAR *et al*., 2008; GRAJZER *et al*., 2015; FROMM *et al*., 2012).

Table 4. Total tochopherols of rosehip seed oils.

Refereces	Zlatanov (1999)	Anwar *et al.* (2008)	Grajzer *et al.* (2015)	Fromm *et al.* (2012)
		Tocopherols (g/kg)		
α-Tochopherols	19.0	0.4±34.9	0.1-0.2	0.2±5.1
β-Tochopherols	nd	nd	-	nd
ɣ-Tochopherols	71.0	0.002±84.0	0.6-0.8	0.9±55.6
δ-Tochopherols	1.8	0.09±10.4	0.2-0.3	0.03±3.7
Total Tochopherols	91.8	0.002±50.2	1.0-1.1	1.0±55.9

4. CONCLUSION

The improvement of food products is directed towards ensuring nutritional and functional benefits.
Therefore, an adequate description of lipid food components of rosehip seed oil was provided in order to develop their possible re-use as bioactive component in the functional food production. Considering the possibility of their combination, which may permit an improved solubility, stability or bioactivity than the single one. In particular, the oil content of rose hip seeds ranges from 5 to 18 % and it basically includes different amount of unsaturated fatty acids as linoleic, linolenic and oleic acid. However, for what

concern the sterols content around 5 g/kg with the most abundant β-Sitosterol and tocopherols amount with γ-tocopherol were observed.

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WINE INTOLERANCE IN ITALY: A PILOT STUDY

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ABSTRACT

Intolerance to wine has been investigated under different aspects. In our study, we explored intolerance/allergy reactions in Italy by means of a survey including 901 questionnaires.

Prevalence of wine or beer intolerance was 6%, of which 11 reported that was verified by a doctor. Odds ratio for wine intolerance with another declared allergy/intolerance is 1.6.

In conclusion, we observed a high prevalence of intolerance to wine in Italy, highlighting that some sections of the population (young people and women) may be more exposed to these phenomena.

Keywords: alcoholic drinks, allergy, intolerance, Italy, survey, wine

1. INTRODUCTION

Over the last few years, consumers have become increasingly interested not only in safety of food and beverages, but also in their healthiness. At the same time, the cultural conception of the consumed products has gained importance in relation to shared cultural and social significance and practice in a given environment or geographical area. When dealing with wine, the cultural connotation is well defined: in Mediterranean culture, the emotional and cultural aspects of wine have always been strongly connected. Moreover, in western culture, wine is often associated with festive and/or convivial moments. The effects of wine consumption have been extensively studied and a moderate alcohol consumption was proven to have positive healthy effects (RONKSLEY *et al*., 2011); however, as for other food, allergic reactions and intolerances were also reported for wine (NIESTIJL JANSEN *et al*., 1994; VALLY *et al*., 1999; ARMENTIA, 2008; BÖHN *et al*., 2013; JAECKELS *et al*., 2015; BANSAL *et al*., 2017; WÜTHRICH, 2018). A recent study reported prevalence of 74% for upper airway symptoms and 51% for lower respiratory symptoms after alcohol ingestion in patients with aspirin-exacerbated respiratory disease (CARDET *et al*., 2014). Additional risk factors include female sex, history of allergic rhinitis, chronic obstructive pulmonary disease, and asthma (NIHLEN *et al*., 2005).

The etiology of this reaction is still unclear, because wine is a complex beverage, with several potential allergens. Potential allergens include proteins from grape, molds, yeasts, as well as proteins from insects that have contaminated the mash. Milk derivatives as caseine and potassium caseinate, and other animal-derived products as isinglass and gelatin are used in the fining process to remove phenolic and tannin compounds from white wine, and egg white (albumin) is used to remove tannin compounds from red wine. Non-grape-derived tannins such as those from the bark and galls of trees are also used. These fining agents are added to the wine and the precipitates removed (ROLLAND *et al*., 2006). Allergic reactions have been described also for components like ethanol, acetaldehyde, acetic acid and sulfites. Ethanol, acetaldehyde and acetic acid, flavonoids (anthocyanins and chatechines), sulfites, histamine and other biogenic amines are the main causative agents of intolerance reactions (pseudoallergic reactions) to wine (VALLY *et al*., 1999; WÜTHRICH, 2018).

Actually, the presence of histamine in red wine as a source of intolerance has been controversial. Several studies observed a clear correlation between the red wine consumption (more rich in histamine than white wine) and the insurgence of the typical symptoms for food intolerance, such as sneezing, flush, headache, diarrhea, skin itch, and shortness of breath (JARISCH and WANTKE, 1996; WANTKE, HEMMER, HAGLMÜLLER, *et al*., 1996; WANTKE, HEMMER, HAGLMULLER, *et al*., 1996). Moreover, well trained wine assessors were able to identify elevated histamine concentrations in wine (ROHN *et al*., 2005). Nevertheless, other studies did not observe such correlation between wine intolerance and histamine content of wine (KANNY *et al*., 2001). In either cases, sample size was very low, and this may be the reason for this contradiction. Similar challenges were conducted to investigate the role of sulfites on the pathogenesis of wine-induced asthma, with no clear correlation, but more likely, this phenomenon appears to be a complex phenomenon, involving several different mechanisms (VALLY *et al*., 1999).

In asthmatics, alcoholic drinks trigger asthma symptoms in more than one third of these patients (AYRES and CLARK, 1983; VALLY *et al*., 2000). With regard to the prevalence of wine intolerance/allergy in general population, two surveys were conducted in Germany and Denmark (LINNEBERG *et al*., 2008; WIGAND *et al*., 2012). The German study was

localized in Mainz, the capital of Rhineland-Palatinate, a wine-producing region of Germany, and was conducted by mean of a questionnaire survey (WIGAND *et al*., 2012). The intolerance to wine was reported to be 3.2%, but only two persons reported that a wine allergy was verified by a physician. A cross-sectional study by means of postal questionnaire was conducted in Copenhagen, Denmark, in 2006, involving over 4,000 respondents out of 6,000 invited people (LINNEBERG *et al*., 2008). The survey investigated the hypersensitivity reactions following consumption of alcoholic beverages divided by symptoms from the nose, lower airways, and the skin, with a prevalence of 7.6%, 3.2%, and 7.2%, respectively, and a cumulative prevalence of 13.9%. This study investigated all types of alcoholic beverages, including beer, red wine, white wine, dessert wine, and spirits.
To the best of our knowledge, no data are available about the prevalence of intolerance/allergy symptoms induced by wine in the Mediterranean countries, like Italy, that have a strong tradition in wine consumption. According to a 2014 FAO report, Italy is at the first place in the world ranking of wine producing countries, followed by Spain and France.
Aim of the study was to explore whether intolerance/allergy reactions are also present in the general population of a wine-producing country, such as Italy, and to estimate the prevalence by means of a questionnaire survey.

2. MATERIALS AND METHODS

The questionnaire about wine consumption and intolerance developed by Wigand an colleagues (WIGAND *et al*., 2012) was translated in Italian, slightly modified and transferred to Google Surveys (https://surveys.google.com). Questionnaires were submitted either by face-to-face interview or self-submission after receiving the link to the Google form. For face-to-face interview, the answers were either recorded on a printed survey and then added later on the Google form by our personnel, or directly entered on the online survey from a portable device.
Subjects were included in the study with a convenience sampling, enrolling for the face-to-face interview 170 people attending to two events held in September 2017 in Piedmont (Cheese 2017: http://cheese.slowfood.it/en/ and European Researchers' Night 2017: http://nottedeiricercatori.piemontevalledaosta.it). Snowball sampling starting from researchers involved in the study was used to enroll additional 731 people, using the online form.
As described by Wigand and colleagues, the questionnaire, in addition to standard questions on age and sex, included questions on whether allergy-like symptoms had occurred after wine consumption: participants had to choose among a list of symptoms (Table 1) experienced after the consumption of red, white and rosé wine. The intensity and the time of occurrence of each symptom was also assessed by the participants (intensity: none, weak, moderate, strong, very strong; time: <15 minutes, 15 minutes to 1 hour, 1 to 2 hours, >2 hours). In addition, participants were asked about intolerance to various known allergens, listed in Table 2. For each reported intolerance, participants had to declare if it was medically diagnosed. According to Wigand and colleagues (WIGAND *et al*., 2012), all reported symptoms, except for headache, were considered as symptoms of intolerance and a score was given (Table 1). Each subject scoring more than 10 was defined as "intolerant to wine".

Table 1. Symptoms of wine intolerance, their score and prevalence with CI 95%.

Symptoms	Score	Number of participants	Prevalence	CI 95%
Circulatory collapse	10	2	0.2	0-0.8
Shortness of breath/asthma	8	25	2.9	1.9-4.2
Tachycardia	7	50	5.9	4.4-7.7
Itching	6	48	5.6	4.2-7.4
Flushed skin	5	160	21.6	18.7-24.7
Low blood pressure	5	51	6	4.5-7.8
Rhinorrea	4	30	3.4	2.3-4.9
Burning sensation in lips, palate, neck	4	47	5.5	4.1-7.3
Stomach or intestinal cramps	3	99	12.3	10.1-14.8
Diarrhea	3	48	5.6	4.2-7.4
Vomiting	3	33	3.8	2.6-5.3
Skin rash, hives, oedema	-	23	2.6	1.7-3.9
Headache	-	353	64.4	60.2-68.4
Other symptoms	-	48	5.6	4.2-7.4

Once ended the survey, we performed a descriptive analysis of the characteristics of participants, of the frequency and of the preferences in alcohol consumption. Then, we calculated the prevalence and the 95% confidence interval (95% CI) of each symptom, and the prevalence of people intolerant to wine. Odds ratio (OR) and their 95% CI were calculated applying a logistic regression model to verify if there was some gender or age difference among people classified as intolerant to wine. The presence of allergies among participants was described and gender or age differences were assessed by calculating OR and their 95% CI. Finally, OR and their 95% CI were calculated to describe the relationship between intolerance to wine and the presence of allergies. All the statistical analyses were carried out using the software STATA 15.1 (StataCorp LLC, College station TX77845, Texas, USA, www.stata.com).

3. RESULTS

A total of 901 questionnaires were included in the study, and sex was equally distributed with 52% (n=486) of females and 48% (n=429) males. The geographical proportion and the proportion for age among participants do not reflect the proportion in the general population, as reported in Fig. 1, with an overrepresentation of people under 55 years of age.

As shown in Fig. 2, most of participants affirmed to drink wine more than once a month, and only a minority declared to have never drank wine. Generally, men drank more than women (OR 2.78, 95% CI 2.09-3.70)

In our survey, 54 (6%) participants declared to have an intolerance to wine or beer, most of which were females; 11 of them reported that the allergy was verified by a doctor. The 36% of participants reported at least one symptom of intolerance after drinking wine. The prevalence of each symptom consequent to drinking wine, reported by participants is summarized in Table 1.

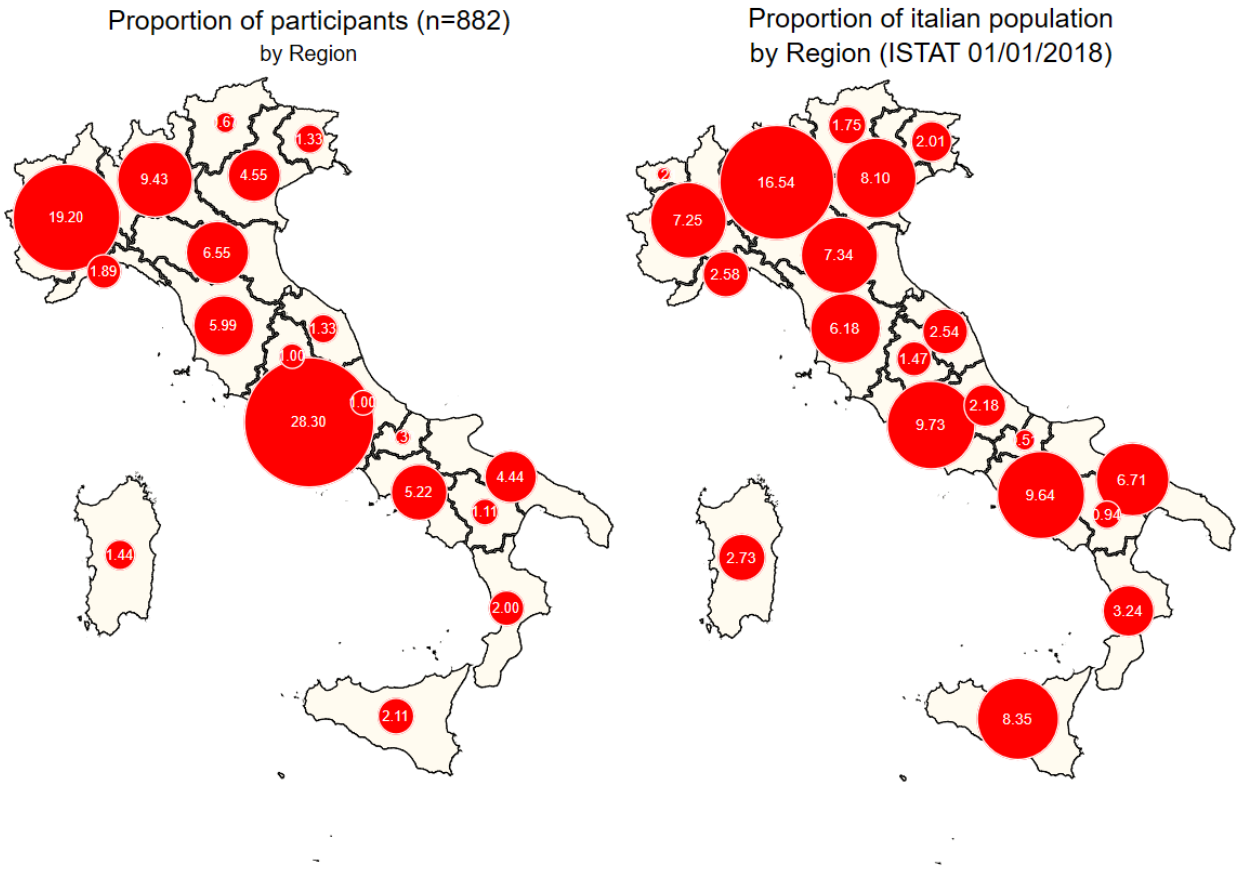

Figure 1. Comparison between the geographical distribution of the Italian general population at Jan, the 1st 2018 (right), and people enrolled in the study (left).

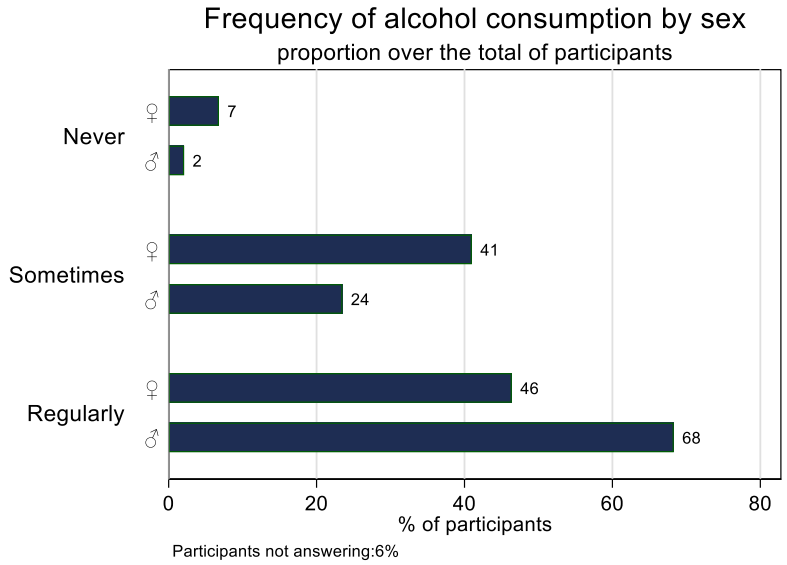

Figure 2. Alcohol consumption: proportion of alcohol consumption by sex among people enrolled in the study. *Never*: never drunk wine; *Sometimes*: once a month; *Regularly*: more than once a month.

Having achieved a wine intolerance score of at least 10, a total of 138 subjects were classified as intolerant to wine (18%, 95% CI 15-21%), with an increased risk of intolerance to wine in females (OR 1.89, 95%CI 1.29-2.75), without differences in the age classes considered or in the type of wine. The 61% of participants declared to have at least one allergy/intolerance, with a risk of reporting at least one allergy/intolerance increased in females (OR 1.96, CI95% 1.49-2.58), without differences in the age classes considered or in the type of wine. The prevalence of each allergy/intolerance self-declared by participants is reported in Table 2.

Table 2. Prevalence of each allergy/intolerance self-declared by participants and declared as medically diagnosed.

	Self-declared		Medically diagnosed	
Allergens	**Prevalence**	**CI 95%**	**Prevalence**	**CI 95%**
Alcohol	4	2.8-5.6	1.2	0.6-2.2
Banana	2.2	1.3-3.3	1.5	0.8-2.5
Beer	3.8	2.6-5.3	1.3	0.7-2.3
Carrots	1.6	0.9-2.6	1.3	0.7-2.3
Celery	1.2	0.6-2.2	1.1	0.5-2.1
Cherries	2.3	1.4-3.5	1.5	0.8-2.5
Crustaceans	3.8	2.6-5.3	1.9	1.1-3.1
Eggs	2.5	1.6-3.8	1.6	0.9-2.6
Fish	1.7	1-2.8	1.3	0.7-2.3
Gluten	5.3	3.9-7	1.9	1.1-3.1
Grapes	1.6	0.9-2.6	1.2	0.6-2.2
House dust	36.1	32.4-39.9	16.9	14.3-19.7
Kiwi	5.1	3.8-6.8	1.8	1-2.9
Latex	4.8	3.4-6.4	1.7	1-2.8
Lupines	1.5	0.8-2.5	1.1	0.5-2.1
Medications	13.6	11.3-16.2	9.2	7.3-11.4
Milk and derivates	15.5	13-18.2	4.2	2.9-5.7
Mustard	1.8	1-2.9	1.1	0.5-2.1
Nickel	13.3	11-15.9	5.3	3.9-7
Nuts	6.9	5.3-8.8	3.3	2.2-4.7
Peaches	4	2.8-5.6	1.9	1.1-3.1
Peanut	3.3	2.2-4.7	1.9	1.1-3.1
Pears	2.2	1.3-3.3	1.5	0.8-2.5
Peppers	4	2.8-5.6	1.5	0.8-2.5
Plums	1.5	0.8-2.5	1.2	0.6-2.2
Pollen	39.7	35.9-43.6	21.4	18.5-24.6
Seafood	4.9	3.5-6.6	2.3	1.4-3.5
Sesame	1.6	0.9-2.6	1	0.5-1.9
Soya	1.8	1-2.9	1	0.5-1.9
Strawberries	4	2.8-5.6	2.3	1.4-3.5
Sulphites	11.9	9.8-14.4	1.6	0.9-2.6
Tomatoes	4.9	3.5-6.6	1.8	1-2.9
Wine	4.5	3.2-6.1	1.2	0.6-2.2

Participants classified as intolerant to wine showed an increased risk to have a medically-diagnosed allergy/intolerance (Table 3).
The OR of being intolerant to wine when declaring one medically diagnosed allergy/intolerance is 1.6 (CI95% 1.1-2.3). Table 3 reports the OR of being intolerant to wine for each medically diagnosed allergy/intolerance.

Table 3. Odds ratio and their 95% confidence interval of having a medically diagnosed allergy/intolerance when classified as intolerant to wine.

	Wine intolerants only	
Allergens	**Odds ratio**	**CI95%**
Banana	33	7-307.4
Carrots	29.7	6.2-280.3
Celery	53.2	7.2-2332.6
Cherries	33	7-307.4
Crustaceans	14.4	4.6-53
Eggs	14.8	4.2-65.4
Fish	29.7	6.2-280.3
Gluten	10.9	3.6-36.5
Grapes	59.5	8.3-2586.2
House dust	2.2	1.3-3.4
Kiwi	26.3	7.1-145.3
Latex	16.4	4.8-71.5
Medications	2.7	1.5-4.6
Milk and derivates	5.5	2.6-11.5
Nickel	3.7	1.8-7.2
Nuts	5.6	2.4-12.8
Peaches	8.4	2.8-26.5
Peanut	10.9	3.6-36.5
Pears	33	7-307.4
Peppers	33	7-307.4
Plums	59.5	8.3-2586.2
Pollen	1.5	0.9-2.4
Seafood	11.2	4.1-33.8
Strawberries	11.2	4.1-33.8
Sulphites	21.9	5.7-123.4
Tomatoes	18.1	5.3-77.7

4. CONCLUSIONS

Wine is one of the oldest beverages, whose production can be traced back to 5,000 BC and it is nowadays often associated with convivial moments.
Intolerance to wine has been investigated under different aspects, albeit the unclear etiology. The prevalence of such intolerance was investigated in Nordic countries like Germany and Denmark, but no information about Mediterranean countries has been

published. Our survey aimed to fill this lack and showed that wine intolerance is present also in Italy. The prevalence of intolerants to wine (6%) is higher than the prevalence reported in Germany (3.2%), but lower than the one reported in Denmark (13%). This high percentage may be explained by the inclusion of all the alcoholic drinks in the Danish study without differentiation, although the Authors reported that red wine was listed as the main cause for hypersensitivity symptoms, as well as spirits (LINNEBERG *et al*., 2008). Both the previous studies reported that symptoms of intolerance were more frequent after drinking red wine rather than white wine, while we found no differences among the type of wine. WÜTHRICH (2018) reported that the most frequent reactions are intolerance reactions to sulfites, which occur particularly after the ingestion of white wine and in asthma patients, and to histamine and other biogenic amines, mainly after ingestion of red wine. Particularly in white wine, allergy-like intolerance reactions are caused by sulfite (VALLY *et al*., 2000; VALLY *et al*., 2001). Asthma patients are especially sensitive.

In agreement with the previous studies, we also found an increased risk of being classified as intolerant to wine in females than in males. In line with what reported in the literature (NIHLEN *et al*., 2005; CARDET *et al*., 2014), our study highlighted a greater risk of being intolerant to wine in people who claim to have allergies or intolerances diagnosed by the doctor. On the other side, participants classified as intolerant to wine showed an increased risk to have a medically-diagnosed allergy/intolerance. Specifically, the highest associated risks (OR>50) were detected for celery, plums, and (as predictable) grapes.

Even in this case, however, it must be borne in mind that the analyzed data were reported by participants and were not investigated through the execution of clinical tests, therefore they might be subject to distortions.

Furthermore, the selection of a sample of convenience could cause a loss of external validity of the study, therefore the quantitative results of this study must be interpreted with caution.

The wine also contains molecules (biogenic amines like histamine and putrescine, and sulphites), which can cause phenomena mimicking allergies, making it difficult to distinguish between false allergy phenomena (for example histamine reactions) and real allergies. In fact, allergy/intolerance to sulfites had a significant OR (21.9) in those persons classified as intolerant to wine.

In conclusion, although with some limitations, our study indicates a high prevalence of the phenomenon of intolerance to wine, highlighting that some sections of the population (young people and women) may be more exposed to these phenomena. However, the relationship between the presence in the wine of molecules that can cause false allergy phenomena must be further investigated.

ACKNOWLEDGMENTS

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PAPER

LACTIC ACID BACTERIA MICROBIOTA OF "PIROT`S KASHKAVAL"

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ABSTRACT

"Pirot`s kashkaval" is an autochthonous dairy product belonging to pasta-filata cheeses. In order to determine the changes in the lactic acid bacteria microbiota during 60 days of ripening, 315 lactic acid bacteria strains were isolated from ewe`s and cow`s milk cheese and identified as *Enterococcus faecium, Pediococcus acidilactici, Pd. pentosaceus, Lactobacillus casei/rhamnosus, Lb. plantaum, Lb. casei, Lb. fermentum, Lb. paracesei, Lb. rhamnosus* and *Streptococcus macedonicus*. Enterococci were the most dominant isolated strains. In the final stages of ripening, the increase of the population of *Lb. casei* and *Pd. acidilactici* was observed for ewe`s and cow`s milk cheese, respectively.

Keywords: cow`s milk cheese, ewe`s milk cheese, molecular identification, lactic acid bacteria, "Pirot`s kashkaval",

1. INTRODUCTION

"Pirot`s kashkaval" cheese is an autochthonous dairy product produced in Pirot, Republic of Serbia and its surroundings by a specific technology. The production process, which differs this type of cheese from other produced in Serbia includes the cooking of partly fermented curd prior to ripening. This type of cheese has light to intensively yellow color, monolith, partially layered and elastically-plastic structure. The combination of cow's and ewe`s milk is, usually, being used for the production. The taste of "Pirot`s kashkaval" is mildly sour and piquant, specific and depends on the type of used milk (OSTOJIĆ *et al*., 2012).
The production is characteristic for the process of cooking of the sliced fermented curd for 5-8 min. at temperature of 72-75°C. The cheese curd is then salted, mixed, molded and additionally ripened for a few months (MANČIĆ and MANČIĆ, 2005). The cooking of curd in hot water has the significant influence on its microbiota. Thermal treatment of fermented curd is a way of pasteurization of cheese dough, which leads to certain biochemical and microbiological processes. During this process the significant drop in bacterial and yeast number occurs, so it can be very important in the cases when it's not possible to get good quality milk and when milk pasteurization is not applied (ALRUBAI, 1979).
The formation of sensory profile of cheeses is the result of metabolic activity of the present microbiota (BENITO de CARDENAS, *et al.*, 1990; MUSTAFA, 2006, DUAN *et al*., 2008). Products of glicolysis, proteolysis of casein and lipolysis of fats, as the main metabolic functions of lactic acid bacteria (LAB), have a great influence on cheese flavor.
Since starter cultures are not added during the production of "Pirot`s kashkaval", cheese flavor originates from metabolic products of autochthonous microbiota. The advantages of autochthonous microbiota are fast growth and development and production of specific sensory characteristics of the products. Furthermore, determination of the microbiota composition is an important step in analyzing traditional fermented dairy products and eventual devinition of autochthonous starter cultures. The aim of this work was the isolation and characterization of LAB present during the process of ripening of "Pirot`s kashkaval" unique in Serbia for the production process. For that purpose, 10 samples of cheese produced from cow`s and ewe`s milk were collected during 60 days of ripening and analysed.

2. MATERIALS AND METHODS

2.1. Cheese samples

"Pirot`s kashkaval" was produced by a traditional process from cow's and ewe`s milk. After the addition of rennet, the whey was separated, and the fresh curd was fermented for a few days. The fermented curd was sliced and cooked in hot water (72-75°C). After that, the curd was salted, molded and ripened in a ripening chamber. Sampling was performed during the ripening process of 10-15 days at 25°C and until the end of the two months ripening period at 10°C.
Samples of cow`s milk cheese (CC) and ewe`s milk cheese (EC), were collected during the process of ripening after 1 (CC1 and EC1), 5 (CC5 and EC5), 20 (CC20 and EC20), 30 (CC30 and EC30) and 60 (CC60 and EC60) days of ripening. The samples were then packed in

vacuum, transported and kept up to 3 days at + 5°C to the moment of microbiological analyses.

2.2. Isolation and determination of LAB number

Cheese samples (10 g) were transferred aseptically to 90 mL of 2% (w/v) sodium citrate solution (t=45°C) and homogenized for 30 minutes. The isolation of LAB was performed by serial dilution method. A volume of 1 mL of appropriate dilution was transferred into a Petri dishes and layered with MRS (Torlak, Belgrade, Serbia), M17 (Merck, Darmstadt, Germany) and MSE agar (tripton 10 g L^{-1}, gelatine 2.5 g L^{-1}, yeast extract 5 g L^{-1}, sucrose 100 g L^{-1}, glucose 5 g L^{-1}, sodium citrate 1 g L^{-1}, sodium azide 0.075 g L^{-1} and agar 13 g L^{-1}) for determination of presumptive lactobacilli, lactococci and leuconostocs, respectively. After solidification, second layer of medium was poured in order to achieve micro-aerophilic conditions preferable for the growth of LAB. Plates with MRS and M17 agar were incubated for 48 h at 30 and 45°C in order to determine both mesophilic and thermophilic LAB strains, while the MSE agar plates were incubated 48h at 30°C. Determination of total number of mesophilic bacteria was performed on nutrition agar (NA) plates (Torlak, Belgrade, Serbia) (48 h, 30°C). The determination of number of bacteria was performed in triplicate and the values are presented as the mean value.
After the incubation and enumeration, at least 30 LAB colonies were selected from each sample and purified. Preliminary characterization of the isolates was done by Gram staining and catalase test. Bacterial cultures were stocked in liquid medium (MRS and M17 broth), with addition of 20% (v/v) glycerol at the temperature of -20°C until further analysis.

2.3. Molecular identification of isolates

The extraction of total DNA, PCR amplification with (GTG)5 primer and electrophoresis were done by already described method (NIKOLIĆ *et al.*, 2008). Sequencing of 16S rRNA genes was performed by multiplying of fragments by U968 (5′-AACGCGAAGAACCTTAC-3′) and L1401 (5′-AACGCGAAGAACCTTAC-3′) primers (RANDAZZO *et al.*, 2002) and using Taq DNA polymerases. Reaction was carried out in PCR System 2700 (Applied Biosystems) with the following parameters: starting denaturation of DNA during 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and the extension of incomplete products for 7 min at 72°C. Electrophoresis of multiplied products was done at 1% (w/v) agar gel with the addition of ethidium bromide. Multiplied fragments were purified using QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany), while their sequencing was done in Macrogen in Seoul, South Korea. BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) was used for determining the most similar sequence from NCBI base.

3. RESULTS AND DISCUSSION

3.1. Determination of number of bacteria

The changes of total mesophilic bacteria and LAB number determined at MRS, M17 and MSE agar plates in analyzed ewe`s and cow`s milk cheese samples are shown in Figs. 1 and 2, respectively. During the first 5 days of ripening, a slight increase of total number of

mesophilic LAB determined at MRS agar plates was noticed, from 7.7 to 7.8 log CFU g^{-1}, after which the number decreases to 6.4 log CFU g^{-1} (Fig. 1).

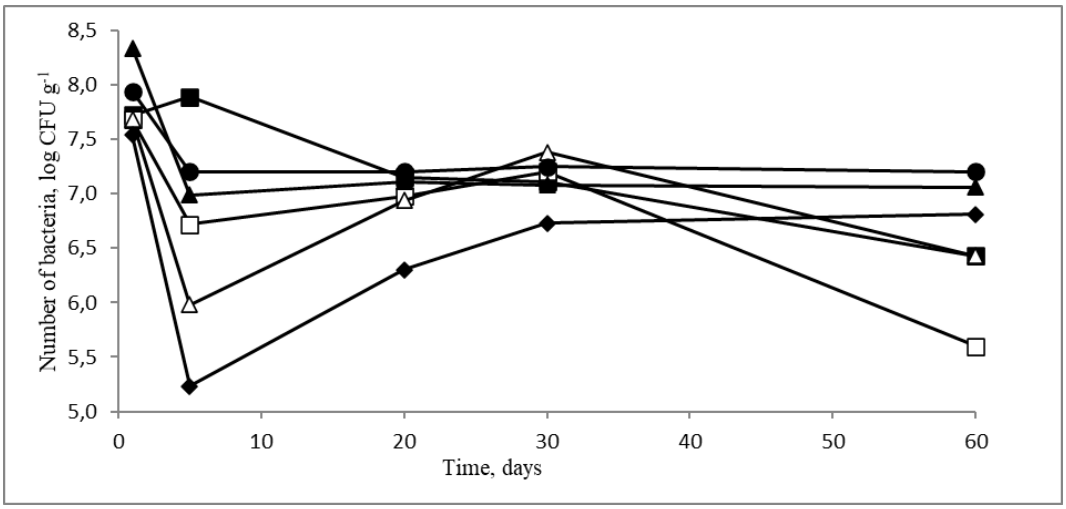

Figure 1. The change of number of bacteria during ripening of Pirot cheese produced from ewe`s milk at MRS (■), M17 (▲), MSE (♦) and NA (●) plates incubated at 30 (full symbols) and 45 °C (empty symbols).

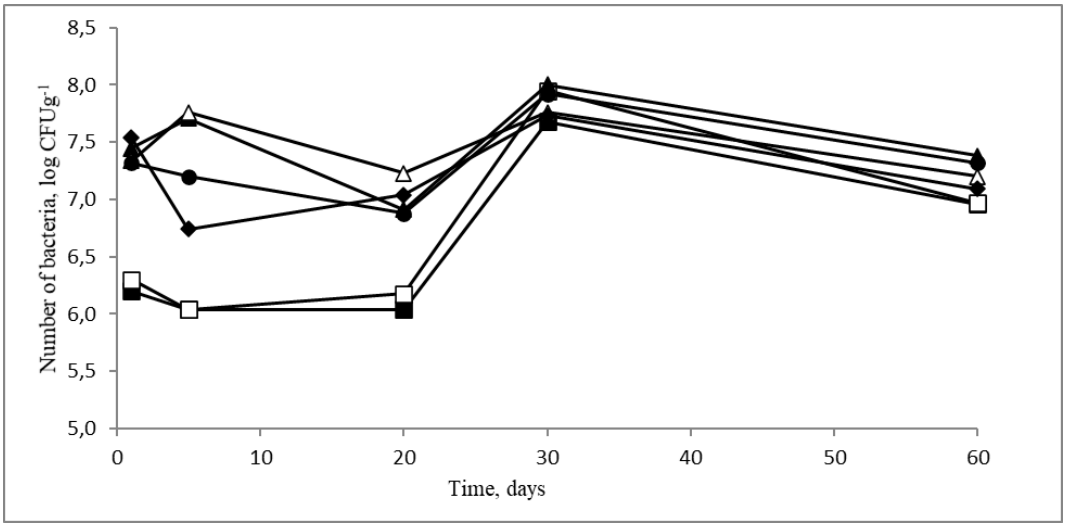

Figure 2. Change of the number of bacteria during ripening of Pirot cheese produced from cow`s milk at MRS (■), M17 (▲), MSE (♦) and NA (●) plates, incubated at 30°C (full symbols) and 45°C (empty symbols).

The number of microorganisms on M17, MSE and nutrition agar plates decreased in the first 5 days of ripening. Thus, number of mesophilic LAB at M17 agar plates decreased from 8.3 to 6.9 log CFU g^{-1} during the first 5 days, after which it was constant with the value of 7.1 log CFU g^{-1}. The number of thermophilic LAB was lower compared to the number of mesophilic LAB, regardless the used medium. Also, number of thermophilic bacteria decreased continually after the 30th day of ripening. The total number of mesophilic bacteria incubated at nutrition agar plates in first 5 days decreased from 7.9 to

7.2 log CFU g^{-1}, and that value maintained to the end of ripening. The number of mesophilic LAB determined on MSE agar plates decreased in first 5 days from 7.5 to 5.2 log CFU g^{-1} and after that it increased to 6.8 log CFU g^{-1} (Fig. 1).
The number of bacteria in cow`s milk cheese samples is shown in Fig. 2. The increase of the LAB number at the beginning of fermentation (to 5th day) was only noticed on M17 agar plates for both mesophilic (from 7.5 to 7.7 log CFU g^{-1}) and thermophilic bacteria (from 7.3 to 7.8 log CFU g^{-1}). In the following 15 days, number of mesophilic LAB decreased to 6.9 log CFU g^{-1}, and thermophilic to 7.2 log CFUg^{-1}. At the end of fermentation, the number of LAB on M17 agar plates was 7.3 log CFU g^{-1}. The number of LAB on MRS agar in the first 20 days decreased for both bacterial groups reaching the value of cca 6.0 log CFU g^{-1}. Mesophilic and thermophilic LAB on MRS agar plates reached maximum on the 30th day of ripening (7.7 log CFU g^{-1}), afterwards a slight decrease was observed (Fig. 2).
High initial number of LAB in both cheese types can be explained by the process of fermentation, which took place before curd cooking. The fermented curd was thermally processed by immersion in hot water before sampling, but it can be assumed that heat stressed cells remained viable. The decrease of the LAB number was observed, regardless the type of milk used for making cheese, in the period from the 1st to the 5th day of ripening due to the adaptation of the microbiota to the ripening conditions. This decrease is in accordance with the literature results (ALRUBAI, 1979; GOBBETTI *et al*., 1997; BARUZZI *et al.*, 2002), which indicate that cooking of curd has a great influence on the microbiota and lead to a significant decrease of total number of bacteria in the first stage of ripening. The differences in the LAB number of cow`s and ewe`s milk cheese are probably the result of the different microbiota composition in these two cheese types. The stagnation of LAB number during the ripening of cow`s milk cheese lasts longer (30 days, Fig. 1) in relation to the samples of ewe`s milk cheese (20 days, Fig. 2). After that, the constant number of bacteria was achieved in the range from 6.5 to 7.4 log CFU g^{-1}, for both type of cheese. At the end of fermentation, the lowest number was observed for thermophilic LAB on MRS agar plates. This can be explained by the ripening conditions (temperature 10°C) which favors the growth of mesophilic bacteria. The domination of mesophilic LAB in the later stages of pasta filata cheese ripening has already been reported by SUCCI *et al*. (2016).
Determined LAB number in analyzed samples is in accordance with earlier researches for "Pirot`s kashkaval" (MIJAČEVIĆ *et al.*, 2005a; MIJAČEVIĆ *et al.*, 2005), Italian cheese Pugliese, Silano and Molise, produced from pasteurized cow milk (GOBBETTI *et al.*, 2002; COPPOLA *et al.*, 2003; PIRAINO *et al.*, 2005), Mozzarella from cow milk (de CANDIA *et al.*, 2007) and Taleggio cheese from Lombardi in Italy (GOBBETTI *et al.*, 1997).

3.2. Identification of lactic acid bacteria isolates

From 5 different samples of Pirot`s ewe`s milk cheese 173 LAB isolates were obtained. The identification was performed by (GTG)5-PCR fingerprinting and 16S rRNA gene sequencing. According to the (GTG)5-PCR fingerprinting low level of diversity was observed among the isolates and representative fingerprints are shown in Fig. 3. Most of the isolates belong to cocci (130), while 43 belong to bacilli. Isolates were identified as the representatives of *Enterococcus faecium, Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus casei/rhamnosus, Lactobacillus plantaum, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus paracesei* and *Streptococcus macedonicus*. Among them, the most dominant were *En. faecium* (50%) and *Pd. acidilactici* (16%), while the least isolated strains were *Lb. fermentum* (1%) and *Lb. paracasei* (0.5%). During 60 days of ripening of Pirot`s

cow`s milk cheese 142 LAB isolates were isolated and identified as *En. faecium* (44.4% of isolates), *Pd. acidilactici* (50%), *Pd. pentosaceus* (0.7%), *Lb. plantarum* (1.4%), *Lactobacillus rhamnosus* (0.7%) and *Lb. casei* (2.8%).
The presence of different LAB strains is important in cheese ripening due to their unique metabolism products (COGAN, 2000). Dominancy of particular LAB strain is directly dependent of milk type, animal breeding, and way of feeding, type and quality of pasture, altitude, and production process, cheese storage conditions and many other (TOPISIROVIĆ *et al.*, 2006). The presence and prevalence of concrete LAB strains in analysed cheese samples is mainly affected by LAB heat resistance during the curd cooking. Among LAB strains isolated from Pirot`s cheese samples, the representatives of genera *Enterococcus* and *Pediococcus* dominate, while in a smaller percentage *Lactobacillus* spp. and *Streptococcus* sp. were present. The obtained results are in accordance with literature data according to which LAB isolated from cheese belong to genera *Enterococcus, Pediococcus, Lactobacillus, Streptococcus* and *Lactococcus* (FLEET, 1999; POZNANSKI *et al.*, 2004; MENG *et al.*, 2018; VANDERA *et al.*, 2019).

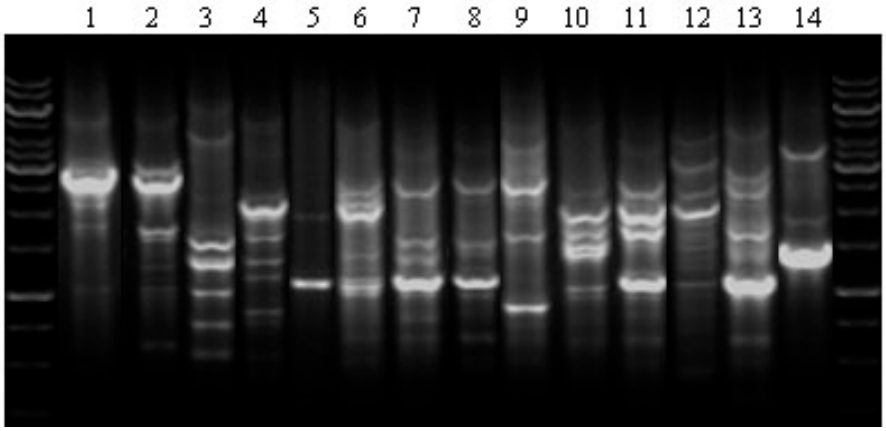

Figure 3. Representative (GTG)5-PCR fingerprints of LAB strains isolated from Pirot cow`s and ewe`s milk cheese: *En. faecium* (1,2), *Pd. acidilactici* (3), *Pd. pentosaceus* (4), *Lb.plantarum* (5), *Lb. casei* (6,7,8), *Lb. fermentum* (9), *Lb. paracesei* (10,11), *Lb. rhamnosus* (12), *Lb. casei/rhamnosus* (13) and *St. macedonicus* (14).

3.3. Microbiota dynamics in "Pirot`s kashkaval"

The results of monitoring changes in microbiota during ripening of cheese made from ewe`s milk are presented on Fig. 4. After 24 hours (sample EC1), the most frequently isolated was *En. faecium* (60%), while *St. macedonicus* (36%) and *Pd. acidilactici* (4%) were isolated as well. In sample after 5 days of fermentation (EC5) the population of *En. faecium* increased (85%), as well as pediococci (9%), while streptococci were not isolated. The percentage of enterococci decreased significantly after 20 days (EC20), while the percentage of pediococci (*Pd. acidilactici* – 47% and *Pd. pentosaceus* – 18%) and lactobacilli increased (*Lb. casei/rhamnosus* 6% and *Lb. plantarum* 9%). In the sample after 60 days of fermentation (EC60) *En. faecium* (55%) was dominant, while *Lb. casei* (20%), *Pd. acidilactici* (14%) and *Lb. casei/rhamnosus* (3%) were also isolated. *Lb. fermentum* (5%) and *Lb. paracasei* (3%) were identified only in this sample.
The change of microbiota during 60 days cow`s milk cheese ripening is shown in Fig. 5.

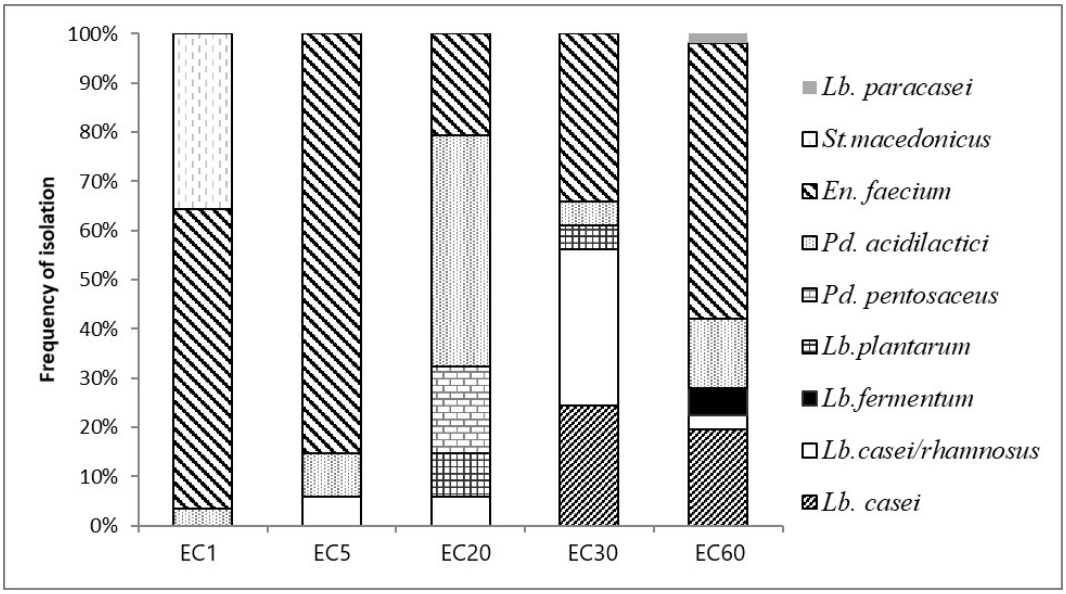

Figure 4. Frequency of isolation of LAB strains after 1 (EC1), 5 (EC5), 20 (EC20), 30 (EC30) and 60 (EC60) days of ripening of Pirot ewe`s milk cheese.

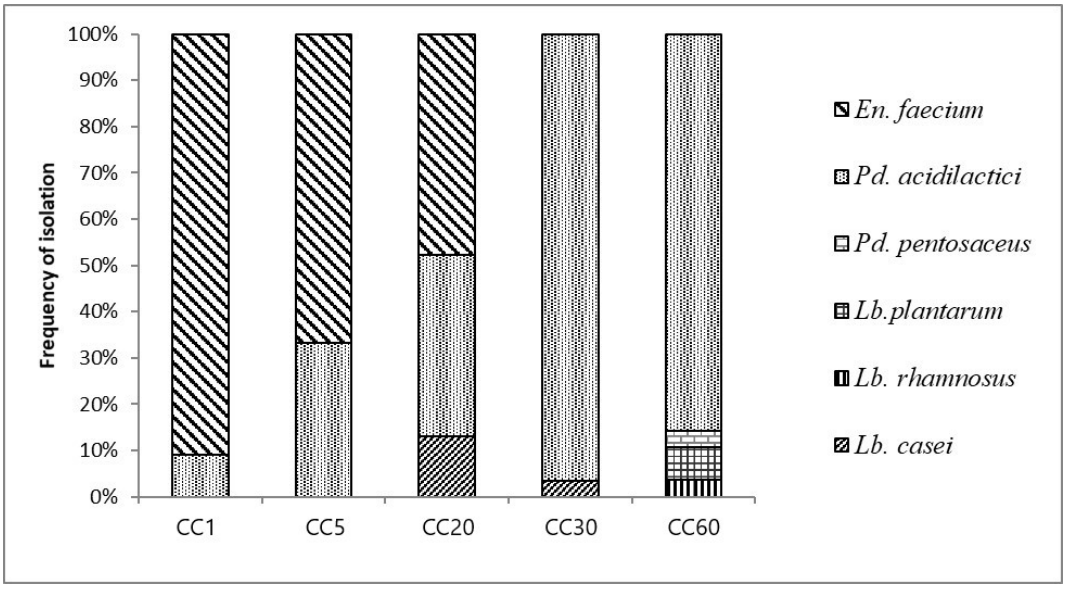

Figure 5. Frequency of isolation of LAB strains after 1 (CC1), 5 (CC5), 20 (CC20), 30 (CC30) and 60 (CC60) days of ripening of Pirot cow`s milk cheese.

At the beginning of ripening, in the sample CC1, two strains of LAB where isolated: *En. faecium* with 91% and *Pd. acidilactici,* 9%. The population of *En. faecium* decreased (66%), while the population of *Pd. acidilactici* (33%) increased in the sample CC5. After 20 days of ripening (CC20) *Pd. acidilactici* was isolated in the frequency of 39%. On the other hand, the presence of *En. faecium* was on the previous level, 47%. In this sample, *Lb. casei* was isolated with 13% of population. To the end of ripening, *Pd. acidilactici* was dominant with 97% in the sample CC30 and 85% in the sample CC60. Also, another representative of

pediococci, *Pd. pentosaceus*, was isolated, with 4% of total population of CC60. Lactobacilli were isolated in low frequency in the samples after 30 days (*Lb. casei* - 3%) and 60 days (*Lb. rhamnosus* - 4% and *Lb. plantarum* - 7%) of ripening.

Generally, enterococci were the most numerous genera in Pirot`s cheese, thus *En. faecium* made 50% of total identified microbiota in ewe`s milk cheese and 44% in cow`s milk cheese. This strain was identified in all phases of ripening in ewe`s milk cheese, and the highest number was observed at the beginning of ripening. In the cow`s milk cheese samples, *En. faecium* dominated in the first stage of ripening, while it was not isolated after 30 days. Enterococci often present the considerable part of LAB microbiota of many traditionally produced types of cheese (FONTECHA *et al*., 1990; COGAN *et al*., 1997; MOREA *et al.*, 1999; DOMINGOS-LOPES *et al.*, 2017; VANDERA *et al*., 2019). The presence of enterococci (especially *En. faecalis* and *En. faecium*) is characteristic for cheese made of ewe`s or goat's milk. As well, these two species have a very important role in lipolysis, proteolysis and production of diacetyl in cheese (GIRAFFA, 2002). The prevalence of enterococci in analysed samples are probably the result of the use of raw milk for the cheese production. The number of enterococci can be up to 60000 times higher in cheese produced from raw milk than from pasteurized milk (PAPPA *et al*., 2019).

Pediococci often take part in autochthonous microbiota of cheese and have a significant impact on ripening of many cheese types (BHOWMIK and MART, 1990). *Pd. acidilactici* was isolated from all analyzed cheese samples. In ewe`s milk cheese the highest percent of isolation was in the sample after 20 days of ripening, while in cow`s milk cheese it was dominant in the final stage of ripening. *Pd. acidilactici* and *Pd. pentocaseus* were identified in cheese produced in the Mediterranean (POZNANSKI *et al.*, 2004; MOREA *et al.*, 2007; AYDEMIR *et al.*, 2015; De PASQUALE *et al.*, 2019), while in ripe Italian cheese Parmigiano Reggiano they are predominant (GOBBETTI *et al.*, 2002). On the other hand, in the analysis of Pirot`s cow`s milk cheese (OSTOJIĆ, 2012), this bacterium was not identified.

The other LAB strains identified in Pirot`s cheese belonged to lactobacilli (*Lb. plantarum, Lb. casei/rhamnosus, Lb. rhamnosus, Lb. casei* and *Lb. paracasei*) and streptococci (*St. macedonicus*). Lactobacilli represent dominant population of many types of cheese where they have a significant effect on formation of aroma compounds (BERESFORD *et al.*, 2001; WOUTERS *et al.*, 2002; De PASQUALE *et al.*, 2019). *Lb. plantarum* was isolated from Italian and Argentinean cheese (ZAGO *et al.*, 2011) and *Lb. fermentum* was isolated from Caciocavallo Pugliese (MOREA *et al*., 2007). *Lb. rhamnosus* was identified in cheese Parmigiano Reggiano (COPPOLA *et al*., 2005; SUCCI *et al.*, 2005; De DEA LINDNER *et al*., 2008; BOVE *et al.*, 2011) and Irish Cheddar cheese (MLALAZI *et al.*, 2011). *Lb. casei* and *Lb. paracasei* were isolated and identified in Spanish cheese Cabrales (BELÉN-FLÓREZ *et al.*, 2006), Turkish cheese Kasar (AYDEMIR *et al*., 2015) and Italian cheese Montasio (MARINO *et al.*, 2003). These lactobacilli can have good probiotic potential and possibility to produce different bacteriocins (MLALAZI *et al.*, 2011; ZAGO *et al.*, 2011; MILIĆEVIĆ *et al*., 2014). The largest diversity of LAB strains in the analysed samples was observed after two months ripening period. High diversity of LAB strains and significant changes in microbiota composition has been reported in various types of pasta filata cheeses as the result of different processing (curd cooking and stretching) and ripening conditions (GOBBETTI *et al*., 2018). Additionally, greater diversity of *Lactobacillus* strains has been noticed in the later stage of ripening, in correlation with the results of SANT`ANNA *et al*. (2019).

In Pirot`s ewe`s milk cheese the population of *St. macedonicus* was also detected. This bacteria was first identified in Greek cheese Kasseri (TSAKALIDOU *et al.*, 1998; GEORGALAKI *et al.*, 2000) and was also isolated from Italian types of cheese from raw

milk: Asiago, Montasio, Monte Veronese, Morlacco, Spressa, Fontina, Ragusano, Mozzarella (LOMBARDI *et al.*, 2004), Nostrano di Primiero (POZNANSKI *et al.*, 2004), Toma piemontese (ZEPPA *et al.*, 2004) as well as from Pirot cow`s milk cheese (OSTOJIĆ *et al.*, 2012). This streptococcus has good acidification, proteolytic and bacteriocin characteristics, thus some authors claim it as multi-functional and very suitable for the production of dairy products (De VUYST and TSAKALIDOU, 2008).
Microbiological profile of LAB population similar to LAB population of Pirot`s cheese was reported for traditionally made types of cheese in the region of the Mediterranean: Caciovallo Molise (COPPOLA *et al.*, 2003), Caciocavallo Pugliese (MOREA *et al.*, 2007), Montasio (MARINO *et al.*, 2003), Toma piemontese (ZEPPA *et al.*, 2004), Nostrano si Primiero (POZNANSKI *et al.*, 2004) and Kasar (AYDEMIR *et al.*, 2015).

4. CONCLUSIONS

"Pirot`s kashkaval" is an autochthonous product traditionally made in Serbia. In order to continue and promote the production of this cheese, is very important to understand the composition and the changes in microbiota which occurs during ripening. Since LAB are being recognized as the most important in cheese ripening, the isolation and identification of LAB from "Pirot`s kashkaval" is of primary importance. LAB microbiota isolated during ripening of "Pirot`s kashkaval" made of cow`s and ewe`s milk was constituted of the genera *Enterococcus, Pediococcus, Lactobacillus* and *Streptococcus*. The most dominant species, in both types of cheese, was *En. faecium*. During the ripening of cow`s milk cheese, the population and diversity of lactobacilli increased. On the other hand, ripening of ewe`s milk cheese was characterized by the increase of pediococci population. Understanding of the microbiological changes which occurred during ripening of "Pirot`s kashkaval" can contribute to the definition of starter cultures suitable for industrial production of "Pirot`s kashkaval".

AKNOLEDGMENTS

This work was performed at the University of Nis, Faculty of Technology in Leskovac.

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PAPER

PHYTOCHEMICAL CHARACTERISTICS AND ANTIOXIDANT ACTIVITY OF SEVERAL FIG (*FICUS CARICA* L.) ECOTYPES

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ABSTRACT

In this study, phenolics and reducing sugar compositions of fig fruits (27 Tunisian ecotypes) were analyzed. In addition, the antioxidant activity was determined by two methods; the ABTS and the DPPH assays. Phytochemical composition of the 27 fig ecotypes was found to be very diverse, as the total polyphenols varied from 51.50 (*'Bouholi'*) to 100.23 (*'Nasri'*) mg gallic acid equivalent/100 g fresh weight. Total flavonoids also varied from 0.33 (*'Bayoudhi1'*) to 17.59 (*'SoltaniAhmar'*) mg quercetin equivalent/100 g fresh weight, and total anthocyanins extended from 1.61 (*'Besbessi'*) to 11.67 (*'Zidi2'*) mg/100 g fresh weight. Additionally, DPPH % inhibition ranged from 11.37 (*'Besbessi'*) to 64.73 % (*'Bouharrag'*) and ABTS from 38.50 (*'Sawoudi5'*) to 676.13 (*'Nemri'*). The ecotypes 'Zergui' and 'Nasri' had the highest contents of glucose (5.68 and 4.83 g/ 100 g FW, respectively) and fructose (5.43 and 4.69 g/ 100 g FW, respectively). The results also showed that fig fruits are a good and valuable source of natural antioxidants that can be used in food and medical sectors.

Keywords: anthocyanins, antioxidant activity, ecotypes, *Ficus carica*, flavonoids, fruits, polyphenols

1. INTRODUCTION

Fig (*Ficus carica* L.), which belongs to the *Moraceae* family, is considered to be one of the oldest cultivated fruit species and an important crop worldwide for both fresh and dry consumption (DUENAS *et al*., 2008; BACHIR BEY and LOUAILECHE, 2015). The world production of figs is about one million tons, and it is mostly concentrated in the Mediterranean area (VEBERIC *et al*., 2008). Tunisia produces about 29 000 tons, which represents 3 % of total world production (FAOSTAT, 2015).
In Tunisia, figs have been grown traditionally for several centuries (ALJANE *et al*., 2018). Local fig ecotypes are numerous and well adapted to the local agro-ecological conditions (ALJANE and FERCHICHI, 2010). Their denominations relate to the fruit color, the period of fruit maturation or to their geographic origin (ALJANE, 2016). Exchange of plant material was frequent between regions of which synonymy and homonymy may be encountered (CHATTI *et al*., 2004; MARS, 2003; ALJANE and FERCHICHI, 2010). Since several decades, the cultivated areas decreased due to the extinction of many ecotypes, the intensive urbanization as well as the biotic and abiotic stresses (MARS *et al*., 1998; MARS, 2003) despite the installation of many new plantations (MARS *et al*., 2008). Whether fresh or dried, figs constitute an important part of the human diet; they are especially rich in fiber, minerals, proteins, sugars, organic acids and antioxidant compounds (ERCISLI *et al*., 2012). Fig fruit is an important source of minerals, vitamins and polyphenols (DUENAS *et al*., 2008; ALJANE and FERCHICHI, 2009; ADILETTA *et al*., 2019). In addition, SOLOMON *et al.* (2006) recorded high polyphenols contents, especially flavonoids and anthocyanins, the highest being their antioxidant activity. The contents of total polyphenols, anthocyanins as well as total antioxidant activity and other properties such as skin color are strongly influenced by the ecotype (SOLOMON *et al*., 2006; VEBERIC *et al*., 2008; CALISKAN and POLAT, 2011; ERCISLI *et al*., 2012). Similarly, several reports have highlighted the influence of fruit variety, harvest season and growing technology in the fields of phenolic contents (TREUTTER, 2010; VALLEJO *et al*., 2012). Moreover, antioxidant activity and phenolic compounds varied considerably depending on the part of the fruit. Indeed, several authors have reported the great contribution of fruit skin (compared to pulp) to these compounds especially in darker varieties (VEBERIC *et al*., 2008; DUENAS *et al*., 2008). The aim of the present work was to study the phytochemical characteristics and sugar composition of 27 fig ecotypes grown in Tunisia.

2. MATERIALS AND METHODS

2.1. Fruit fig material

Ripe Fig fruits from 27 Tunisian fig ecotypes (different fig-growing traditional geographic regions) were harvested in 2015 from the experimental field for germplasm collection of the Institute of Arid Regions (IRA) of Medenine, Tunisia (Table 1). The experimental orchard of 10 years old, included 3 replicates of 5 x 5 m cultivated understandard cultural practices. Within 2 h after harvest, whole fruits were stored at - 20°C for further analysis. Triplicate of 10 frozen fruits samples from each ecotype were homogenized in a blender and used for phytochemical and nutritional analysis.

Table 1. Ecotype's name, types, localities of origin of the studied 27 Tunisian fig fruits.

Ecotype's name	Types	Localities of Origin (Governorate)
Bither1	San Pedro	Ghadhabna (Mahdia)
Jebali1	Smyrna	Islands of Kerkenah (Sfax)
Mahdoui	Smyrna	Islands of Kerkenah (Sfax)
Bayoudhi1	Common	Beni Kheddache (Médenine)
Bayoudhi2	Common	Toujen (Gabès)
Besbessi	San Pedro	MasjedAissa (Sousse)
Bither2	San Pedro	Islands of Kerkenah (Sfax)
Jemâaoui	Smyrna	Beni Kheddache (Médenine)
Rogabi	Smyrna	Beni Kheddache (Médenine)
Gaa Zir	Smyrna	Gafsa (Gafsa)
Temri	Smyrna	Islands of Kerkenah (Sfax)
Zergui	Smyrna	Djébba (Béja)
Baghali2	Smyrna	Ghadhabna (Mahdia)
Baghali3	Smyrna	Islands of Kerkenah (Sfax)
Chetoui Akhal	Common	Ghadhabna (Mahdia)
Croussi	Smyrna	Beni Kheddache (Médenine)
Kahli2	Smyrna	Islands of Kerkenah (Sfax)
Nemri	Smyrna	Djébba (Béja)
Soltani Ahmer	Smyrna	Djébba (Béja)
Wedlani	Smyrna	Beni Kheddache (Médenine)
Bouharrag	Smyrna	Djébba (Béja)
Bouholi	San Pedro	Djébba (Béja)
Kahli1	Smyrna	Ghadhabna (Mahdia)
Nasri	Smyrna	Toujen (Gabès)
Sawoudi3	Smyrna	Bir Amir (Tataouine)
Sawoudi5	Smyrna	Gafsa (Gafsa)
Zidi2	Smyrna	Djébba (Béja)

2.2. Determination of phenolics composition of fig fruits

2.2.1 Methanolic Extraction

A total of 1 g of fruit samples was homogenized in 25 ml of extraction solution and 80% methanol. It was stirred for 2 h in the dark at room temperature. The obtained mixture was centrifuged two sequential times for 15 min at 3500 rpm, and supernatant was filtered and taken for further analysis.

2.2.2 Total Polyphenols (TP)

Total polyphenols (TP) contents of fig fruits were determined spectrophotometrically using the Folin-Ciocalteu method as previously described by SLINGARD and SINGLETON (1977) with some modifications. The absorbance of each sample was measured at 760 nm using a spectrophotometer (Shimadzu 1600-UV, Japan).

Quantifications were calculated using a calibration curve daily prepared with known concentrations of gallic acid standards, and results are expressed as mg gallic acid equivalents (GAE) on fresh weight (FW) basis (mg GAE/100 g FW).

2.2.3 Total anthocyanins (TA)

Total anthocyanins (TA) contents were quantified in accordance with the pH differential method using two buffer systems as previously described by CHENG and BREEN (1991). In brief, methanolic extract were diluted with two buffer solutions of pH 1 and 4.5. Anthocyanins were estimated using absorbance measurement at 530 and 657 nm in buffers at pH 1.0 and 4.5, respectively; where Absorbance (A) was measured using this formula:

$$A = [(A530 – A657)\ pH\ 1.0 - (A530 – A657)\ pH\ 4.5]$$

with a molar extinction coefficient of cyanidin-3-glucosid of 29.600. Total anthocyanin quantities were expressed as mg of cyanidin-3-glucoside equivalents (CGE) per g fresh weight of fig fruit (mg CGE/100 g FW).

2.2.4 Total flavonoïds (TF)

Total flavonoïds were determined using a colorimetric method previously described by KARADENIZ *et al.* (2005). Methanolic extract (1 ml) was added to 5 ml of distilled water and mixed. Then, 5% sodium nitrite solution (0.3 ml) was added, followed by 10% aluminium chloride solution (0.3 ml), mixed and incubated at room temperature for 5 min. After incubation, 2 ml of 1M sodium hydroxide were added to the mixture and thenthe volume of reaction mixture was made up to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance was determined at 510 nm. Flavonoid contents were calculated using a standard calibration curve, prepared from quercetinand expressed as quercetin equivalent in mg per g fresh weight of fruit (mg quercetin/100 g FW).

2.3. Determination of antioxidant properties of fig fruits

The DPPH (1,1 diphenyl 2 pycrilhydrazil (DPPH) radical-scavenging activity of the extract was measured as described by REBAI et al. (2012) and BACHIR BEY et al. (2013). An aliquot (200 μl) of the extract was added to 1 ml of a methanolic DPPH solution (500 μM). The decolorizing process was measured at 517 nm after 30 min of reaction. The scavenging activity percentage of DPPH (%) of the fig extract was calculated using this formula: A = (A blank – A sample)/ (A blank) * 100.
For the standard TEAC (Trolox equivalent antioxidant capacity) assay, ABTS (2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) was dissolved in methanolic solution (14 mM) and prepared with 10 ml ammonium persulfate ($NH_2\ 2S_2O_8$) (4.9 mM) as described by OZGEN *et al.* (2009). The mixture was diluted in methanol to an absorbance of 1.00±0.01 at 734 nm for long stability (OZGEN *et al.*, 2009). For the spectrophotometric assay, 30 μl of fig fruit extract and 2.97 ml of ABTS+ solution were mixed and incubated for 1 h in darkness. The absorbance was determined at 734 nm using a spectrophotometer (SPECORD 210 Plus-Analytik Jena, Japan). The TEAC was expressed as mg equivalent vitamin C (Acid ascorbic) per 100 g fresh weight of fig fruit (mg EVC/100 g FW).

2.4. Determination of reducing sugars of fig fruits

Reducing sugars (glucose and fructose) were determined according to the method described by MELGAREJO *et al.* (2003) and GUNDOGDU *et al.* (2011). Briefly, 10 g fruit was centrifuged at 12000 rpm for 2 min at 4°C, thereafter, the supernatant was filtered and transferred into a vial and used for analysis. Analysis of glucose and fructose was performed by HPLC (KNAUER type) with Eurospher 100 NH_2 column and refractive index detector (RI Detectors K-2301) using 80% acetonitrile as a mobile phase. The calculation of concentrations was based on standards solutions of glucose (2%) and fructose (2%). The results were expressed in g/100 g FW and all the samples were analysed in triplicate.

2.5. Statistical analysis

All analyses were performed with R software (R Core Team, 2019). DPPH inhibition %data were arcsine transformed to meet assumptions of analysis of variance (ANOVA) for homogeneity of variance and normality and are reported in tables as untransformed values. Data were analyzed using one-way analysis of variance (ANOVA) considering them as factor ecotypes or ecotype groups, followed by post-hoc Tukey multiple comparison to determine if differences ($P < 0.05$) between fig ecotypes were significant. Additionally, Pearson's correlation coefficients were also performed based on phytochemical compositions and antioxidant activity of the 27 fig ecotypes.

3. RESULTS AND DISCUSSION

3.1. Fruit skin color

The 27 Tunisian local fig ecotypes revealed great morphological variability in their external fruit color (Fig. 1) and consequently were classified into 6 groups which are: (green yellowish, green, red greenish, brown purplish, purple greenish and purple blackish). Among the studied fruit fig ecotypes, fifteen had variably intense purple skin (eight had purple-greenish and seven purple blackish). Additionally, seven ecotypes showed skin color ranging from green to yellow. The remaining ecotypes: 'Jemâaoui' and 'Rogabi' presented red greenish and 'Gaa Zir', 'Temri' and 'Zergui' were brown purplish (Table 2). Color is one of the most important indicators of maturity and quality of fruits, which is influenced by the concentration and distribution of various anthocyanins (GAO and MAZZA, 1995).

3.2. Fruit phenolic compound contents

The level of phenolic compounds of the 27 Tunisian fig ecotypes are given in Table 2, while the mean values obtained for each skin color group are shown in Fig. 2. The one-way ANOVA analysis followed by post-hoc Tukey multiple comparison test of total polyphenols, total anthocyanins and total flavonoids showed highly significant differences ($p < 0.001$) among the 27 fig ecotypes. When we applied ANOVA analysis to the six skin color groups, the total anthocyanins showed highly significant differences ($p<0.001$),

whereas the total flavonoids were only significant ($p<0.05$). Unlike these compounds, the total polyphenols revealed no significant differences among the six groups.

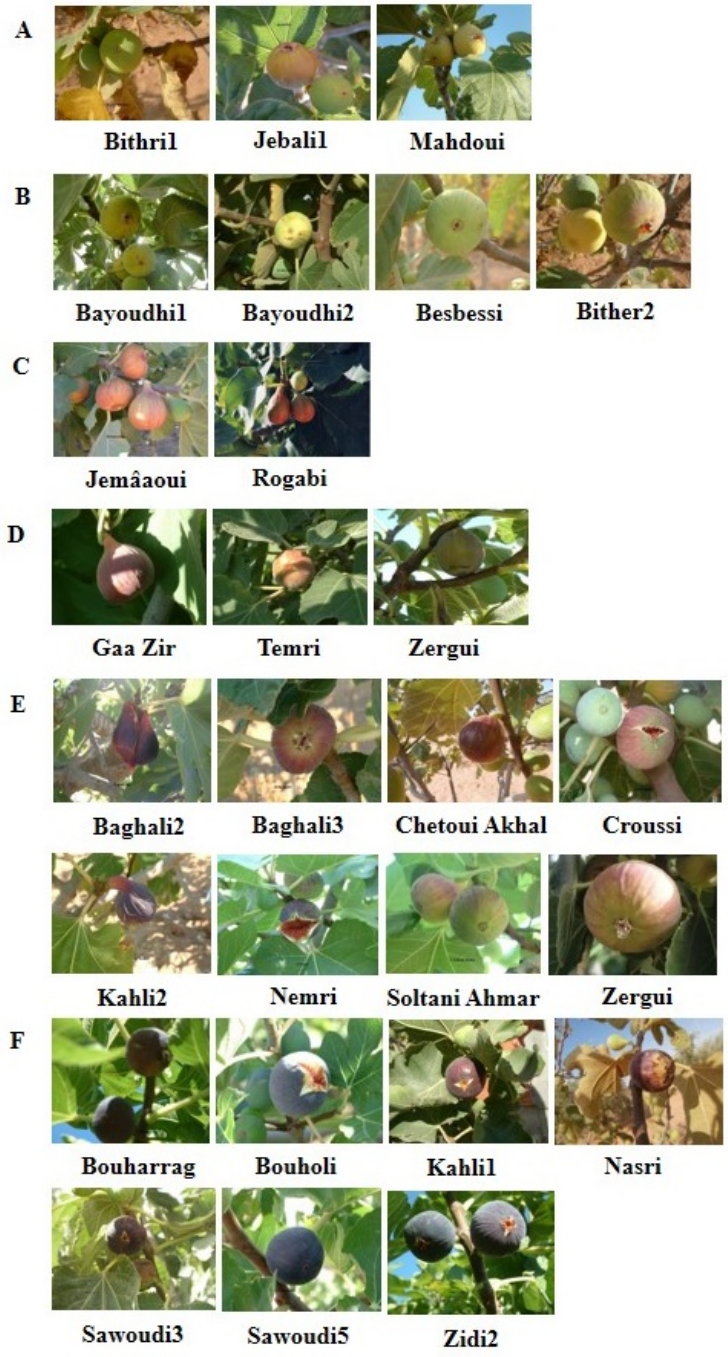


Figure 1. Morphological variability in external fruit color of the 27 studied fig ecotypes (A: Green yellowish, B: Green, C: Red greenish, D: Brown purplish, E: Purple greenish and F: Purple blackish).

3.2.1 Total polyphenols

The total polyphenols (TP) have been reported to be the main phytochemical responsible for the antioxidant activity of figs. The TP contents of fig ecotypes varied from 51.50 ('Bouholi') to 100.22 ('Nasri') mg GAE/ 100 g FW. The highest TP levels were observed, in descending order, in the following ecotypes ('Nasri', 'Bayoudhi2', 'Zidi2', Baghali3', 'Rogabi', Sawoudi5') (Table 2). The results of the total polyphenols contents are higher than those obtained in previous studies conducted by ALJANE and SDIRI (2014). Nevertheless, thesecontents are inferior to those found by VALLEJO et *al.* (2012) and CAPANOGLU (2014), who reported concentrations of 331.93 and 169.4 mg GAE/ 100 g FW in Indian and Turkish figs, respectively, but are comparable to the results of PIGA *et al.* (2008). On the contrary SOLOMON *et al.* (2006), CALISKAN and POLAT (2011) and DEBIB *et al.* (2014) showed that the dark fig fruits contain higher total polyphenols than the light ones. We did not obtain significant differences in total polyphenols based on fruit skin color groups (Fig. 2). This discrepancy might be explained by the fact that total polyphenols contents are greatly influenced by various parameters such as weather conditions, ripening stage, degree of fruit maturation, and postharvest storage conditions (VALLEJO *et al*., 2012; BACHIR BEY and LOUAILECHE, 2015).

3.2.2. Total anthocyanins

The total anthocyanins (TA) are natural pigments belonging to the flavonoid family and are responsible for the red, blue and purple color of many fruits. The total anthocyanins amounts of the studied fig ecotypes varied from 2.57 ('Baghali3') to 11.67 ('Zidi2') mg CGE/100 g FW (Table 2). 'Zidi2' ecotypes had the highest contents (11.67) followed by 'Sawoudi3' (9.7) and then 'Bouholi' (8.17). It is apparent that purple blackish ecotypes contain more anthocyanins, with average value of 7.11 mg CGE/ 100 g FW. The other fruit ecotypes varied within 3.17 in green-yellowish fruit skin color group to 5.08 mg CGE/100 g FW in red greenish (Fig. 2). These levels are similar to those obtained in our previous study on Tunisian fig varieties, where we found TA to be between 0.55 and 9.16 mg CGE/100 g FW (ALJANE and SDIRI, 2014). SOLOMON *et al.* (2006) reported that the dark fig 'Mission' variety has eight times higher total anthocyanins (10.9 mg CGE/100 g FW) than the red-brown Turkey one (1.3 mg CGE/ 100 g FW), while these compounds were not detected in 'Brunswick' and 'Kadota' ecotypes, which have light fruit skin color. The TA content of the majority purple-blackish ecotypes is higher than that found by OUCHEMOUKH *et al.* (2012) in black figs (5.9 mg CGE/ 100 g FW). In addition, the total anthocyanins content of our samples was lower than that of other studies on commercial fig ecotypes (DEL CARO and PIGA, 2007; PIGA *et al*., 2008; DUENAS *et al*., 2008; ERCISLI *et al*., 2012). The results showed that total anthocyanins (TA) contents were strongly influenced by fruit skin color. Indeed, the purple blackish fig ecotypes ('Zidi2', 'Sawoudi3' and 'Bouholi') had the highest contents and might be used as good sources of anthocyanins. Such result is in good agreement with those advanced by SOLOMON *et al.* (2006), who reported a large contribution of fig fruit skin to the total anthocyanins accumulation.

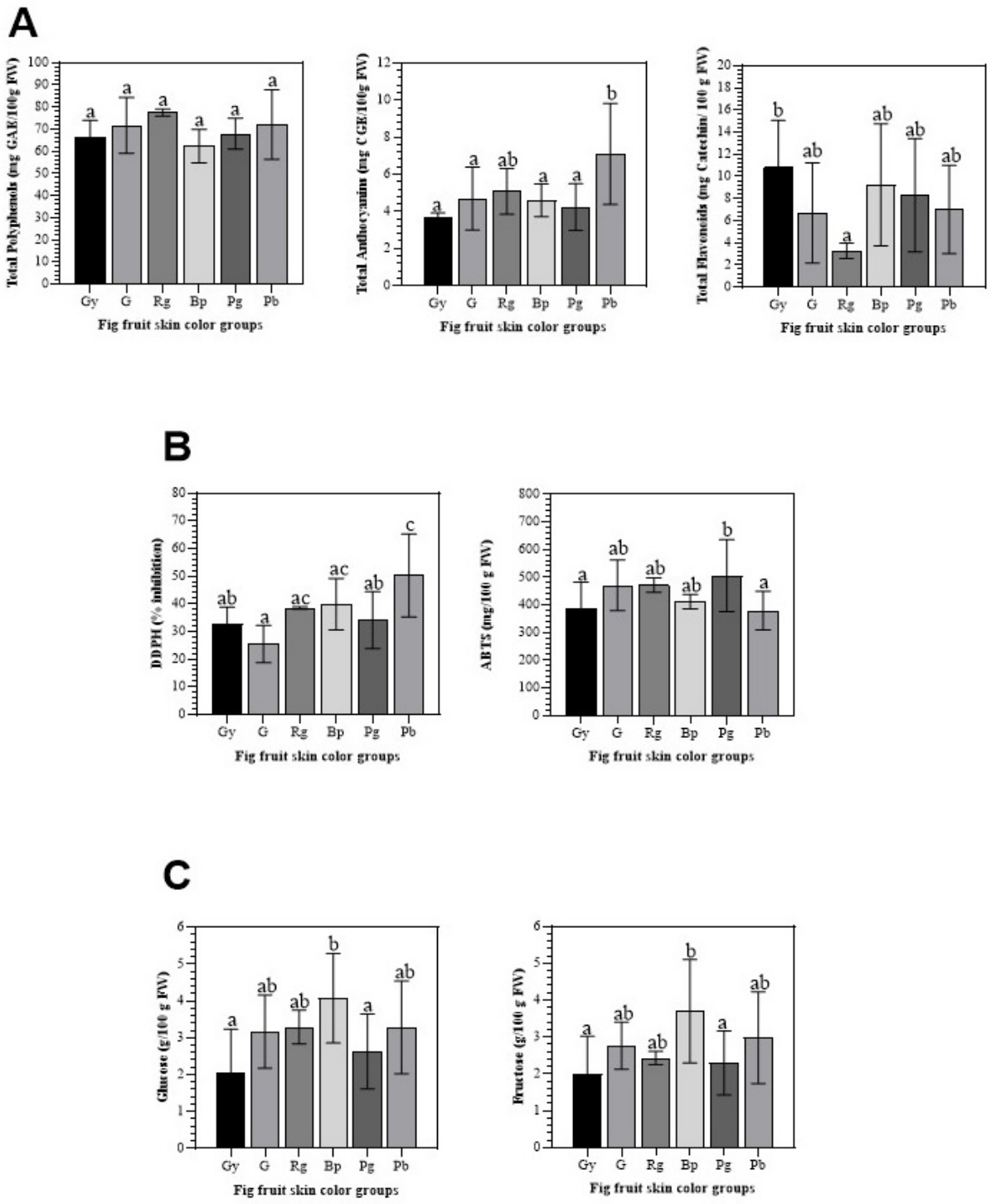
Table 2. Total Polyphenols, total anthocyanins and total flavonoids of 27 Tunisian fig ecotypes.

Ecotype's name	Total polyphenols mg GAE/ 100 g FW	Total anthocyanins mg CGE/ 100 g FW	Total flavonoids mg QE/ 100 g FW	Fruit skin color group
Bither1	60.50±0.74 ef	3.75±0.19 ade	5.68±0.30 ef	
Jebali1	76.47±0.10 lm	3.43±0.40 ad	11.50±0.90 i	Green yellowish
Mahdoui	63.21±0.31 g	3.73±0.12 ade	15.26±0.9 j	
Bayoudhi1	76.62±0.05 lm	3.00±0.1 ab	0.33±0.11a	
Bayoudhi2	88.45±0.47 p	5.61±0.10 ghi	5.68±0.30 ef	Green
Besbessi	56.29±0.76 bc	3.33±0.27 ac	12.16±0.30 i	
Bither2	65.53±1.45 h	6.80±0.82 ij	8.59±0.3 h	
Jemâaoui	76.15±0.24 lm	6.20±0.1 hj	2.77±0.68 bc	Red greenish
Rogabi	79.03±0.15 no	3.96±0.24 bcdf	3.76±0.19 cd	
Gaa Zir	71.75±0.07 ij	4.54±0.38 cdfg	5.42±0.11 def	
Temri	60.61±0.03 ef	3.67±0.47 ade	5.68±0.3 ef	Brown purplish
Zergui	54.60±1.36 b	5.57±0.08 ghi	16.57±0.14 jk	
Baghali2	69.93±0.10 i	3.75±0.08 ade	6.14±0.9 fg	
Baghali3	79.42±0.61 no	2.57±0.04 a	4.36±0.36 ce	
Chetoui Akhal	73.35±0.59 jk	7.03±0.72 jk	12.29±0.19 i	
Croussi	74.57±0.52 kl	4.28±0.24 cdf	5.68±0.41 ef	Purple greenish
Kahli2	62.33±0.09 fg	4.21±0.08 bcdf	12.75±0.30 i	
Nemri	59.34±0.56 de	3.78±0.34 ade	5.76±0.82 eg	
Soltani Ahmer	61.59±0.66 eg	4.67±0.12 dfg	17.59±0.14 k	
Wedlani	63.56±0.3 gh	3.55±0.10 c	1.78±0.19 ab	
Bouharrag	58.20±1.58 cd	5.12±0.24 fh	7.47±0.24 gh	
Bouholi	51.50±1.49 a	8.17±0.90 i	1.78±0.90 ab	
Kahli1	61.29±0.62 eg	6.21±0.06 hj	11.70±0.07i	
Nasri	100.22±0.38 q	3.95±0.94 bcdf	1.85±0.94 ab	Purple blackish
Sawoudi3	75.44±0.41 km	9.70±0.47 l	8.99±0.48 h	
Sawoudi5	77.37±0.44 mn	4.90±0.25 efg	11.70±0.26 i	
Zidi2	81.25±0.99 o	11.67±0.15 m	5.62±0.16 ef	
Total mean	69.58±11.14	4.08±2.11	7.73±4.72	
F value	731.6	85.1	222.1	
P value	***	***	***	

'***' 0.001 '.Values in the same column with different lower- case letters are significantly different at P<0.05 according to post-hoc Tukey multiple comparison, GAE: Gallic acid equivalent, CGE: cyanidin-3-glucoside equivalent, QE: quercetin equivalent, FW: Fresh weight.

3.2.3 Total flavonoids

The purple-greenish ecotype 'Soltani Ahmar' had the highest contents (17.59 mg QE/100 g FW) followed by 'Zergui' from the brown purplish group (16.57 mg QE/100 g FW) and 'Mahdoui' from the green-yellowish with an amount of 15.26 mg QE/100 g FW. Whereas, the lowest contents were observed in the following ecotypes ('Bayoudhi1', 'Bouholi', 'Wedlani', 'Nasri', 'Jemâaoui' and 'Rogabi') (Table 2).



Gy: Green yellowish, G: Green, Rg: Red greenish, Bp: Brown purplish, Pg: Purple greenish, Pb: Purple blackish

Figure 2. Total phenolic content (A): total polyphenols, total anthocyanins, total flavonoids, antioxidant capacity (B): DPPH: 1.1 Diphenyl 2 pycril hydrazil, ABTS: acid 2.2-azino-bis-3 ethylbenzothiazoline-6-sulfonique and sugar compositions (C): Glucose and Fructose of 6 fig fruit skin color groups. Different letters indicate significant differences by post-hoc Tukey multiple comparison at $p< 0.05$.

The obtained values of total flavonoids are lower than those found by BACHIR BEY and LOUAILECHE (2015) who have advanced contents of 87.24 and 126.55 mg/100 g FW for Algerian light and dark varieties, respectively. The green yellowish group, which is light figs, has the highest total flavonoid contents, followed by green, brown purplish, purple greenish and purple blackish groups (Fig. 2). Such result is quite different from those reported by SOLOMON et *al.* (2006) and VALLEJO et *al.* (2012) who found that the total flavonoids contents of dark-purple fig varieties were greater than those of light ones.

3.3. Antioxidant activities

The antioxidant activities of the 27 Tunisian fig ecotypes are summarized in Table 3. The one -way ANOVA analysis of ABTS and DDPH followed by post-hoc Tukey multiple comparison test indicated highly significant differences among the 27 fig ecotypes and also between the six groups.

3.3.1 DPPH radical-scavenging activity

Data of the scavenging activity against DDPH indicated that the best antiradical effect was achieved by the 'Bouharrag' ecotypes (64.73%), whereas, 'Besbessi' had the least activity (14.59%) (Table 3). The results clearly revealed a stronger DPPH scavenging activity in purple blackish ecotypes compared to green ones, with average values of 50.25% and 26.95%, respectively (Fig. 2). These results are in accordance with those obtained by BACHIR BEY and LOUAILECHE (2015), who reported a DDPH radical scavenging activity varying from 28.33% to 45.25% in 'Taghanimt' and 'Bouankik' varieties, respectively. The study of DDPH scavenging activity of Algerian fig varieties clearly showed that dark varieties have stronger DDPH scavenging activities than the light one, with mean values of 41.63 and 31.3%, respectively (BACHIR BEY AND LOUAILECHE, 2015).

3.3.2 ABTS radical cation scavenging activity

The results of the scavenging activity of ABTS radical ranged from 'Mahdoui' (263.7 EVC mg/100 g FW) to 'Nemri' (676.13 EVC mg/100 g FW). It is apparent that antioxidant activity (ABTS) was lower in green yellowish and purple-blackish groups, whereas, the purple greenish showed the highest value (Fig. 2). The current results are comparable to the data obtained by SOLOMON *et al.* (2006), who indicated that dark fig varieties had high ABTS antioxidant capacities.

3.4. Reducing Sugars compositions

The analyses of variance for glucose (GLUC) and fructose (FRUC) revealed significant differences among the 27 studied ecotypes and within the fruit skin color groups. The ecotypes 'Zergui' and 'Nasri' had the highest contents of glucose (5.68 and 4.83 g/100 g FW, respectively) and fructose (5.43 and 4.69 g/100 g FW) values. Nevertheless, GLUC and FRUC were very low for the 'Mahdoui' ecotype (1.12 and 0.86 g/100 g FW, respectively) (Table 3). These results were lower than those obtained by MELGAREJO *et al.* (2003), as the glucose contents of 'Tio Antonio' and 'Calar' variety were 15.89 and 13.41 g/100 g FW, respectively. Similarly, CALISKAN and POLAT (2012) reported that GLUC and FRUC contents obtained in 'Sarilop' variety were 10.7 and 7.8 mg 100/ g FW,

respectively. The sugar composition of figs, especially fructose, can influence perceived fruit sweetness (SETSER, 1993).

Table 3. Effects of genotype on antioxidant activity (DPPH and ABTS) and sugar compositions for 27 Tunisian fig ecotypes.

Ecotype name	DPPH inhibition %	ABTS mg EVC/ 100 g FW	GLUC g/ 100 gFW	FRUC g/ 100 gFW	Fruit skin color group
Bither1	40.74±0.65 l	412.96±6.60 def	1.79±0.29 ab	1.96±0.21 acd	Green yellowish
Jebali1	28.99±0.99 f	480.26±26.04 hi	3.30±0.90 bde	3.16±0.42 cef	
Mahdoui	28.63±0.54 f	263.70±9.31 a	1.12±0.88 a	0.86±0.12 a	
Bayoudhi1	30.38±0.53 g	496.76±5.68 ij	4.66±0.10 ef	3.52±0.09 eg	Green
Bayoudhi2	26.55±0.50 e	376.40±5.55 cd	2.52±0.30 ad	2.47±0.10 bce	
Besbessi	14.59±0.52 b	407.96±3.61 de	3.20±0.31 bde	2.89±0.28 bcef	
Bither2	30.37±0.54 g	601.13±1.02 k	2.30±0.29 ad	2.18±0.81 ae	
Jemâaoui	38.49±0.50 j	448.60±10.28 fgh	3.37±0.67bde	2.45±0.11bce	Red greenish
Rogabi	38.49±0.50 j	493.76±6.26 ij	3.21±0.23 bde	2.41±0.26 bce	
Gaa Zir	45.49±0.50 m	384.06±5.47 cd	3.32±0.11 bde	3.34±0.31 cef	Brown purplish
Temri	27.42±0.51 e	441.56±7.76 eg	3.32±0.30 bef	2.32±0.45 bce	
Zergui	46.61±0.53 n	409.36±10.96 de	5.68±0.16 f	5.43±0.10 h	
Baghali2	29.47±0.50 fg	378.73±1.55 cd	2.00±0.90 abc	1.96±0.10 acd	Purple greenish
Baghali 3	26.48±0.50 e	658.96±10.15 l	2.57±0.90 ad	2.39±0.08 bce	
Chetoui Akhal	15.46±0.50 b	465.16±4.19 gi	2.56±0.30 ad	1.98±0.80 ade	
Croussi	35.54±0.50 i	575.86±3.58 k	2.52±0.40 ad	2.32±0.30 acd	
Kahli2	45.30±0.60 m	275.60±39.57 a	2.26±0.30 ad	2.15±0.10 ae	
Nemri	41.05±1.07 l	676.13±13.85 l	4.33±0.12 ef	3.96±0.35 fg	
Soltani Ahmer	48.07±1.00 o	490.43±10.50 ij	1.73±0.18 ab	1.56±0.11 ab	
Wedlani	31.52±0.50 h	519.70±1.47 j	3.63±0.20 cde	2.90±0.12 bcef	
Bouharrag	39.63±0.65 k	347.16±4.07 bc	3.58±1.05 cde	3.29±0.95 deg	Purple blackish
Bouholi	64.73±0.55 s	264.70±7.59 a	3.19±0.22 bde	3.05±0.95 cef	
Kahli1	19.62±0.54 d	407.80±5.63 de	2.25±0.90 ad	2.19±0.10 ae	
Nasri	56.55±0.51 q	465.16±4.19 gi	4.83±0.12 ef	4.69±0.95 ghe	
Sawoudi3	52.42±0.52 p	462.83±7.00 gi	3.12±0.30 ade	2.52±0.45 bcef	
Sawoudi5	62.45±0.51 r	383.50±15.05 cd	2.24±0.90 ad	1.82±0.25 ac	
Zidi2	56.37±0.54 q	322.20±1.92 b	3.84±0.30 de	3.22±0.17 cef	
Total mean	36.62±13.42	441.13±105.74	3.00±1.15	2.63±1.04	
F value	1454	763.1	10.47	13.47	
P value	***	***	***	***	

0 '***' 0.001. Values in the column with different lower-case letters are significantly different at $p< 0.05$ according to post-hoc Tukey multiple comparison. DPPH: 1.1 Diphényl 2 PycrilHydrazil. ABTS: acide 2.2-azino-bis-3-ethylbenzothiazoline-6-sulfonique, EVC: equivalent vitamin C, GLUC: Glucose, FRUC: Fructose, FW: Fresh weight.

It is more likely that the GLUC and FRUC contents depended on fruit skin color (Fig. 2). Similarly, CALISKAN and POLAT (2012) observed that fig genotypes with green or brown fruit skin color had higher GLUC and FRUC than the genotypes with black skin fruit. ABIDI *et al.* (2011) and CALISKAN and POLAT (2011) have also mentioned that several parameters like: climate variables, cultural practices and harvest time could introduce variability among sugar compositions of fig fruits.

3.5. Correlations between phytochemical and antioxidant activities parameters

Obtained results revealed the existence of a significant positive correlation between GLUC and FRUC (r = 0.889). Similar results between fructose and sucrose contents in fig fruits havealso been reported by CALISKAN and POLAT (2011; 2012). In addition, we detected slightly positive correlations between GLUC and DPPH (r = 0.374) and between TA and DDPH antioxidant activity (r = 0.292). The later correlation was not significant as reported by BACHIR BEY and LOUAILECHE (2015) and SOLOMON *et al*. (2006), who recorded a high correlation (r=0.91).It is also worthy to mention a slightly negative correlation between TP and TF, with value of r=-0.370 (Table 4).

Table 4. Pearson's linear correlation coefficients between total polyphenols (TP), total anthocyanins (TA), total flavonoids (TF), antioxidant capacity (DPPH and ABTS) and sugar composition (GLUC and FRUC) in fig fruits (n =30).

TF	-0.370					
TA	0.053	0.037				
DPPH	0.144	-0.038	0.292			
ABTS	0.187	-0.225	-0.294	-0.213		
GLUC	0.185	-0.171	0.012	0.374	0.104	
FRUC	0.082	-0.236	-0.118	0.284	0.154	0.889*
Parameters	TP	TF	TA	DPPH	ABTS	GLUC

DPPH: 1.1 Diphényl 2 PycrilHydrazil, ABTS: acide 2.2-azino-bis-3-ethylbenzothiazoline-6-sulfonique; *, P<0.05.

4. CONCLUSIONS

Since all fig trees were grown under the same environmental and edaphic conditions and subjected to uniform cultural practices (irrigation, fertilization, pruning), the observed differences in the phytochemical composition, antioxidant activity and sugar contents on fig fruits are largely dependent on the biochemical characteristic of each ecotype and to a lesser extent on the ripening stage and postharvest storage conditions. Our results revealed a considerable variation in the phytochemical, antioxidant activity and sugar compositions were observed in the 27 Tunisian fig ecotypes. The ecotypes with purple-blackish skin 'Bouholi', 'Sawoudi3' and 'Zidi2' had the highest contents of TA. Skin color had a highly significant effect on total anthocyanins and was the major tissue that contributed to anthocyanin compositions in figs fruits. Among all studied ecotypes, 'Nasri' showed the highest amount of TP. In addition, 'Bouholi' ecotype presented the highest antioxidant activity of DDPH and 'Nemri', 'Baghali3' and 'Bither2' ecotypes

showed the highest ABTS radical scavenging activity. Regarding the sugar contents, the ecotypes with higher values of GLUC and FRUC were 'Zergui' and 'Nasri', respectively. Due to high contents of bioactive substances and antioxidant activities, figs (particularly dark varieties) are an interesting alternative for antioxidant additives that could be used in pharmaceutical and food industry.

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PAPER

LARVAL STAGES OF *SULCASCARIS SULCATA* (NEMATODA: ANISAKIDAE) IN SCALLOPS FROM THE NORTHERN ADRIATIC SEA: IMPLICATIONS FOR SEAFOOD CONTROL AND SURVEILLANCE

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ABSTRACT

A survey of fresh great Mediterranean scallops (*Pecten jacobaeus*) and frozen queen scallops (*Aequipecten opercularis*) collected from the northern Adriatic Sea demonstrated the presence of nematode larvae that were morphologically and molecularly identified as *Sulcascaris sulcata*. The presence of this nematode in the northern Adriatic Sea has been described in the loggerhead sea turtle, which is the final host, but it was only recently described in the intermediate molluscan host. Affected scallops present rust-brown lesions in the adductor muscle. In this study, necroscopic and histopathological lesions are described in affected scallops. Furthermore, the detrimental effects of this parasite on the commercialization of scallops under the current European legislation concerning the hygiene of seafood products are evaluated.

Keywords: emerging parasites, food legislation, nematode, RFLP, scallop, *Sulcascaris sulcata*

1. INTRODUCTION

Sulcascaris sulcata (RUDOLPHI, 1819) is a nematode parasite of sea turtles that belongs to the Anisakidae family. This nematode was first described by COBB (1930) in *Pecten* spp. in North Carolina and was named *Paranisakis pectinis*. The adult stage of *S. sulcata* has been recorded in loggerhead sea turtles (*Caretta caretta*) and green sea turtles (*Chelonia mydas*) in the Mediterranean and Caribbean Seas and in the South Atlantic, western Atlantic, and western Pacific oceans (LICHTENFELS *et al*., 1978; LESTER *et al*., 1980; BERRY and CANNON, 1981; WERNEK *et al*., 2008; SANTORO *et al*., 2010). In the western Atlantic, *S. sulcata* has also been recorded in Kemp's Ridley turtles (*Lepidochelys kempii*) (GREINER, 2013). Since 1998, *S. sulcata* infection has been extensively investigated in *C. caretta* from the Adriatic Sea (MANFREDI *et al*., 1998; GRAČAN *et al*., 2012; SANTORO *et al*., 2019; MARANGI *et al*., 2020).

Intermediate hosts for *S. sulcata* have been identified from natural or experimental infections in several mollusc species, including both bivalves and gastropods, as reviewed in SANTORO *et al.* (2020). The occurrence of larval stages of *S. sulcata* in molluscs from the Mediterranean basin has been only recently described in *Mytilus galloprovincialis* from the Tyrrhenian Sea (SANTORO *et al*., 2020) and in two pectinidae scallops, *Pecten jacobaeus* and *Aequipecten opercularis,* from the northern Adriatic Sea (MARCER *et al*., 2020).

Adults of *S. sulcata* reside in the gastric lumen of sea turtles, which become infected by consuming parasitized molluscs (BERRY and CANNON, 1981). As adult nematodes undergo sexual reproduction, eggs are released into the water column in the faeces (BERRY and CANNON, 1981; DEARDOFF, 1989). Nematode larvae progress through the first two stages of development (L1 and L2) in the water column/benthos and enter marine molluscs through inhalation in the siphon, where L3 larvae grow and moult into the L4 stage (BERRY and CANNON, 1981; DEARDOFF, 1989), appearing as tubular worms coiled within a sheath. These larvae encapsulate in the adductor muscle, which exhibits brown, rust or yellow-coloured lesions or cysts with an elongated shape (LICHTENFELS *et al*., 1978; BERRY and CANNON, 1981; DEARDOFF, 1989).

From a food hygiene perspective, the colour of the L4 larvae can be whitish and difficult to identify in the adductor muscle, although its presence is easily detectable because of the rust–brown lesions created by the encysted nematode. This pigmentation has been associated with the bivalve's response to the excretory/secretory liquid produced by the worm (DEARDOFF, 1989).

While the adductor muscle itself shows no signs of quality loss, its aesthetics may cause an economic problem in the scallop market, which has been involved in raw seafood consumption, a trend that has spread in the last several years. Traditionally cooked molluscs, such as pectinid, are now increasingly consumed raw. Therefore, the gross lesions caused by *S. sulcata* in edible molluscs may be perceived as unacceptable quality defects.

The aim of this study is to describe the macroscopic and histological appearance of the lesions caused by *S. sulcata* in the intermediate hosts *P. jacobaeus* and *A. opercularis* collected in the northern Adriatic Sea. Implications regarding the commercialization of scallops under the enforcement of European legislation on seafood safety are also considered.

2. MATERIALS AND METHODS

2.1. Sampling and necroscopic observation

A total of 54 wild-caught great Mediterranean scallops (*P. jacobaeus*) and 10 frozen queen scallops (*A. opercularis*) from the northern Adriatic (FAO zone 37.02.01) were collected between 2017 and 2019 by the Public Health Authority (Local Veterinary Services, Venice) at the wholesale fish market and were submitted to the IZSVe laboratory. Using a dissecting microscope, researchers evaluated the specimens for the presence of rust-brown lesions and *S. sulcata* larvae. All organs were observed with a focus on the adductor muscle and were dissected with parallel cuts to evaluate deep lesions in the muscular mass. Dissected organs (visceral mass, gonad, adductor muscle, and mantle) were artificially digested in a sodium chloride-pepsin solution (0,85% sodium chloride with pepsin added to a concentration of 10 mg/L) according to JACKSON *et al*. (1981). The pH of the solution was adjusted by adding HCl 6N, obtaining a final value of pH 2.0, before incubation using a magnetic stirrer at 37°C for 24 h.

2.2. Morphological examination and viability of the larvae

Larval length and morphology were assessed with a Leitz Diaplan light microscope (Leica, UK) and compared with the identification keys proposed by BERRY and CANNON (1981). Digital images and measurements were obtained using an integrated LEICA MC170HD (Leica, UK) camera and LAS 4.5.0 (Leica, UK) software. The viability test was performed by transferring the larvae in a Petri dish with saline solution at room temperature (20°C) for 2 h to verify any spontaneous movement and reaction to tactile stimulation.

2.3. Biomolecular analysis

DNA extraction was performed on 20 whole larvae using the QIAamp® DNA Mini Kit according to the manufacturer's instructions. The amplification of the entire rDNA fragment comprising ITS1, 5.8S, and ITS2 was performed with the forward primer NC5 (5′ -GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and the reverse primer NC2 (5′ -TTA GTT TCT TTT CCT CCG CT-3′) (ZHU *et al*., 2001) following the protocol of MARCER *et al.* (2020). PCR amplicons (~1050 bp) were digested with 2 restriction enzymes, *HhaI* and *HinfI,* as described by D'Amelio *et al.* (2000). DNA restriction fragments were evaluated by electrophoresis on a 2% w/V agarose gel stained with ethidium bromide at 4,5/6 V/cm for 40 min. Another four Anisakidae species (*Anisakis physeteris*, *A. pegreffii*, *A. sensu stricto,* and *Pseudoterranova* spp.) and a Raphidascarididae (*Hysterothylacium aduncum*) were included in the restriction fragment length polymorphism (RFLP) analysis for comparison. Subsequently, amplification of the *cox2* mtDNA gene (measuring 609 bp) was performed with the primers 211F (5′ -TTT TCT AGT TAT ATA GAT TGR TTY AT-3′) and 210R (5′ -CAC CAA CTC TTA AAA TTA TC-3′) (GARBIN *et al*., 2011; MARCER *et al*., 2020); the amplicons were sequenced and compared with the GenBank database using BLAST software. For further details, see the work of MARCER *et al*. (2020).

2.4. Histological examination

Portions of the adductor muscle that contained encysted larvae were fixed in Carson's fixative for 24 h, dehydrated and embedded in Paraplast® applying standard histological protocols (HOWARD *et al*., 2004). Sections measuring 3 μm were stained with Harris's haematoxylin and eosin-floxin. Slides were observed with a Leitz Diaplan microscope at 40-1000X magnification.

3. RESULTS AND DISCUSSION

3.1. Necroscopic observation

Upon visual examination, affected scallops presented rust-brown to orange/brown singular or multifocal areas with elongated shapes (measuring 2-7 mm in length and 1-3 mm in width) on the surface of the adductor muscle. Nearly all lesions were observed along the exterior edge of the adductor muscle (Fig. 1A) on the opposite side of the digestive gland. Not all the lesions that were observed contained encysted nematodes.
Nematode larvae were observed in 35 out of 54 *P. jacobaeus* with an overall number of 277 larvae, while in *A. opercularis,* only 1 specimen out of 10 was infested by two nematodes. The number of parasites recorded for each specimen ranged between 1 and 10 nematodes.
The artificial digestion method, which was performed after carefully checking the scallops by visual examination, did not indicate the presence of additional larvae.
The presence of fewer nematode larvae compared with the number of brownish lesions has been explained by the relocation and re-encystment of the larvae in the muscle as their growth progresses (RUDDERS *et al*., 2019).

3.2. Morphological examination and viability of the larvae

The morphology of the larvae and their dimensions (9-21 mm in length, 0.5 mm in width) were consistent with *S. sulcata* specimens in the L4 larval stage (BERRY and CANNON, 1981). Larvae showed a well-differentiated oesophageal ventriculum that was characterized by shrinkage of the proximal junction with the oesophagus, absence of lateral diverticula and a rounded flask-like distal fund (Fig. 1D); in some specimens, cranial development of an intestinal caecum was visible as a rudimental sac lateral to the ventriculum.
The lack of ventricular appendix and noticeable intestinal caecum differentiate *S. sulcata* from the genera *Hysterothylacium, Contracaecum, Phocascaris and Pseudoterranova,* while its ventriculum morphology differentiates it from the genus *Anisakis* (Fig. 1E). All the nematodes evaluated were viable with the exception of 2 larvae in frozen *A. opercularis*.

3.3. Biomolecular analysis

Biomolecular analyses confirmed the species identification of *S. sulcata*. Consensus sequences of the *cox2* gene displayed 100% nucleotide similarity to the *S. sulcata* sequence (HQ328505) available in the GenBank database; see also MARCER *et al.* (2020).

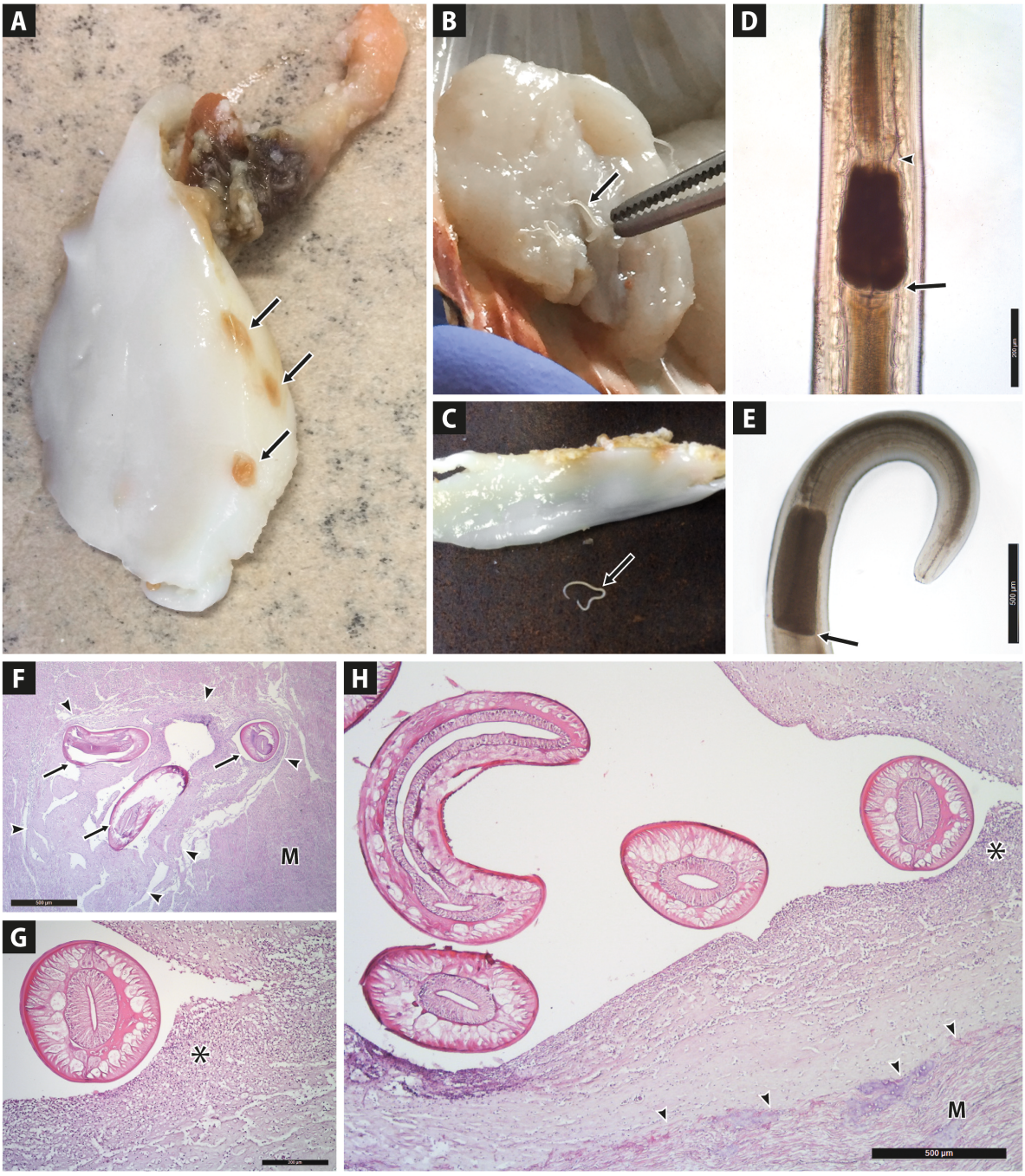

Figure 1. A) Cross-section of the adductor muscle of *Pecten jacobaeus*, showing on its surface rust-brown spots (arrows) containing encysted nematodes; B and C) cream-white *Sulcascaris sulcata* larvae (arrows); D) microscopic observation of the oesophageal ventriculum of *S. sulcata* larvae, shrinkage of the proximal junction with the oesophagus (arrowhead), rounded flask-like distal end of the ventriculum (arrow); E) comparison with the ventriculum of *Anisakis simplex* showing blunt distal fund (arrow); F) histological observation of encysted and coiled *S. sulcata* (arrows) with the external margin of the connective sheath highlighted (arrowheads), normal adductor muscle (M)(4X); G) higher magnification of a cross-section of the nematode, showing haemocytic infiltration (*) (25X); H) connective sheath (encapsulating *S. sulcata*, haemocytic infiltration (*) is present in the inner layer, deposition of small pigmented granules is observed at the periphery of the connective capsule (arrowheads), normal musculature (M) (10X).

All 20 restriction profiles obtained with RFLP were analogous. Restriction with *HhaI* produced three fragments measuring approximately 100 bp, 250 bp and 400 bp; conversely, restriction with *HinfI* produced only one fragment of 500 bp. The restriction profile of *S. sulcata* appeared different from those of the other evaluated *Anisakidae* species (Fig. 2). RFLP results were also compared with the restriction profiles of other species: *Anisakis simplex/pegreffii, A. paggiae, A. typica, A. brevispisculata, A. ziphidarum, A. sp.* A and *Contracaecum rudolphii* according to D'AMELIO *et al.* (1999) and ZHU *et al*. (2000). The profile of *S. sulcata* did not match any of these species.

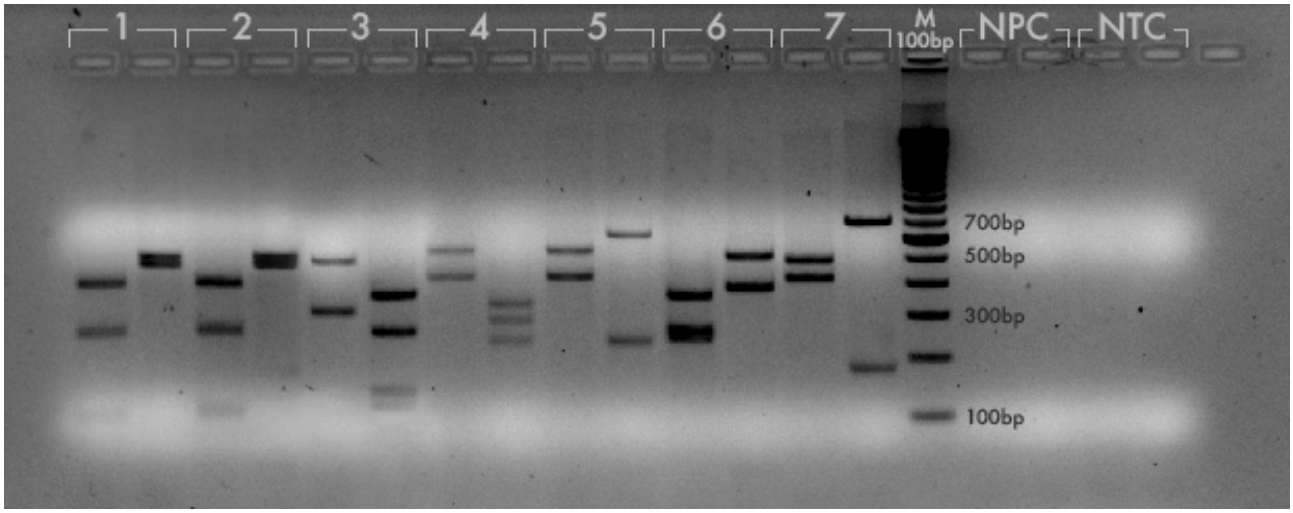

Figure 2. Restriction fragment length polymorphism (RFLP) patterns of the rDNA region spanning ITS-1, the 5.8S gene and ITS-2 with two restriction enzymes (*HhaI, HinfI*). Lanes: 1 and 2, *Sulcascaris sulcata*; 3, *Anisakis physeteris*; 4, *A. pegreffii*; 5, *A. sensu stricto*; 6, *Hysterothylacium aduncum*; *7*, *Pseudoterranova* spp.; M, marker (*Bio-Rad* 100-bp molecular ruler); NPC, negative process control; NTC, negative template control.

3.4.Histological investigation

Microscopic observation of the affected adductor muscle indicated moderate haemocytic infiltration (Fig. 1G-H) and intense fibrotic reaction around the encysted larvae, which resulted in the encapsulation of the parasite (Fig. 1F). The muscle tissue around the connective capsule did not appear to be affected (Fig. 1F-H). The presence of light brown pigmented granules in the outer layer of the connective sheath around the larvae was observed, as reported by DEARDOFF (1989).

3.5. Regulation overview

European legislation concerning consumer protections for fishery and aquaculture products permits the consumption of raw products (finfish and cephalopods) only if previously frozen or properly treated with other systems of proven efficacy against the parasites, except for fish farmed with special guarantees (Reg. EU 1276/2011, amending Annex III to Regulation EC 853/2004). No reference is made to other aquatic animals, such as bivalve molluscs.

Scallops are traditionally consumed cooked, and heat treatment rapidly inactivates *S. sulcata* (1-6 seconds at 95°C is sufficient) (RUDDERS *et al*., 2019). However, *P. jacobaeus,* a Mediterranean scallop, is increasingly consumed raw among other seafood according to current consumer eating habits (DASCHNER, 2016). No cases of *S. sulcata* infection in humans have been reported to date, although it cannot be excluded that similar to other Anisakidae, humans may act as paratenic hosts (LAUCKNER, 1983). Experimental infection trials performed in homeotherm species (chickens and cats) failed to establish L3 larvae, suggesting that infection by *S. sulcata* involves only poikilotherm sea turtles as the final host (BERRY and CANNON, 1981). *Hysterothylacium aduncum* (family Raphidascarididae), which has predatory sea fish as its final host, has recently been reported in humans and may be considered to be an exception (GONZÁLEZ-AMORES *et al*., 2017). It must be considered that L3 larvae of *S. sulcata* experimentally demonstrated a reduced viability at 37°C (3.25-6.75 h), thereby appearing unlikely to be pathogenic in humans (RUDDERS *et al*., 2019).

No information regarding possible allergic reactions caused by *S. sulcata* has been reported, unlike other Anisakidae (AIBINU *et al*., 2019). However, it should be considered in the application of European legislation ("*food business operator must ensure that the raw material or finished product undergoes a freezing treatment in order to kill viable parasites that may be a risk to the health of the consumer*") that the residual risk of *S. sulcata* to public health cannot be ruled out at present. Moreover, the ban on marketing fishery products with clearly visible parasites remains to be applied (Reg. EC 853/2004, section VIII, chap. V). Although it is difficult to establish a tolerance for the number of parasites, the presence of 3-4 larvae/scallop is immediately evident, causing a noticeable colour change in the area where the parasite is located.

The authorities supervising the presence of parasites in fishery products should consider possible accidental transfers of such parasites as *Anisakis* from a nearby parasitized fish or fillet. This transfer could occur during the handling of fresh pectinid molluscs, which are usually sold with the pulp exposed, especially when the product is placed on counters in the fish market. A simple field control with a light microscope would quickly and safely differentiate *S. sulcata* from *Anisakis* spp., considering the morphology of the ventriculum ending with a rounded fund (BERRY and CANNON, 1981).

It is advisable for food business operators to enforce, in their companies' internal quality-control systems, an evaluation of the degree of infestation of the scallops collected from different fishing sites. Both the prevalence and intensity of infestation should be considered in the evaluation, excluding products supplied from highly infested fishing sites, as provided for fish from EC Reg. no. 2074/2005, annex II, chap. II.

This report describes the presence of *S. sulcata* in pectinidae in the northern Adriatic Sea. In this area, *P. jacobaeus* and *A. opercularis* are reported as part of loggerhead sea turtle diets (LAZAR *et al*. 2011). The prevalence of the infestation recorded in *C. caretta* in the Adriatic Sea is higher than in other areas of the Mediterranean Sea, where bivalves constitute a marginal food source (SANTORO *et al.* 2019). The northern Adriatic Sea offers a large number of neritic zones for *C. caretta* foraging, where benthic invertebrates, such as scallops, are abundant (LAZAR *et al*. 2011). This trophic network could explain the abundance of *S. sulcata* in both final and intermediate hosts.

4. CONCLUSIONS

New eating habits promoting raw seafood consumption combined with the preference for local products and ecological conditions favouring the diffusion of *S. sulcata* can create new challenges for inspectors of seafood products, as well as retailers.
Scallops are collected in unclassified areas and are therefore already subject to analysis by food operators for such parameters as biotoxins, heavy metals and pathogenic microorganisms. The possibility that *S. sulcata* may also be present in the product must be considered by food business operators in their internal quality control systems (Reg. EC 853/2004, section VIII, chap. IX).

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PAPER

BIOACTIVE COMPOUNDS IN YELLOW, LIGHT YELLOW, AND CREAM-COLOURED POTATO TUBERS AFTER SHORT-TERM STORAGE AND BOILING

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ABSTRACT

This study measured the changes in bioactive compounds [L-ascorbic acid (AA) and total phenolic (TP) compounds] and antioxidant activity (measured in Trolox equivalents, TE) in six potato (*Solanum tuberosum* L.) varieties with yellow, light-yellow, and cream-coloured flesh after several different treatments. The experimental materials included raw tubers and both peeled and unpeeled tubers that had been boiled. Analyses were conducted immediately after harvest and after 3 months of storage at 5°C and 8°C. Flesh colour significantly affected the AA and TP contents in tubers. The difference in AA content was 0.195 $mg \cdot g^{-1}$ DM between cream- and yellow-coloured tubers and 0.086 $mg \cdot g^{-1}$ DM between cream and light-yellow tubers. Differences in TP contents between tubers with different flesh colours did not exceed 33%. Significant losses in AA were detected in yellow- and light-yellow-fleshed tubers that had been peeled and cooked after harvest (44 and 46%, respectively). Cooking peeled tubers significantly decreased the antioxidant activity in potatoes regardless of flesh colour and storage treatment. Unpeeled cooked tubers had significantly higher antioxidant activity than raw tubers after harvest. Irrespective of flesh colour, high linear correlations were found between (AA)×(TE) for cooked peeled tubers. A significant determination coefficient (R^2) was observed between (TPs)×(TE) for raw and cooked unpeeled light-yellow and yellow-coloured tubers. The linear relationship between TPs and TE after cooking was significant for unpeeled tubers. The greatest matching of the model characteristics of the interdependence of features (ϕ^2) was 75% for (AA) × (TE) and 80% for (TPs) × (TE).

Keywords: antioxidant activity, ascorbic acid, phenolics, boiling, potato, storage

1. INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are a rich source of nutrients, particularly complex carbohydrates (starch), phenolic compounds (HEJTMÀNKOVA *et al*., 2009, LACHMAN *et al*., 2012, NAVARRE *et al*., 2010, RUMBOA *et al*., 2009, TEOW *et al*., 2007), and vitamin C (L-ascorbic acid, AA), with AA levels ranging from 14 to 25 $mg \cdot 100^{-1}$ g fresh matter (FM) depending on the variety (BURGOS *et al*., 2009, GRUDZIŃSKA and ZGÓRSKA, 2011, HAN *et al*., 2004, VALCARCEL *et al*., 2015). The importance of these compounds in the human diet has been emphasized by recent studies on their health-promoting properties (WELCH *et al*., 2005, CAHILL *et al*., 2009). Until recently, it was thought that the processing of potatoes, such as cooking, degrades antioxidants and reduces their activity. However, the impact of processing on antioxidant activity is not always straightforward. For example, RUMBOA et al. (2009) found that a reduction in the natural antioxidant content in a food product (potato extracts) may be accompanied by an increase in antioxidant activity.

Research on the bioactive compounds in potato (LACHMAN and HAMOUZ, 2005, REYES *et al*., 2005, LACHMAN *et al*., 2012, HEJTMÀNKOVA *et al*., 2009, BELLUMORI *et al*., 2017) has focused on variations in potatoes with different flesh colours (e.g., white, red, pink, and purple), growing locations (JANSEN and FLAMME, 2008, LACHMAN *et al*., 2008, VALCARCEL *et al*., 2015, PERLA *et al*., 2012, SILVEIRA *et al*., 2016), and cultivation systems (BRAZINSKIENE *et al*., 2014, GRUDZIŃSKA *et al*., 2016) and the effects of cooking (MULINACCI *et al*., 2008, LACHMAN *et al*., 2012, BURGOS *et al*., 2013, BELLUMORI *et al*., 2017) and blanching conditions (MARANGONI *et al*., 2019). However, these studies have not unequivocally demonstrated a correlation between changes in the levels of bioactive compounds and antioxidant activity in potato tubers after storage or non-peeled tubers after cooking using a best-fit model. In particular, such studies have not been performed in potato varieties with cream, light yellow, and yellow flesh, the most popular flesh colours in Europe.

The aim of this study was to determine the changes in bioactive compound levels and antioxidant activity in raw potatoes and in peeled and unpeeled boiled potato tubers with yellow, light-yellow, and cream-coloured flesh after short-term storage at 5°C and 8°C. We also developed a model describing the association of these changes.

2. MATERIALS AND METHODS

2.1. Chemicals

All reagents used in this study, including 2,6-dichloroindophenol (puriss p.a 97.0%), oxalic acid (puriss p.a ≥99.0%), acetone (puriss p.a 99.5%), L-ascorbic acid (L-AA) standard solution (puriss p.a ≥90%), Trolox ((±)-6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (97%), 2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (activity 90–110%), potassium persulfate (puriss p.a 98%), ethanol (puriss p.a 96%), Folin–Ciocalteu reagent, chlorogenic acid (puriss p.a ≥98.0%), and sodium carbonate (puriss p.a 99%) were purchased from Sigma Aldrich, Fluk, Poch, or Linegal Chemicals.

2.2. Plant materials

The experimental materials were six varieties of potato (*Solanum tuberosum* L.). The varieties and their characteristics are shown in Table 1.

Table 1. Characteristics of the potato varieties examined in this study.

Variety	Origin of seed tubers	Skin colour	Flesh colour	Average Yield [t ha^{-1}][1]	Cooking type
Ametyst	Poland	yellow	cream	64.4	medium meal
Aruba	Poland	yellow	cream	34.4	slightly floury
Ingrid	Netherlands	yellow	light yellow	41.2	slightly floury
Flaming	Poland	red	light yellow	33.3	slightly floury
Altesse	France	yellow	yellow	44.9	intermediate type
Elanda	Poland	yellow	yellow	44.0	slightly floury

[1]Characteristics from the *National Register of Varieties of Potato ed. XV*

Potatoes were grown in experimental fields at the experimental station of the Plant Breeding and Acclimatization Institute, Research Division Jadwisin, Poland. Agronomic inputs used in conventional systems are shown in Table 2.

Table 2. Agronomic inputs used in conventional systems.

Crop production practice	Conventional system
Fertilisation	4–5 t ploughed rye straw + 1 kg mineral nitrogen per 100 kg straw + mustard as a catch crop; N: 100 kg.ha^{-1}, P: 53 kg.ha^{-1}, K: 150 kg.ha^{-1}
Weed control	Mechanical tillage + herbicides: 2012: Afalon-1.9 l.ha^{-1}, Titus+Trend (60 g.ha^{-1} + 0.5 l.ha^{-1}) 2013: Linurex-1.8 l.ha^{-1}, Titus + Trend (60 g.ha^{-1} + 0.5 l.ha^{-1})
Colorado potato beetle control	Chemical insecticides: 2012: Actara -60 g.ha^{-1} 2013: Actara 2 times per season -70 g.ha^{-1}, Apacz-40 g.ha^{-1}
Late blight control	Chemical fungicides 2012: Ridomil-2 l.ha^{-1}, Revus-0.6 l.ha^{-1}, Ranman-0.2 l.ha^{-1}, Altima-0.4 l.ha^{-1}, Ranman-0.2 l.ha^{-1}, 2013: Revus-0.6 l.ha^{-1}

After harvest, the potato tubers (whole crop) were placed in an experimental storage room under the following conditions: 1) during the preparatory period for the first two weeks after harvest, the temperature was maintained at 15°C with 95±2% relative humidity; 2) subsequently, over a two-week period, the temperature was gradually lowered to 8°C (chamber I) or 5°C (chamber II) while maintaining the same relative humidity (95%).
Post-harvest potato samples were collected for analysis immediately after harvest (the third set of ten days [decade] of September). Post-storage samples were collected after 3 months of storage at 5°C and 8°C (the third decade of January). In each test, the samples were collected at random times (each laboratory sample consisted of ca. 50 tubers ~40 mm in size).

2.3. Sample preparation

The samples were prepared for analysis in the following manner: 1) tubers were left raw, unpeeled, and uncut; 2) uncut tubers were peeled (cut slit width 1.33 mm) and boiled in water in a beaker (standard proportions of 0.5 kg of potatoes and 0.7 dm^3 of boiling water without added salt) for approximately 15±2 min (beginning when the tubers were placed into boiling water); and 3) tubers were left unpeeled and boiled.

2.4. Measurement of dry matter

The dry matter content was determined using a two-stage drying-weighing method involving drying at 60°C for 12 hours, followed by 105°C until the sample maintained a constant weight.

2.5. Extraction of hydrophilic fractions

Freeze-dried samples were ground into a fine powder with a Freezer Mill 6770. Five grams of freeze-dried powder was vortexed for 2 min in 25 ml hexane, and the mixture was filtered through a Buchner funnel. The hexane extraction step was repeated twice, and the combined lipophilic extracts were evaporated to dryness at 50°C in a vacuum evaporator. The residue produced after hexane extraction was extracted twice in 25 ml of acidified methanol (7% acetic acid in 80% methanol) to obtain the hydrophilic fraction. The final volume of the hydrophilic fraction was adjusted to 50 ml with acidified methanol.

2.6. Measurement of ABTS radical-scavenging activity

The ABTS radical-scavenging activity of the hydrophilic fractions was determined as described by RICE-EVANS *et al*. (1997) using the modifications described by RE *et al*. (1999). The ABTS_+ solution consisted of 7 mM ABTS salt and 2.45 mM potassium persulfate (final concentration) in 25 ml of distilled water. The mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The ABTS_+ solution was diluted with 95% ethanol (approximately 600 μl ABTS to 40 ml 95% ethanol) to obtain an absorbance of approximately 0.7 (±0.02) at 734 nm. Fresh ABTS_+ solution was prepared for each analysis. Antioxidant or standard solutions (20 μl) were mixed with 1 ml of diluted ABTS_+ solution and incubated at 30°C. The absorbance at 734 nm was read every minute for 30 min. Ethanol (95%) was used as a blank. Trolox (0 to 500 μM) was used as a standard. Free radical scavenging activity was expressed as μmoles of Trolox per 100 grams of sample (μmol TE·100 g^{-1}).

2.7. Measurement of total phenolics

Total phenolic contents were measured by the Folin-Ciocalteu method using the modifications described by Singleton *et al*. (1999). The hydrophilic extract (0.5 ml) was diluted with distilled water to 5 ml, to which 0.5 ml Folin-Ciocalteu reagent was added and allowed to react at room temperature for 3 min. After the addition of 1 ml of 1 N sodium carbonate, the mixture was incubated at room temperature for 1.5 h. The absorbance was measured at 725 nm using a spectrophotometer (T70+ UV/VIS) with distilled water as a blank. Chlorogenic acid was used as a standard. Total phenolic contents were reported as milligrams per gram dry matter (mg TPs·g^{-1} DM).

2.8. Measurement of L-ascorbic acid

L-ascorbic acid (AA) concentrations were measured using a standard spectrophotometric method (Polish standard PN-A04019) based on the ability of AA to reduce the dye 2,6-dichloroindophenol. Briefly, a 10-g sample of potato tuber was extracted in 0.4% oxalic acid by homogenizing the sample in an Ultra Turrax T25 for 3 min at 13,500 rpm. The extract was filtered through filter paper under a vacuum and adjusted to 100 ml with the same extraction solution. Next, 5 ml of the extract was reacted with 2 ml of 2,6-dichloroindophenol (1.6%) for 2 min. The absorbance was measured at 500 nm using a spectrophotometer (T70+ UV/VIS) with oxalic acid and 2 ml of 2,6-dichloroindophenol (1.6%) as a blank. The AA concentration was quantified via comparison with a standard curve of L-AA. The AA content was reported as milligrams per gram dry matter (mg AA·g^{-1} DM).

2.9. Statistical analysis

Two and three-way analyses of variance (ANOVAs) based on fixed model and multiple regression analysis were conducted to determine if the studied factors significantly differed from the analysed features. Significant differences between means for the objects (after confirming the existence of these differences using F-test in analysis of variance) were determined using Tukey's multiple comparison procedure with $P \leq 0.05$. Relationships (after confirming the existence of these relationships using multiple regression model analysis) were described using determination coefficients (R^2) and convergence coefficients (φ^2). Calculations were performed using Statistica software (v.12).

3. RESULTS AND DISCUSSION

3.1. Ascorbic acid (AA)

According to BURGOS *et al.* (2009), MURNIECE *et al.* (2011), HAMOUZ *et al.* (2008), and VALCALCER *et al.* (2015), the AA content in potato tubers after harvest is dependent on the variety, place of cultivation, and environmental conditions during growth. According to HAMOUZ *et al.* (2008), the AA content in raw potatoes can vary from 14 to 1.093 mg·g^{-1} DM, while according to BURGOS *et al.* (2009), it can vary from 295 to 1.677 mg·g^{-1} DM In the current study, the highest AA content was detected in potato tubers after harvest (light yellow-fleshed potato, 1.142 mg·g^{-1} DM) (Table 3).
Flesh colour significantly affected AA content in tubers. We observed significant differences in AA content between tubers with yellow-coloured flesh (1.100 mg·g^{-1} DM) and cream-coloured flesh (0.952 mg·g^{-1} DM). By contrast, HEJTMÁNKOVA *et al.* (2009) showed that white and yellow potatoes did not differ in terms of AA content, whereas HAMOUZ *et al.* (2008) determined that AA content was 2.9-times higher in red and purple potatoes than in potatoes with yellow and white flesh. VALCALCER *et al.* (2015) studied 5 potato varieties with white flesh, 7 with yellow flesh, 20 with light-yellow flesh, and 25 with cream-coloured flesh and found that the AA content in tubers is determined by both environmental conditions (location, climate) and variety. In the current study, the highest AA content was detected in raw tubers of the Altesse variety (yellow flesh) and the lowest was detected in potatoes of the Ametyst variety (cream-coloured flesh). The difference in AA content between these varieties reached 26%.

Short-term storage at both 5°C and 8°C significantly affected the AA content in raw potatoes. Potato tubers contained approximately 40% more AA immediately after harvest than potatoes stored for 3 months. Consistent with this finding, KEIJBETS and EBBENHORST-SELLER (1990) recorded AA losses of 20–60% in potatoes during the first 4 months of storage. Such patterns were also observed by ABONG *et al.* (2001), who showed that the losses of AA in raw potatoes were much higher during the first months of storage than during the final storage period. Similar studies were carried out by GRUDZIŃSKA and ZGÓRSKA (2011), who showed that in potato tubers stored through autumn (up to 90 days), AA losses reached 10%, while in potatoes stored through winter (up to 150 days), AA losses reached 22%. RIVERO *et al.* (2003) showed that the reductions of AA levels after 20 weeks of storage (140 days) reached 50% in some varieties. We found that AA contents were significantly lower in tubers stored at 5°C (0.702 $mg \cdot g^{-1}$ DM) vs. 8°C (0.967 $mg \cdot g^{-1}$ DM). Similar associations were observed by KÜLEN *et al.* (2013) who recorded a loss of AA in raw potato tubers in the first months of storage at 4°C. These observations are consistent with the conclusions of SAPEI and HWE (2014), whose study on the kinetics of vitamin C degradation revealed that losses of vitamin C in products stored at lower temperatures could be reduced by the simultaneous presence of sucrose. Potatoes stored at lower temperatures accumulate this sugar GRUDZIŃSKA at el. (2016). No interactions between flesh colour and the time of tuber storage in relation to storage temperature have been demonstrated.

Statistical analysis of our results revealed that boiling had a significant effect on AA contents in potato tubers (Table 4). The greatest changes in AA content were observed in boiled potatoes directly after harvest (~40% loss of AA), while the smallest changes were observed in boiled potatoes after 3 months of storage at 5°C.

In addition, peeling tubers before thermal processing increased the losses of AA by approximately 7%. Peeling had a significant effect on the AA content in boiled tubers with cream-coloured flesh after harvest (peeled 0.715 $mg \cdot g^{-1}$ DM; unpeeled 0.466 $mg \cdot g^{-1}$ DM) and in yellow-coloured potatoes (peeled 0.607 $mg \cdot g^{-1}$ DM; unpeeled 1.009 $mg \cdot g^{-1}$ DM). These differences were not observed in tubers that were boiled after storage. Similar association were noted by RYTEL and LISIŃSKA (2007). LACHMAN *et al.* (2013) detected significant losses (up to 69%) of AA in boiled peeled potatoes compared to raw tubers. In the current study, such large differences were not observed. The greatest differences between AA losses were recorded after harvest in potatoes with yellow and light-yellow flesh that were peeled and boiled (44% and 46%, respectively).

Statistical analysis of the results revealed significant interactions between flesh colour, cooking, and storage (Table 4). The lowest AA content was detected in unpeeled boiled potatoes with cream-coloured flesh after harvest (0.466 $mg \cdot g^{-1}$ DM), while the highest AA content was detected in unpeeled boiled yellow-coloured potatoes after harvest (1.009 $mg \cdot g^{-1}$ DM) and in peeled boiled yellow-coloured potatoes after storage at 8°C (0.955 $mg \cdot g^{-1}$ DM).

Table 3. AA content [mg g $^{-1}$ DM] in potato tubers under different treatments.

Variety	After harvest				After storage							
					5°C				8°C			
		After cooking			After cooking				After cooking			
	Raw	Unpeeled	Peeled	Mean	Raw	Unpeeled	Peeled	Mean	Raw	Unpeeled	Peeled	Mean
CREAM-COLOURED FLESH												
Ametyst	0.889	0.455	0.685	0.676^{ab}	0.695	0.540	0.615	0.616^{a}	0.840	0.800	0.740	0.793^{bc}
Aruba	1.016	0.518	0.746	0.760^{b}	0.655	0.524	0.592	0.590^{a}	0.885	0.592	0.743	0.740^{b}
Mean	*0.952*	*0.466^{A}*	*0.715^{C}*	*0.711*	*0.675*	*0.532^{B}*	*0.603^{BC}*	*0.603*	*0.862*	*0.696^{C}*	*0.741^{C}*	*0.767*
LIGHT YELLOW FLESH												
Ingrid	1.344	0.786	0.585	0.905^{cd}	0.798	0.559	0.560	0.639^{a}	0.986	0.740	0.659	0.795^{bc}
Flaming	0.941	0.482	0.647	0.690^{ab}	0.468	0.603	0.533	0.534^{a}	0.821	0.389	0.879	0.696^{ab}
Mean	*1.142*	*0.634^{BC}*	*0.616^{BC}*	*0.797*	*0.633*	*0.581^{B}*	*0.546^{B}*	*0.587*	*0.903*	*0.564^{B}*	*0.769^{C}*	*0.746*
YELLOW FLESH												
Altesse	1.147	0.830	0.614	0.863^{c}	1.014	0.705	0.624	0.781^{bc}	1.132	0.816	0.860	0.936^{d}
Elanda	1.054	1.188	0.601	0.947^{d}	0.582	0.823	0.750	0.718^{b}	1.136	0.920	1.051	1.035^{e}
Mean	*1.100*	*1.009^{E}*	*0.607^{BC}*	*0.906*	*0.798*	*0.764^{C}*	*0.687^{C}*	*0.750*	*1.134*	*0.868^{D}*	*0.955^{E}*	*0.986*
Mean	1.065	0.703	0.646		0.702	0.626	0.612		0.967	0.709	0.822	
Mean of cooked		*0.674a*				*0.619 a*				*0.765 b*		

Homogenous groups are denoted by letters (a, b, c and A, B, C). Means with different letters are significantly different.
a, b, c Means with different letters are significantly different between varieties after harvest and storage.
A, B, C Means with different letters are significantly different between varieties with different flesh colours after harvest and storage.

Table 4. Sources of variation and ANOVA results for AA content in potato tubers under different treatments (statistical analyses of the data shown in Table 3).

Sources of variation	ANOVA results					
	Sum of the squares	Degrees of freedom	Mean square	F statistic	p-value	Significance
Variety (V)	0.965	5	0.193	16.363	0.0001	***
Storage temperature (S)	0.603	2	0.302	25.562	0.0001	***
Boiling (B)	0.920	2	0.460	38.982	0.0025	**
(V) × (S)	0.382	10	0.038	3.237	0.0001	***
(V) × (B)	0.558	10	0.056	4.733	0.0001	***
(S) × (B)	0.544	4	0.136	11.518	0.0001	***
(V) × (S) × (B)	0.887	20	0.044	3.759	0.0001	***
Flesh colour (FC)	0.769	2	0.384	21.372	0.0001	***
(FC) × (S)	0.076	4	0.019	1.051	0.3861	n.s
(FC) × (B)	0.233	4	0.058	3.238	0.0162	*
(FC) × (S) × (B)	0.332	8	0.041	2.307	0.0279	*

n.s. not significant; *, significant at α=0.05; **, significant at α=0.01; ***, significant at α=0.001.

3.2. Total phenolics (TPs)

Table 5 shows the TP contents in the potato tubers under different treatments.
Flesh colour had a significant effect on TP content. The highest TP content was found in tubers with yellow flesh both after harvest and after storage (3.766 mg·g^{-1} DM after harvest to 6.350 mg·g^{-1} DM after storage). The TP content was significantly lower in tubers with cream-coloured flesh (f2.633 to 3.831 mg·g^{-1} DM) and light-yellow flesh (3.363 to 4.335 mg·g^{-1} DM). Similar results were obtained by VALCARCEL *et al.* (2015) and TIERNO *et al.* (2015).
The differences in TP contents between raw tubers with yellow- and cream-coloured flesh were 1.125 mg·g^{-1} DM for tubers after harvest, 1.268 mg·g^{-1} DM for tubers stored at 5°C, and 2.520 mg·g^{-1} DM for tubers stored at 8°C. For raw tubers with light-yellow and yellow flesh, the differences in TP contents were much lower, ranging from 0.395 mg·g^{-1} DM after harvest to 2.016 mg·g^{-1} DM after storage at 8°C. Differences in TP contents between tubers with different flesh colours (yellow, light yellow, and cream) were no greater than 33%. LACHMAN *et al.* (2008) observed much greater differences in TP contents (58%) between potato tubers with purple and yellow flesh.
Storage temperature significantly affects TP contents in tubers (GRUDZIŃSKA and ZGÓRSKA, 2011, KÜLEN *et al.*, 2013). We found that the TP content was significantly higher in tubers stored at the higher temperature (8°C) (3.830 to 6.350 mg·g^{-1} D.M) than at 5°C (2.982 to 4.250 mg·g^{-1} D.M). KUMAR and EZEKIEL (2009), GRUDZIŃSKA and ZGÓRSKA (2011), and GRUDZIŃSKA and BARBAŚ (2017) found that at high storage temperatures, tubers germinate more frequently and lose turgor. AL - WESHAHY *et al.* (2013) found that the TP content in tubers significantly decreased during the first 4 weeks of storage (regardless of temperature) and significantly increased after 8 weeks of storage. At the higher storage temperature (8°C), we observed significant variation in the

differences in TP content, with the greatest differences observed in raw tubers with light-yellow flesh (Ingrid and Flaming) and yellow flesh (Altesse and Elanda).

Table 5. TP contents [mg g $^{-1}$ DM] in potato tubers under different treatments.

Variety	After harvest				After storage							
					5°C				8°C			
	Raw	After cooking		Mean	Raw	After cooking		Mean	Raw	After cooking		Mean
		peeled	peeled			peeled	peeled			peeled	peeled	
CREAM-COLOURED FLESH												
Ametyst	2.433	3.043	2.511	2.662^{a}	2.907	5.414	4.194	4.171^{de}	3.455	4.125	3.875	3.818^{cd}
Aruba	2.834	3.242	2.461	2.845^{ab}	3.058	6.105	4.134	4.432^{ef}	4.208	4.963	3.316	4.162^{de}
Mean	*2.633*	*3.142*	*2.486*	*2.754^{B}*	*2.982*	*5.760*	*4.164*	*4.302^{CD}*	*3.831*	*4.544*	*3.595*	*3.990^{C}*
LIGHT YELLOW FLESH												
Ingrid	3.547	4.029	2.916	3.497^{bc}	3.980	6.869	4.726	5.191^{ef}	5.106	6.138	4.400	5.214^{ef}
Flaming	3.180	2.786	1.978	2.648^{a}	2.127	5.102	2.802	3.343^{bc}	3.565	4.276	2.643	3.494^{bc}
Mean	*3.363*	*3.407*	*2.447*	*3.072^{BC}*	*3.053*	*5.985*	*3.764*	*4.267^{CD}*	*4.335c*	*5.207*	*3.521*	*4.354^{CD}*
YELLOW FLESH												
Altesse	3.510	3.870	3.847	3.742^{bc}	4.316	7.067	5.904	5.762^{fg}	5.343	6.276	4.790	5.469^{ef}
Elanda	4.007	4.075	3.257	3.779^{bc}	4.184	7.747	5.118	5.683^{f}	7.363	8.938	5.047	7.116^{g}
Mean	*3.758*	*3.973*	*3.552*	*3.761^{C}*	*4.250*	*7.407*	*5.511*	*5.722^{D}*	*6.35l*	*7.607*	*4.918*	*6.292^{D}*
Mean	3.251	3.507	2.828		3.428	6.384	4.479		4.840	5.786	4.011	
Mean of cooked	*3.167 a*				*5.431 b*				*4.898 ab*			

Homogenous groups are denoted by letters (a, b, c and A, B, C). Means with different letters are significantly different.
a, b, c Means with different letters are significantly different between varieties after harvest and storage.
A, B, C Means with different letters are significantly different between varieties with different flesh colours after harvest and storage.

In unpeeled tubers, TP contents were significantly higher in cooked vs. raw potatoes, regardless of flesh colour and whether the tubers were stored or analysed after harvest. Similar results were obtained by NAVARRE *et al.* (2010) and BURGOS *et al.* (2013), who reported an increase in the contents of phenolic compounds in potato tubers after cooking, which varied depending on the method of cooking (boiling, steaming, baking). According to BLESSINGTON *et al*. (2010), the increase in TP contents in potato tubers after cooking may be related to the higher extractability of these compounds from the cooked tuber cell matrix compared to the uncooked matrix. In the current study, the greatest difference in TP content was detected between raw and unpeeled cooked tubers after storage at 5°C (2.778 to 3.157 mg·g^{-1} DM, respectively) regardless of flesh colour, and the smallest difference was detected in tubers after harvest (0.044 to 0.509 mg·g^{-1}, respectively).
Higher TP levels were observed in unpeeled boiled tubers regardless of whether they were stored at either temperature or measured immediately after harvest. TP contents were significantly lower in peeled vs. unpeeled potatoes. Similar results were obtained by LECHMANA *et al.* (2008), who measured 30.4–38.7% differences in phenolic compound

contents as a result of peeling. Similarly, DAO and FRIDMAN (1992) detected substantial amounts of phenolic compounds just below the skin to ~2 mm into the flesh of potato tubers. TIERNO *et al.* (2015) reported that peeling allows for the migration and degradation of phenolic compounds during cooking. Therefore, cooking potatoes without peeling them is an effective method for reducing the loss of phenolic compounds.
Statistical analysis of our results showed no significant interaction between the factors flesh colour, cooking, and storage (Table 6).

Table 6. Sources of variation and ANOVA results for TP content in potato tubers under different treatments (statistical analysis of the data shown in Table 5).

Source of variation	ANOVA results					
	Sum of the squares	Degrees of Freedom	Mean square	F statistic	p-value	Significance
Variety (V)	73.607	5	14.721	167.538	0.0001	***
Storage (S)	60.932	2	30.466	346.723	0.0001	***
Boiling (B)	51.210	2	25.605	291.401	0.0001	***
(V) × (S)	14.851	10	1.485	16.901	0.0001	***
(V) × (B)	7.792	10	0.779	8.867	0.0001	***
(S) × (B)	32.893	4	8.223	93.587	0.0001	***
(V) × (S) × (B)	5.495	20	0.275	3.127	0.0050	***
Flesh colour (FC)	52.628	2	26.314	55.612	0.0001	***
(FC) × (S)	7.068	4	1.767	3.734	0.0077	**
(FC) × (B)	1.796	4	0.449	0.949	0.4401	n.s
(FC) × (S) × (B)	2.821	8	0.353	0.745	0.6514	n.s

n.s., not significant; *, significant at α=0.05; **, significant at α=0.01; ***, significant at α=0.001.

3.3. Antioxidant activity

Table 7 shows changes in antioxidant activity in potato tubers under different treatments. Flesh colour had no significant effect on the antioxidant activity of raw tubers. Higher antioxidant activities were observed in potatoes with light-yellow flesh after harvest (0.589 μmol TE·g^{-1}), and lower antioxidant activities were observed in potatoes with cream-coloured flesh (0.460 μmol TE·g^{-1}), but these differences were not significant (Tables 7 and 8).
After harvest, the antioxidant activity ranged from 0.460 μmol TE·g^{-1} in cream-coloured tubers to 0.554 μmol TE·g^{-1} in tubers with yellow flesh. After 3 months of storage at both temperatures, the antioxidant activity of tubers significantly decreased by 0.264 μmol TE·g^{-1} for light-yellow tubers and 0.168 μmol TE·g^{-1} for cream-coloured tubers. Similar observations were made by ROSENTHAL and JANSKY (2008), who detected higher antioxidant activity in potato tubers directly after harvest than after storage. According to their study, antioxidant activity did not changed after up to 135 days of storage. In the current study, significant changes in antioxidant activity were observed in tubers after 90 days of storage at 5°C. At the higher storage temperature (8°C), the antioxidant activity in potatoes was similar to that after harvest.

Table 7. Antioxidant activity [μmol TE·g^{-1}] in potato tubers under different treatments.

Variety	After harvest				After storage							
					5°C				8°C			
	After cooking				After cooking				After cooking			
	Raw	Unpeeled	Peeled	Mean	Raw	Unpeeled	Peeled	Mean	Raw	Unpeeled	Peeled	Mean
CREAM-COLOURED FLESH												
Ametyst	0.553	0.348	0.442	0.447 *bc*	0.145	0.354	0.267	0.255 *a*	0.557	0.388	0.496	0.480 *c*
Aruba	0.429	0.718	0.294	0.480 *c*	0.240	0.354	0.395	0.329 *a*	0.496	0.685	0.321	0.500 *cd*
Mean	*0.481^{C}*	*0.533^{D}*	*0.368^{B}*	*0.460*	*0.192^{A}*	*0.354^{B}*	*0.331^{B}*	*0.292*	*0.526^{CD}*	*0.538^{D}*	*0.408^{BC}*	*0.490*
LIGHT YELLOW FLESH												
Ingrid	0.678	0.990	0.348	0.672 *d*	0.297	0.111	0.476	0.294 *a*	0.462	0.449	0.233	0.380 *b*
Flaming	0.456	0.641	0.405	0.500 *cd*	0.432	0.294	0.347	0.357 *ab*	0.307	0.429	0.503	0.413 *bc*
Mean	*0.567^{D}*	*0.815^{E}*	*0.376^{B}*	*0.589*	*0.362^{B}*	*0.202^{A}*	*0.411^{BC}*	*0.325*	*0.384^{CD}*	*0.439^{D}*	*0.368^{BC}*	*0.397*
YELLOW FLESH												
Altesse	0.440	0.523	0.532	0.498 *c*	0.294	0.381	0.321	0.332 *ab*	0.350	0.510	0.489	0.449 *bc*
Elanda	0.746	0.577	0.510	0.611 *d*	0.415	0.361	0.422	0.399 *b*	0.658	0.617	0.368	0.547 *cd*
Mean	*0.593^{D}*	*0.550^{D}*	*0.521^{CD}*	*0.554*	*0.354^{B}*	*0.371^{B}*	*0.371^{B}*	*0.365*	*0.504^{CD}*	*0.563^{D}*	*0.428^{C}*	*0.498*
Mean	0.550 *b*	0.632 *c*	0.421 *ab*		0.303 *a*	0.309 *a*	0.371 *a*		0.471*b*	0.513*b*	0.401*ab*	
Mean of cooked	*0.527 c*				*0.340 a*				*0.457 b*			

Homogenous groups are denoted by letters (a, b, c and A, B, C). Means with different letters are significantly different.
a, b, c Means with different letters are significantly different between varieties after harvest and storage.
A, B, C Means with different letters are significantly different between tubers with different flesh colours after harvest and storage.

Table 8. Sources of variation and ANOVA results for antioxidant activity in potato tubers under different treatments (statistical analysis of the data shown in Table 7).

Sources of variation	ANOVA results					
	Sum of the squares	Degrees of freedom	Mean square	F statistic	p-value	Significance
Variety (V)	0.164	5	0.033	156.747	0.0001	***
Storage (S)	0.788	2	0.394	1876.919	0.0001	***
Boiling (B)	0.135	2	0.068	322.795	0.0001	***
(V) × (S)	0.252	10	0.025	120.186	0.0001	***
(V) × (B)	0.309	10	0.031	147.103	0.0001	***
(S) × (B)	0.245	4	0.061	291.976	0.0001	***
(V) × (S) × (B)	0.705	20	0.035	168.099	0.0001	***
Flesh colour (FC)	0.063	2	0.031	2.778	0.6810	n.s
(FC) × (S)	0.149	4	0.037	3.303	0.0147	**
(FC) × (B)	0.016	4	0.345	0.345	0.8471	n.s
(FC) × (S) × (B)	0.304	8	3.379	3.379	0.0022	**

n.s., not significant; *, significant at α=0.05; **, significant at α=0.01; ***, significant at α=0.001.

The cooking of peeled tubers led to a decrease in antioxidant activity regardless of flesh colour and storage condition. The antioxidant activity was significantly higher in cooked unpeeled tubers (0.632 μmol TE·g^{-1}) than in raw potatoes after harvest (0.550 μmol TE·g^{-1}) and after storage at 8°C (0.512 μmol TE·g^{-1}).
According to BLESSINGTON *et al.* (2010), PERLI *et al*. (2012), and LACHMAN *et al.* (2013), cooking changes the antioxidant activity in tubers. These changes depend on the cooking method and pre-treatment of potatoes (peeling). As a result of peeling, the contents of the phenolic compounds flavonoids, flavones, anthocyanins, and lutein in potato tubers decreased by approximately 46–54%, leading to a significant reduction in antioxidant activity in tubers after cooking (PERLA *et al.*, 2012). NARA *et al*. (2006) indicated that potato tuber peels have high antioxidant activity, suggesting they could be used as a new source of natural antioxidants.
Statistical analysis of our results revealed a significant effect of variety on antioxidant activity (Table 8). The highest antioxidant activity was detected in raw tubers of the Elanda variety directly after harvest (0.748 μmol TE·g^{-1}) and in unpeeled cooked tubers of the Aruba variety (0.718 μmol TE·g^{-1}), while the lowest antioxidant activity was detected in raw tubers of the Ametyst variety (0.145 μmol TE·g^{-1}) after storage at 5°C and in unpeeled cooked tubers of the Ingrid variety (0.111 μmol TE·g^{-1}).

3.4. Correlation analysis

The correlation between phenolic compound content and antioxidant activity is widely known (LACHMAN *et al.*, 2008, REYES *et al.*, 2005, RUMBOA *et al.*, 2009, TEOW *et al.*, 2007, NZARAMBA *et al.*, 2013). The correlation coefficients between the parameters examined in the above-mentioned studies ranged from 0.430 (TEOW *et al.*, 2007, AL-WESHAHY *et al.*, 2013) to 0.930 (REDDIVARI *et al.*, 2007), with the size of the coefficient

depending on the research material, crop location, and climatic conditions during the growing season.
Table 9 shows the coefficient of determination and convergence coefficient between antioxidant activity and the AA and TP contents depending on flesh colour. The coefficient of determination between (AA) × (TE) in raw potatoes ranged from $R^2 = 0.563$ for light-yellow tubers to $R^2 = 0.737$ for yellow tubers.

Table 9. Determination and convergence coefficients between antioxidant activity (TE) and L-ascorbic acid (AA) and total phenolic compounds (TP) contents in raw, unpeeled, and peeled potatoes after cooking depending on flesh colour.

Flesh colour	(AA)×(TE)			(TPs)×(TE)		
	Raw	Unpeeled	Peeled	Raw	Unpeeled	Peeled
	Determination coefficient (R^2)					
Cream	0.688^{***}	0.758^{***}	0.743^{***}	$0.109^{n.s}$	$0.019^{n.s}$	$0.182^{n.s}$
Light yellow	0.563^{**}	$0.277^{n.s}$	0.702^{***}	0.646^{**}	0.781^{***}	$0.266^{n.s}$
Yellow	0.737^{***}	$0.197^{n.s}$	0.613^{**}	0.793^{***}	0.669^{***}	$0.399^{n.s}$
	Convergence coefficient (ϕ^2)					
Cream	31.2	24.2	25.7	89.1	98.1	81.8
Light yellow	43.7	72.3	29.8	35.4	21.9	73.4
Yellow	26.3	80.3	38.7	20.7	33.1	60.1

n.s., not significant; *, significant at α=0.05; **, significant at α=0.01; ***, significant at α=0.001.

In unpeeled cooked tubers, significant dependencies ($R^2 = 0.758$) were observed only in tubers with cream-coloured flesh. In potatoes with yellow and light-yellow flesh, such relationships were not observed ($R^2 = 0.197$; $R^2 = 0.277$, respectively). Other dependencies were observed for peeled cooked potatoes: regardless of flesh colour, the determination coefficients were statistically significant (0.613 to 0.743). The highest convergence of features was observed for peeled and unpeeled cooked tubers with cream-coloured flesh (24.2 and 25.7%, respectively).
Different relationships were obtained for the (TPs) × (TE) model. For cream-coloured tubers, the determination coefficient (R^2) was not statistically significant (0.019 for unpeeled cooked tubers to 0.182 for peeled cooked tubers). The convergence coefficient ranged from 98 to 81%, indicating that the antioxidant activity in cream-coloured tubers was shaped by other factors and not by TP content. Non-significant relationships (0.182 to 0.399) were also obtained for peeled cooked tubers irrespective of flesh colour. These results are consistent with the finding of DAO and FRIDMAN (1992) that significant amounts of phenolic compounds are present just below the peel to ~2 mm of the potato tuber.
SEIJO-RODRÍGUEZ *et al.* (2018) found that due to the high correlation between TP content and antioxidant activity, TP content could be used as an indicator of the antioxidant activity of a tuber. Our findings do not fully confirm this notion, as this indicator could be determined only in raw and unpeeled cooked tubers but would be difficult to determine using peeled cooked tubers due to the different level of phenolic compound accumulation under the peel.

Fig. 1 shows the relationship between the contents of AA and TPs and antioxidant activity in raw tubers and in unpeeled and peeled cooked tubers regardless of flesh colour. The higher the AA content in boiled peeled potatoes, the higher the antioxidant activity (Fig. 1).

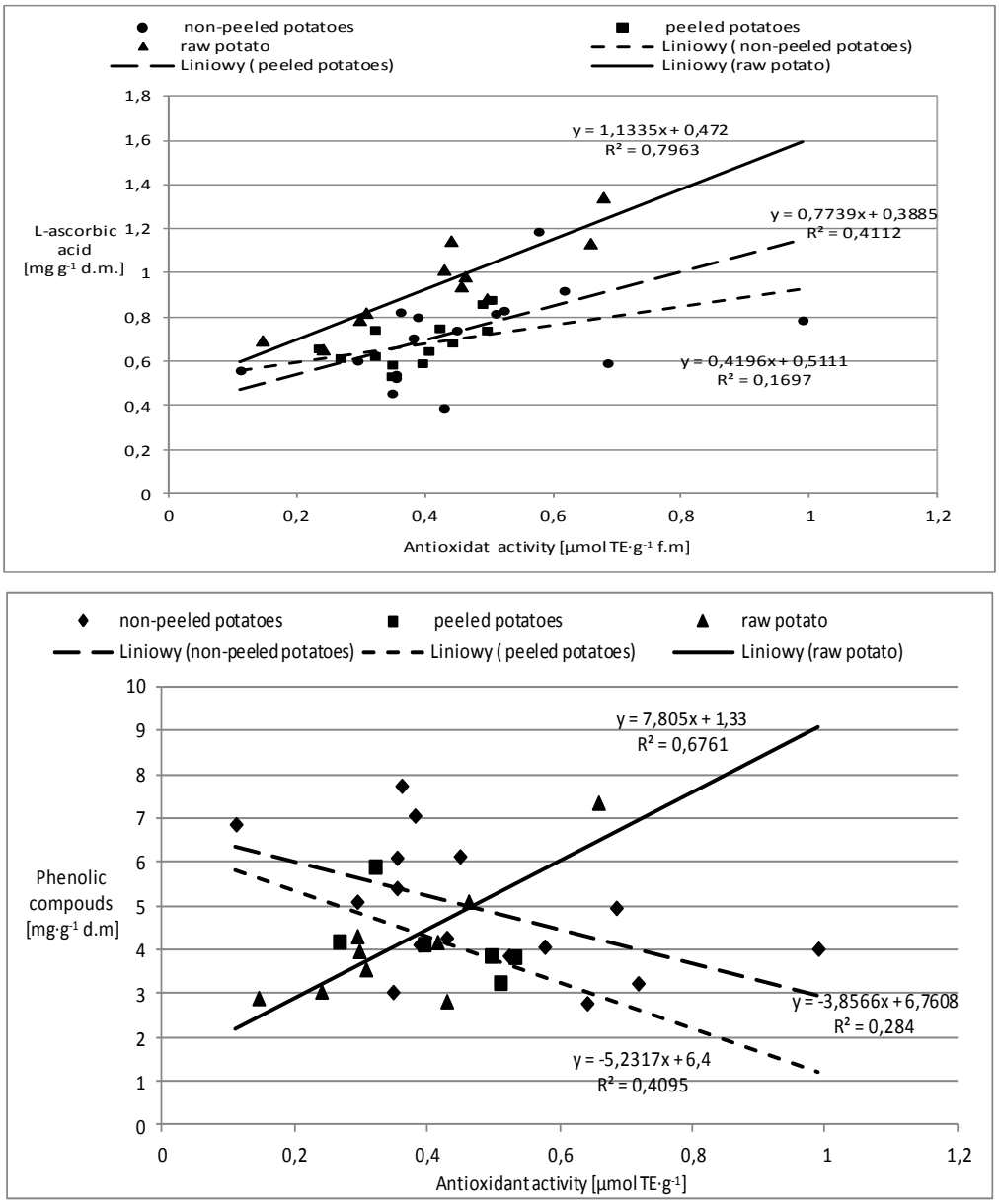

Figure 1. The relationship between antioxidant activity and the contents of L-ascorbic acid and total phenolic compounds in raw potatoes and in unpeeled and peeled potatoes after cooking.

The correlation coefficient between antioxidant activity and AA content for peeled boiled potatoes was $R^2 = 0.411$. HEJTMÁNKOVÁ *et al.* (2009) did not detect a significant correlation between AA content and antioxidant activity ($R^2 = 0.08$) in unpeeled raw potatoes. We obtained similar results, but our results were for unpeeled boiled potatoes (R^2

= 0.169). The highest correlation for AA content and antioxidant activity was obtained for raw tubers (R^2 = 0.796).
In the current study, the correlation between the content of TP compounds and antioxidant activity was inversely proportional in boiled potato tubers (Fig. 1): the lower the TP content, the higher the antioxidant activity. A similar relationship was detected by RUMBOA *et al.* (2009) (R^2 = 0.56). Such irregularities might indicate that the antioxidant activity in tubers after boiling is also shaped by other bioactive compounds.
There was a significant dependence between TP content and antioxidant activity in potatoes after boiling for unpeeled cooked tubers. In peeled cooked tubers, such relationships were not observed (R^2 = 0.023) (Fig. 1B). AL-WESHAHY and RAO (2009) showed that phenolic compound levels are often higher just below the peel of potato tubers than deeper inside the tuber, but this depends on the variety and the colour of the peel itself. However, according to NARA *et al.* (2006), phenolic compounds found in the peel (in both free and bound form) show high antioxidant activity, whereas those in the flesh show low antioxidant activity.

4. CONCLUSIONS

Here we demonstrated that flesh colour has a significant effect on AA and TP contents in tubers. The difference between the AA contents was 12.5% in cream- vs. yellow-coloured tubers and 16.5% between cream- and light-yellow-coloured tubers. Differences in TP contents between potatoes with different flesh colours did not exceed 33%. Significant AA loses were found in peeled boiled yellow and light-yellow tubers after harvest (44 and 46%, respectively). Significant interactions were observed between AA contents and flesh colour, boiling, and storage.
Both storage treatments significantly decreased the antioxidant activity of tubers irrespective of flesh colour. Boiling significantly decreased antioxidant activity in peeled tubers regardless of flesh colour and storage treatment. Unpeeled boiled tubers had significantly higher antioxidant activity than raw tubers after harvest. Regardless of flesh colour, high correlations were observed between (AA) × (TE) for peeled boiled tubers. Significant R^2 values were observed between (TPs) × (TE) for raw and unpeeled boiled light-yellow and yellow tubers. The relationship between TP content and TE in potatoes after boiling was significant for unpeeled tubers. The greatest matching of the model characteristics of the interdependence of features (ϕ^2) was 75% for the model (AA) × (TE) and 80% for the model (TPs) × (TE).

ABBREVIATIONS

AA - ascorbic acid.
DM - dry matter.
TPs - total phenolics.
TE - antioxidant activity, Trolox equivalents.

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PAPER

A CONTINUOUS STUDY ON QUALITATIVE ASSESSMENT OF REHYDRATED 'ANNURCA' APPLE: INFLUENCE OF PROCESS CONDITIONS AND DRYING PRE-TREATMENT

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ABSTRACT

In a previous paper the effect of chemical pre-treatment on quality attributes of 'Annurca' apple slabs dried at different temperatures was investigated. Herein, we evaluated the effect of the same pre-treatment on the quality attributes of the same dried 'Annurca' apple samples rehydrated at two temperatures. Specifically, slabs were initially pre-treated in a dipping solution containing trehalose, sodium chloride, sucrose. Then, they were dried by using a convective dryer at 50°, 55°, 60°, and 65°C, and rehydrated at 30° and 70°C by immersion in water. The combination of pre-treatment, drying at 65°C and rehydration temperature of 30°C enabled to obtain the best preservation of rehydration indices (i.e, water absorption capacity), structure and colour properties. On the contrary, the highest antioxidant activity (EC_{50}) in treated samples was found at the lowest drying temperatures (50° and 55°C) among those investigated and rehydration temperature of 30°C. The PCA provided different behaviours among untreated and treated dried apples when rehydrated at 30° and 70°C, demonstrating that this pre-treatment combined with drying/rehydration temperatures influenced the quality attributes of rehydrated samples.

Keywords: 'Annurca' apple, pre-treatment, rehydration, drying, PCA

1. INTRODUCTION

The 'Annurca' apple (*Malus* x *domestica* Borkh. cv Annurca Rossa del Sud) is a temperate and traditional fruit, cultivated in Southern Italy, in particular in the Campania Region. 'Annurca' appleis rich in bioactive components such as polyphenols, flavonoids and anthocyanins which can contribute to fruit quality in terms of nutritional values, flavour and colour (D'ABROSCA *et al.*, 2017).

Drying is a common method used for apple's preservation and for their consumption over long periods of time on the global markets. Drying contributes to extend the shelf life (more than one year) (NOWACKA*et al.*, 2014) and improves food stability by reducing content-microbial growth, water activity, and minimizing chemical deterioration. Furthermore, drying process creates new processed products, such as rehydrated apples, and reducing the cost of transportation and storage (PROIETTI *et al.*, 2018; ROJAS and AUGUSTO, 2018; WANG *et al.*, 2018; BAEGHBALI *et al.*, 2019; RUSSO *et al.*, 2019;).

However, drying can cause some undesirable changes in fruits including browning and oxidation reactions, case hardening and degradation of nutritional compounds. These changes affect the overall quality of dried fruits, as well as the consumer acceptability (WANG *et al.*, 2018; ÖNAL *et al.*, 2019). Moreover, the structure of tissue is partially destroyed, which may affect water permeability, and as a result the rehydration ability and the product texture (KROKIDA *et al.*, 2000; COX *et al.*, 2012).

Most of the dried foods and particularly apples, are usually rehydrated before their consumption, i.e, in bakery products, instant products (soup), milk products (yogurt, ice-cream), fruit tea – infusion and liqueurs. Rehydration is a complex process, which aims for reconstituting the fresh food's properties by contacting dried product with water or other liquids, i.e, fruit juice, sucrose or glucose solution. The rehydration process consists of three steps at the same time: dried food absorbs water, swelling occurs in the rehydrated product, and soluble components are lost or are diffused through the solution (LEWICKI, 1998; MOREIRA *et al.*, 2008; COX *et al.*, 2012). Rehydration characteristics of dried fruits are considered as quality parameters. Such characteristics are influenced by the samples' composition (i.e, protein content, volume and density of dried products), the drying conditions (i.e, temperature and type of process), and the pre-treatment. Moreover, rehydration temperature is the factor that plays major role on rehydration rate, water uptake and volume changes. On the other hand, during rehydration, immersion of the dried food products in water could cause loss of nutrients (i.e, phenolic content, antioxidant activity) and colour pigments (AMIN *et al.*, 2006; MOREIRA *et al.*, 2008; TUNDE-AKINTUNDE, 2008). In this context, the determination of the best rehydration conditions can be appropriate for deeper understanding of rehydration process. Besides, the improvement of quality attributes such as rehydration indices, volume changes, colour, antioxidant activity seems to be crucial to produce high quality rehydrated new products.

The combination of drying temperatures and pre-treatments has been widely implemented in literature leading to improvements in the drying/rehydration process and the quality of final products (i.e, structure, colour, bioactive compounds, sensorial evaluation) and energy savings (LEWICKI, 1998; VEGA-GÁLVEZ *et al.*, 2008; ADILETTA *et al.*, 2015; ADILETTA *et al.*, 2016a; DA COSTA RIBEIRO *et al.*, 2016; ADILETTA *et al.*, 2018; DERMESONLOUOGLU *et al.*, 2018; ÖNAL *et al.*, 2019). In literature there are many studies focused on the application of different dipping pre-treatment solutions to improve fruits and vegetables' rehydration process: ethyl oleate alkaline solution for tomatoes

(DOYMAZ, 2007); citric acid solution and blanching for apple slices (DOYMAZ, 2010); ethyl oleate and sodium carbonate for cape gooseberries (JUNQUEIRA *et al.*, 2017).
Nevertheless, to our knowledge, no work has fully investigated the effect of a pre-treatment on the quality attributes of the rehydrated food product. In this sense, an-in-depth understanding of rehydration process and how it is affected by pre-treatment conditions is essential for the improvement of process design and rehydrated product quality, as well as, for development of new products.
In this work carbohydrate/salt solution is investigated as alternative process protective agents of food products during the drying/rehydration processes. The novel aspect of this work is to clarify the effect of carbohydrate/salt solution on the rehydration phase of dried apples. Trehalose is a naturally occurring and known as one the non-reducing sugars. Trehalose substitutes water molecules in membrane and thereby preventing the phase transition. Trehalose has unique properties on the preservation of the biostructures during the drying process. Moreover, trehalose plays an important role in the minimization of quality deteriorations such as loss of nutrients, protect the colour and flavour caused by Maillard browning reactions (PATİST and ZOERB, 2005; ADİLETTA *et al.*, 2016a; AKTAŞ *et al.*, 2017). The mixture of trehalose with other compounds or trehalose alone becomes important because of its numerous advantages in food drying application (i.e, shorter drying time, enhancement of drying rate, protection of flavour and colour, improvement of reconstitution ofdried foods properties, inhibition of protein denaturation and higher nutritional content) (PATIST and ZOERB, 2005; ATARÉS*et al.*, 2008; OHTAKE and WANG, 2011; XIN *et al.*, 2013; BETORET*et al.*, 2015; ÖNAl*et al.*; 2019).
Most of works are concentrated on the use of trehalose for osmo-dehydrated or freeze-dried food materials (DERMESONLOUOGLOU *et al.*, 2007; XİN *et al.*, 2013). However, detailed information on the effects of trehalose of the rehydrated dried fruits is still lacking, particularly for apple. Therefore, in this paper, trehalose was used as a stabilizer in the preparation of a chemical pre-treatment solution. The addition of sodium chloride salt to this solution aims for improving the texture preservation and the rehydration ability (Lewicki, 1998; DERMESONLOUOGLOU *et al.*, 2007).
Finally, regarding to rehydration process, several papers deal with the modelling of rehydration process of foodstuffs dried with different methods: hot air drying GARCÍAPASQUAL *et al.*, 2006, ZURA-BRAVO *et al.*, 2013); freeze-drying (LOPEZ-QUİROGA *et al.*, 2019; WALLACH *et al.*, 2011); swell drying and vacuum multi flash drying BENSEDDİK *et al.*, 2019). However, limited information is currently available on the effect of rehydration process temperature on the quality attributes of dehydrated products, such as dried edible Irish Brown seaweed (COX *et* al., 2012) and dried apple slices (ZURA-BRAVO *et al.*, 2013).
Therefore, the present work aims to study the quality of rehydrated 'Annurca' apples, both untreated and treated, at two rehydration temperatures (30° and 70°C) (DOYMAZ, 2010). These temperatures resemble the rehydration at approximately room temperature (e.g. milk) and in hot water (e.g. soup, tea, infusion).
In this framework, a combined pre-treatment of trehalose, sodium chloride and sucrose solution was here utilised and its effect, and that of drying and rehydration temperatures, was investigated on the rehydration indices, colour, volume changes and antioxidant activity of the rehydrated apples.
From an industrial perspective, the development of dried foods is a key to provide for commercialization of innovative rehydrated products and to increase consumers' demand of healthier, convenience and ready-to-eat-foods.

2. MATERIALS AND METHODS

2.1. Raw material preparation

'Annurca' apples were obtained from supermarket in Campania Region, Italy, after reddening–ripening treatment (LO SCALZO *et al*., 2001).
Uniform size, without mechanical damage and freshness were used to select the best samples. Several apple fruits were washed with tap water, peeled by using knife and cylinders (30±0.22 mm for diameter and 5±0.01 mm for thickness) were prepared.
Two different type of samples were analysed in this research: apple slabs without any pre-treatment (UTR) and apple slabs treated (TR) by dipping in carbohydrate/salt solution (0.8% trehalose, 0.1% NaCl and 1.0% sucrose); dipping temperature of 25°C and dipping time of 15 min (ÖNAL *et al*., 2019).

2.2. Drying experiments

The treated (TR) and untreated (UTR) slabs were dried in a convectional drier (Zanussi FCV/E6L3) at four different drying temperatures (50°, 55°, 60°, and 65°C), with a constant air velocity of 2.3 m/s. Drying experiments were stop when the moisture content of slabs was about 0.04 kg water/kg db. In order to evaluate the drying kinetics, for each type of samples, three slabs were continuously weighted using a sensor (Phidgets INC., Canada). This weight sensor is a load cell composed of a transducer, which is able to convert mechanical force into electrical signals. The samples' weight loss was recorded online every 5 min.

2.3. Rehydration experiments

Rehydration experiments of apple slices, previously dried, were performed at the specified rehydration temperatures in distilled water by using water bath to evaluate rehydration capacities (DOYMAZ, 2010; BARRERA *et al*., 2016). The rehydration temperatures were selected as 30°C and 70°C on the basis of the literature (DOYMAZ, 2010) for the experimental design of rehydration process. The water to apple ratio was about 100:1 (weight basis). At specific time, samples were taken out from liquid, blotted with tissue paper and measured by using an electronic balance. Slabs were weighted every 15 min in the initial phase of rehydration process (up to 120 min) and then every 30 min.
The rehydration test was performed in triplicate for each apple sample. The rehydration capacity was calculated as percentage water gain (ADILETTA *et al*., 2016a), as follows:

$$\text{Weight gain (\%)} = \frac{\text{(weight of rehydrated samples-weight of dried samples)}}{\text{(weight of dried samples)}} \times 100 \qquad (1)$$

In order to have more information about the amount of absorbed water, the amount of removed solutes, and the degree of cellular and structural disruption during rehydration, four quality indices were investigated (LEWICKI, 1998; BARRERA *et al*., 2016), as follows: (1) water absorption capacity, WAC; (2) dry matter holding capacity, DHC, (3) rehydration ability, RA; and (4) water holding capacity, WHC. The WAC index explains the ability of food material to absorb water that replaces the water removed during drying. The DHC index is a measure of food material ability to hold soluble solids after rehydration; it gives information on tissue destruction and on tissue permeability to solutes. The RA index

describes the rehydration ability of dried product, and it indicates the total tissue destruction caused by both drying and rehydration conditions (MALDONADO *et al.*, 2010). The WHC index measures the ability of product to maintain its own and added water during the rehydration process. Also, WHC has an important role in the food structure modifications (ZAYAS, 1997).
Three indices were calculated by using Eq. (2) to Eq. (4), which proposed to describe the food's behaviour after rehydration process (Barrera et al., 2016). These indices are water absorption capacity (WAC), dry matter holding capacity (DHC), rehydration ability (RA), defined as follows:

$$WAC = \frac{M_R . x_R^W - M_D . x_D^W}{M_0 - M_D} \quad (2)$$

$$DHC = \frac{M_R . (1 - x_R^W)}{M_D . (1 - x_D^W)} \quad (3)$$

$$RA = WAC \cdot DHC \quad (4)$$

where: M is the total mass in g, and x^i is the mass fraction of i component in g/g, the subscripts 0, D and R state the fresh sample, the completely dried and rehydrated samples, respectively, while superscript w states water.
It was also calculated the water holding capacity (WHC) of the rehydrated structure from the soluble solids content of its liquid phase (z) and the amount of liquid (MCF) removed by centrifugation at 4000 rpm for 10 min (Eq. 5).

$$WHC = \frac{M_R . x_R^W - M_{cr} (1 - . z_R^{SS})}{M_R . x_R^W} \quad (5)$$

2.4. Surface colour measurement

The colour parameters of both untreated and treated fresh and rehydrated apple slabs were measured using a colourimeter (Chroma Meter II Reflectance CR-300 triple flash mode aperture 10 mm Minolta, Japan), calibrated previously with a white standard ceramic plate. To analyze the colour change of fresh and rehydrated samples, CIE lab colour coordinates (L*, a* and b*) were recorded and the average values were calculated for each sample. The lightness value (L*) indicates the lightness/darkness of the sample, a* index represents green when negative and red when positive, and b* index represents blue when negative and yellow when positive.
White index (WI), the whiteness degree of samples, was determined as follows (ADILETTA *et al.*, 2016b):

$$WI = 100 - \sqrt{(100 - L*^2) + (a*^2) + (b*^2)} \quad (6)$$

2.5. DPPH radical scavenging activity

Extracts from fresh and rehydrated apple samples were obtained according to D'ABROSCA *et al.* (2017) with some modifications. Methanol solution (10 mL, 80% v/v) (CHROMASOLV®, for HPLC, ≥99.9, Sigma-Aldrich, USA) was added to fresh (5 g ± 0.01) and rehydrated samples (3 g ± 0.01), after reducing to small pieces. The mixture was homogenized throughout an ultraturrax at 10 rpm and then stirred by vortex for 10 min. Supernatant was filtered by using a Whatman No:2 filter paper. All extractions were performed in triplicate.
The total antioxidant activity of allapple slabs was determined by the DPPH radical scavenging method (BRAND-WILLIAMS *et al*., 1995). Different extracts volumes were mixed with 3.5 mL of 6×10-5 M of DPPH methanol solution in cuvettes. The obtained solution was shaken properly and left for 30 min at room temperature in the dark. The absorbance of solution was measured at 517 nm by using UV-Vis spectrophotometer at 517 nm (Lambda Bio 40; PerkinElmer, Waltham, MA, USA). Methanol was used as the blank, while the control sample was without adding any extract. Percentage of inhibition of DPPH radical was calculated as follows:

$$\% \text{ Antioxidant Activity: } (Abs_{control} - Abs_{sample} / Abs_{control}) \times 100 \qquad (7)$$

Where $Abs_{control}$ is the absorbance of control and Abs_{sample} that of the sample.
The results were showed in terms of the EC_{50} value, which was identified as the sample concentration (mg/mL) required to inhibit 50% of the DPPH radical scavenging activity. EC_{50} was determined from a graph of antioxidant capacity (%) versus extract concentration (mg/mL).

2.6. Diameter and thickness evolution

To determine the reconstitution of volume of dried apples, diameter and thickness were measured every 30 min during the rehydration experiments at 30° and 70°C. The average thickness and diameter of UTR and TR samples were calculated as the average of the measured values of 5 slabs for each sample through image analysis (NH Image/ Image J Software 1.8.0). Both dimensions were measured at different positions of the slices and their average value was considered. In particular, the measurement positions on rehydrated apple slabs were as follows: four positions for diameter (along two perpendicular axes and two diagonal axes) and eight positions for thickness (PONKHAM *et al.*, 2012).

2.7. Statistical analysis

All results were repeated three times and they were reported as the mean±standard deviation (S.D). One way analysis (ANOVA) and Tukey test were applied for comparing mean values by using SPSS 24 Software statistics program (SPSS Inc., Chicago, USA). Any statistical difference was considered significant with $p<0.05$ and it was indicated with different letters. Principal component analysis (PCA) was used to identify the principal components contributing to most of the variations within the dataset, evaluating the impact of dipping pre-treatment and drying/rehydration temperatures on the quality

characteristics of rehydrated apples (UTR and TR). All analyses were performed with SPSS software package, version 24 (SPSS Inc., Chicago, USA).

3. RESULTS AND DISCUSSION

In our previous paper (ÖNAL *et al.*, 2019), the impact of a novel and natural dipping pre-treatment containing trehalose, sucrose and sodium chloride, and air drying temperatures (50°, 55°, 60° and 65°C) was evaluated on drying characteristics and quality properties of dried apples. The results demonstrated that, the dipping pre-treatment containing trehalose allowed to decrease drying times, to better retain physico-chemical, nutritional and sensorial attributes (i.e, colour, shrinkage, microstructure, total phenolics compound and antioxidant activity) and obtain high quality of dried apple snacks.
This study is a continuation of our work already published (ÖNAL *et al.*, 2019), and it will provide information on rehydration characteristics of hot-air dried 'Annurca' apple, as well as, rehydrated apples' quality attributes.

3.1. Rehydration kinetics and rehydration indices

The rehydration is an important process, which is used for understanding the quality of dried food products. The physico-chemical changes and structural modifications that occurred during drying, generate cell collapse and volume reductions, reduce the absorption of water, thereby avoiding the complete rehydration of dried products (LEWICKI, 1998; KROKIDA *et al.*, 2003; MOREIRA *et al.*, 2008; ARAL and MEŞE, 2016). This rehydration process is hence affected by several factors, for instance, physico-chemical properties of food, pre-treatment, drying process and conditions.
Results reported in the following refer to apple samples (UTR and TR) dried at four different temperatures (50°, 55°, 60°, and 65°C), and rehydrated at 30° and 70°C.
In order to evaluate the damage of drying and the impact of rehydration temperature on the rehydration behaviour, the weight gain (%) during rehydration was shown in Fig. 1A-B and Fig. 2A-B for both UTR and TR dried apples at temperatures of 30° and 70°C, respectively.

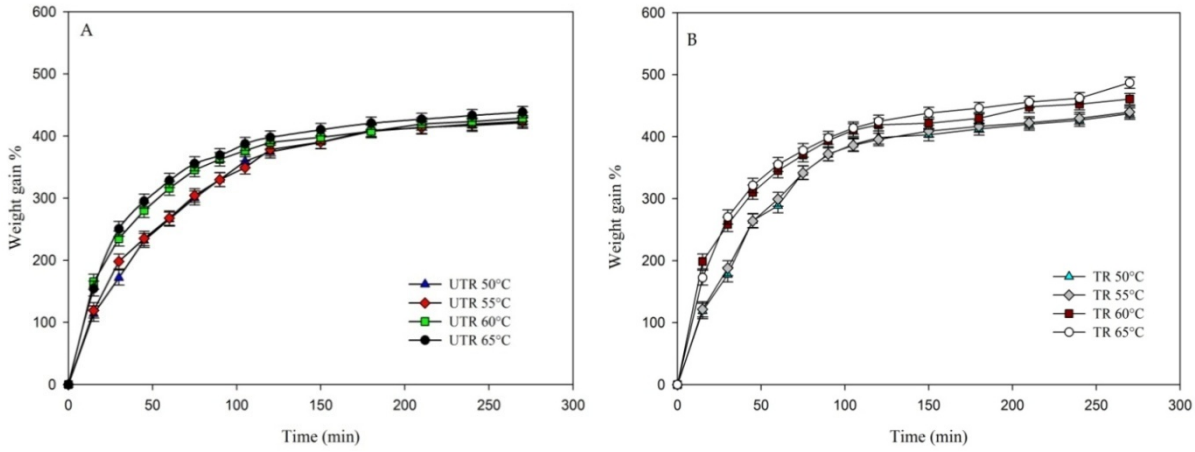

Figure 1. Rehydration kinetics of untreated (A) and treated (B) dried samples (50°, 55°, 60° and 65°C) at rehydration temperature of 30°C.

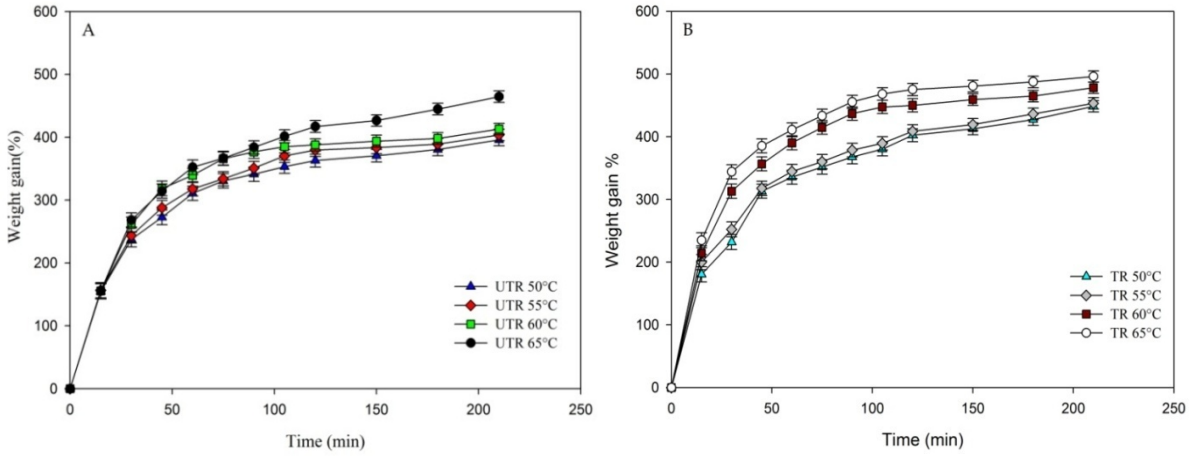

Figure 2. Rehydration kinetics of untreated (A) and treated (B) dried samples (50°, 55°, 60° and 65°C) at rehydration temperature of 70°C.

It was noticed that at any rehydration temperature all the samples showed the same trend. All rehydration curves showed a clear logarithmic trend, and as expected, the rehydration time decreased with increasing temperature from 30° to 70°C. The total rehydration time, that is the time at which a constant water amount was reached, was found as 270 and 210 min for both treated and untreated dried samples, and for rehydration temperatures of 30° and 70°C, respectively.

At the end of the experiments at 30° and 70°C, untreated and treated samples reached almost the same water gain of 420% and 460%, respectively. When rehydration time proceeded, it was observed a reduction in the driving force for water transfer and the system slowly reached equilibrium.

The initial rapid water uptake lasted different times: for both samples at 70°C it was about 30 min with respect to 30°C where it was about 100 min. The fast initial water uptake of curves was detected at rehydration times of 30 and 100 min and that the moisture content on the apples' surface attains the saturation value, almost instantaneously. Hot air dried apple samples exhibited an initial high rate of water uptake followed by a slower rehydration stage that lead to equilibrium moisture values in the rehydration curves– approximately after those times.

The fast initial water uptake is due to the filling of cavities and capillaries near the surface (ÖNAL *et al.*, 2019). Then, the diffusion of water in the pores inside the sample is dominant.

Regarding to the drying conditions, in a previous work (ÖNAL *et al.*, 2019), which analyzed the effect of drying temperatures on drying kinetics and quality properties of dried apples, the optimal drying temperature was found 65°C for preserving the principal quality attributes (i.e, colour, shrinkage, sensorial evaluation and rehydration capacity).

The TR slices showed higher rehydration capacity compared with the UTR ones at both rehydration temperatures. Less rehydration capacity of untreated apples was correlated to the collapse of tissue by higher exposure time during drying. The structure of untreated ones was significantly modified by drying. In other words, the carbohydrate/salt solution containing trehalose here proposed is able to protect apple structure during drying. This is in agreement with the findings reported by ATARÉS *et al.* (2008), DOYMAZ (2010),

VÁSQUEZ-PARRA *et al.* (2013), JUNQUEIRA *et al.* (2017) and ADILETTA *et al.* (2016a,b; 2018) that found higher rehydration capacity during the rehydration experiments, when different pre-treatments were used to preserve the food structure during drying. On the contrary, some studies have mentioned that an increment of rehydration capacity of dried fruits such as for hawthorn (ARAL and MEŞE, 2016), apple (DOYMAZ, 2010; ZURA-BRAVO *et al.*, 2013), red pepper (VEGA- GALVEZ *et al.*, 2008) and lemon (WANG *et al.*, 2018) was observed by increasing the rehydration temperature. This because the higher rehydration temperatures cause the tissue collapse and cell damage, creating larger spaces in dried fruits and in this way enhancing the rehydration ability of the dried materials (WANG *et al.*, 2018).

In Tables 1 and 2, the rehydration quality indices (WAC, DHC, RA and WHC) of both TR and UTR dried apples (50°, 55°, 60° and 65°C) at rehydration temperatures of 30° and 70°C, respectively, were reported. At 30°C, the WAC and DHC indices showed significantly ($p<0.05$) higher values for treated samples dried at 60° and 65°C. This trend indicates that the treated samples were able to absorb more water at low rehydration temperature with regard to the untreated ones. Similar findings were reported by Barrera *et al*. (2016), which found higher values of WAC and DHC indices in rehydrated apples treated previously with vacuum impregnation with sucrose solution. They stated that these higher indices are correlated to higher rehydration ability of apple samples. Furthermore, the highest ability to rehydrate (RA index) and the highest WHC values were observed in treated samples dried at 60° and 65°C and rehydrated at 30°C. Accordingly, increasing rehydration temperature (70°C) leads to texture damage likely due to the fact that the breaking or the denaturation of polysaccharides of cell wall promotes a remarkable reduction of mechanical resistance in the apples. Similar WHC results were obtained by ZURA-BRAVO *et al.* (2013), which found that the highest rehydration temperature (60°C) resulted in lower WHC of rehydrated apple slices.

Table 1. Rehydration indices of both untreated (UTR) and treated (TR) dried samples (50°, 55°, 60° and 65°C) at rehydration temperatures of 30°C.

Rehydration at 30°C	WAC	DHC	RA	WHC
UTR 50°C	0.781 ± 0.03^{a}	0.233 ± 0.007^{a}	0.182 ± 0.02^{a}	0.794 ± 0.013^{a}
TR 50°C	0.806 ± 0.02^{ab}	0.243 ± 0.004^{a}	0.196 ± 0.009^{ab}	0.822 ± 0.02^{ab}
UTR 55°C	0.784 ± 0.024^{a}	0.240 ± 0.008^{a}	0.188 ± 0.005^{ab}	0.796 ± 0.06^{a}
TR 55°C	0.827 ± 0.03^{ab}	0.247 ± 0.003^{a}	0.204 ± 0.014^{ab}	0.859 ± 0.002^{abc}
UTR 60°C	0.816 ± 0.014^{ab}	0.241 ± 0.005^{a}	0.197 ± 0.03^{ab}	0.844 ± 0.03^{abc}
TR 60°C	0.847 ± 0.02^{b}	0.268 ± 0.003^{b}	0.227 ± 0.02^{ab}	0.902 ± 0.015^{c}
UTR 65°C	0.831 ± 0.02^{ab}	0.243 ± 0.006^{a}	0.202 ± 0.009^{ab}	0.877 ± 0.02^{bc}
TR 65°C	0.853 ± 0.013^{b}	0.274 ± 0.004^{b}	0.234 ± 0.012^{b}	0.911 ± 0.02^{c}

Data are the average of three replicates±standard deviation. Different superscript letters in the same column mean significant differences ($p<0.05$).

BARRERA *et al.* (2016) revealed that the vacuum impregnation (VI) with an isotonic sucrose solution significantly improved rehydration process of apple. This explanation was confirmed by higher values of WAC, DHC and WHC reached by VI sucrose samples.

After rehydration experiments at higher temperature 70°C, no significant differences ($p>0.05$) were found in the following indices: DHC, RA, WHC between all untreated and treated samples; except for the treated samples dried at 60°C which showed the highest WAC value.
The combination of lower rehydration temperature (30°C) and higher drying temperatures (60° and 65°C) with this pre-treatment was proven to be useful to preserve the rehydrated apple structure by reducing cellular damage and promoting the absorption of great amount of water.

Table 2. Rehydration indices of both untreated (UTR) and treated (TR) dried samples (50°, 55°, 60° and 65°C) at rehydration temperature of 70°C.

Rehydration at 70°C	WAC	DHC	RA	WHC
UTR 50°C	0.767 ± 0.02^{a}	0.222 ± 0.02^{a}	0.177 ± 0.005^{a}	0.807 ± 0.02^{a}
TR 50°C	0.811 ± 0.023^{ab}	0.236 ± 0.003^{a}	0.191 ± 0.012^{a}	0.825 ± 0.02^{a}
UTR 55°C	0.799 ± 0.03^{ab}	0.213 ± 0.003^{a}	0.170 ± 0.02^{a}	0.813 ± 0.14^{a}
TR 55°C	0.822 ± 0.02^{ab}	0.237 ± 0.02^{a}	0.195 ± 0.03^{a}	0.823 ± 0.03^{a}
UTR 60°C	0.801 ± 0.02^{ab}	0.218 ± 0.014^{a}	0.181 ± 0.02^{a}	0.821 ± 0.02^{a}
TR 60°C	0.830 ± 0.01^{b}	0.241 ± 0.02^{a}	0.200 ± 0.003^{a}	0.870 ± 0.015^{a}
UTR 65°C	0.790 ± 0.015^{ab}	$0,240\pm0.02^{a}$	0.190 ± 0.004^{a}	0.843 ± 0.009^{a}
TR 65°C	0.817 ± 0.01^{ab}	0.239 ± 0.01^{a}	0.195 ± 0.008^{a}	0.879 ± 0.005^{a}

Data are the average of three replicates±standard deviation. Different superscript letters in the same column mean significant differences ($p<0.05$).

3.2. Diameter and thickness evolution

Diameter and thickness evolution is another important factor that should be analyzed during the rehydration tests of apples.
The average diameter and thickness of all dried apples were evaluated during the rehydration. All samples had similar increasing trend of the diameter and thickness during the experiments at 30° and 70°C. In Figs. 3A-B and 4A-B the diameter and thickness of UTR and TR samples dried at 65°C were compared. In all the conditions investigated, they did not reach the diameter and thickness of fresh samples, which were respectively 30 mm and 5 mm.
Obviously, the fastest increment of diameter and thickness took place in the initial period of the rehydration process. In the further stage of process, water absorption slowed down since rehydrated samples got close to the state of balance with equilibrium moisture content.
There were significant increments of diameter and thickness of both samples (TR and UTR) up to 120 min of the rehydration process (for 30° and 70°C). Higher increases in diameter and thickness were observed in treated samples than untreated ones at both temperatures: at 30°C the recovered volume (with respect to fresh one) of TR samples was 78% while that of UTR samples was 71%. In additon, Table 3 showed the diameter and thickness values of untreated (UTR) and treated (TR) apple samples dried at 65°C reached at the end of rehydration step at 30° and 70°C. The final diameter values were significantly different from each other. The '65 TR 30°C' sample exhibited higher diameter than the samples '65 UTR 30°C', '65 UTR 70°C', and '65 TR 70°C'.

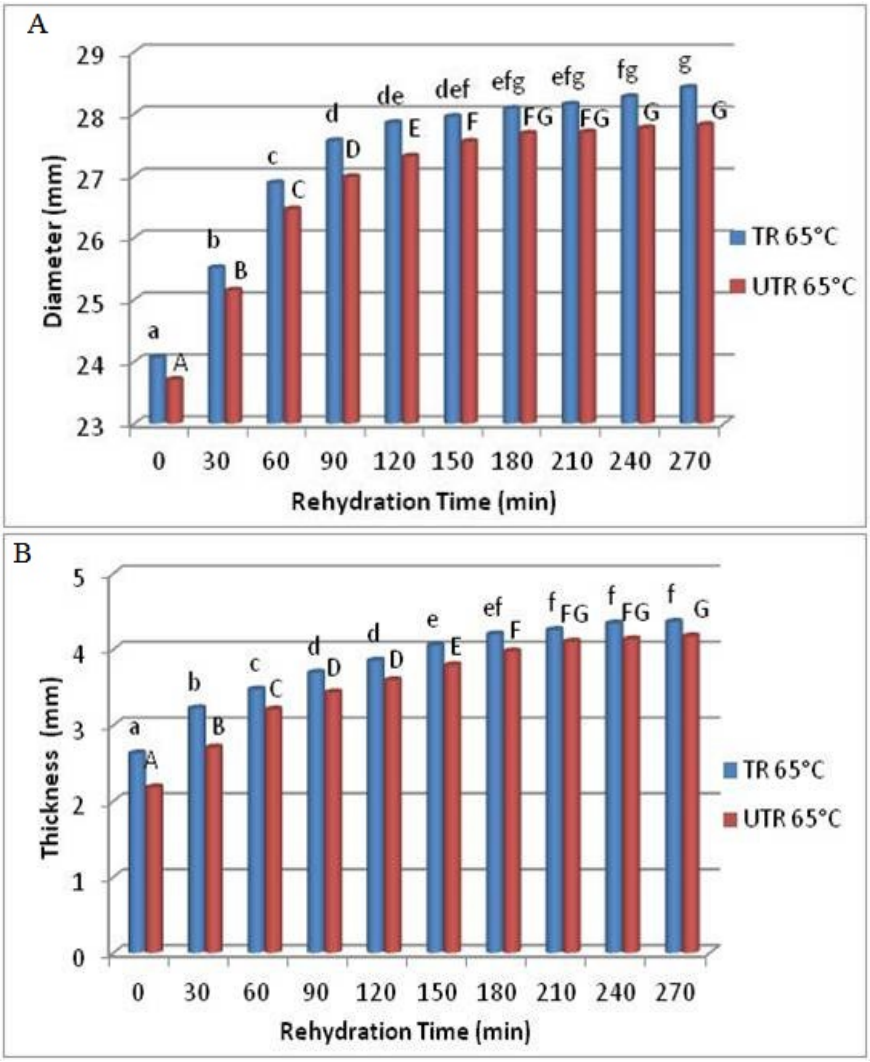

Figure 3. Diameter (A) and thickness (B) of untreated (UTR) and treated (TR) rehydrated samples dried at 65°C during rehydration at 30°C.
Mean values in treated samples with different lower letters are significantly different ($p<0.05$) during the rehydration time, and mean values in untreated samples column with different capital letters are significantly different ($p<0.05$) during the rehydration time.

These results indicated that the pre-treatment solution and rehydration temperature had great impact on final size, shape and appearance of apple samples, therefore on the consumer acceptability. Similarly, in concern with final thickness, no significant differences were found between the samples '65 UTR 70°C' and '65 TR 70°C', while the'65 TR 30°C' sample had the highest final thickness values. Such behaviour is probably due to use of dissacharides, particularly trehalose which effect on pectic cell components results mainly on protecting functionality of proteins and stabilisng the three-dimensional structure of protein (LEWICKI, 1998). In this way, the cell membrane is protected and upon rehydration its functionality is restored. The structure changes and loss of nutritional compounds in untreated samples may be associated with the observation of crack internally in the later stages of rehydration at 70°C. Moreover, the diameter changes were more significant than thickness ones in all investigated samples because the

shrinkage due to the drying occurs preferentially along the diameter than the thickness of the slabs. Among the two investigated temperatures, the best temperature was 30°C with regards to the increment of diameter-thickness of samples.

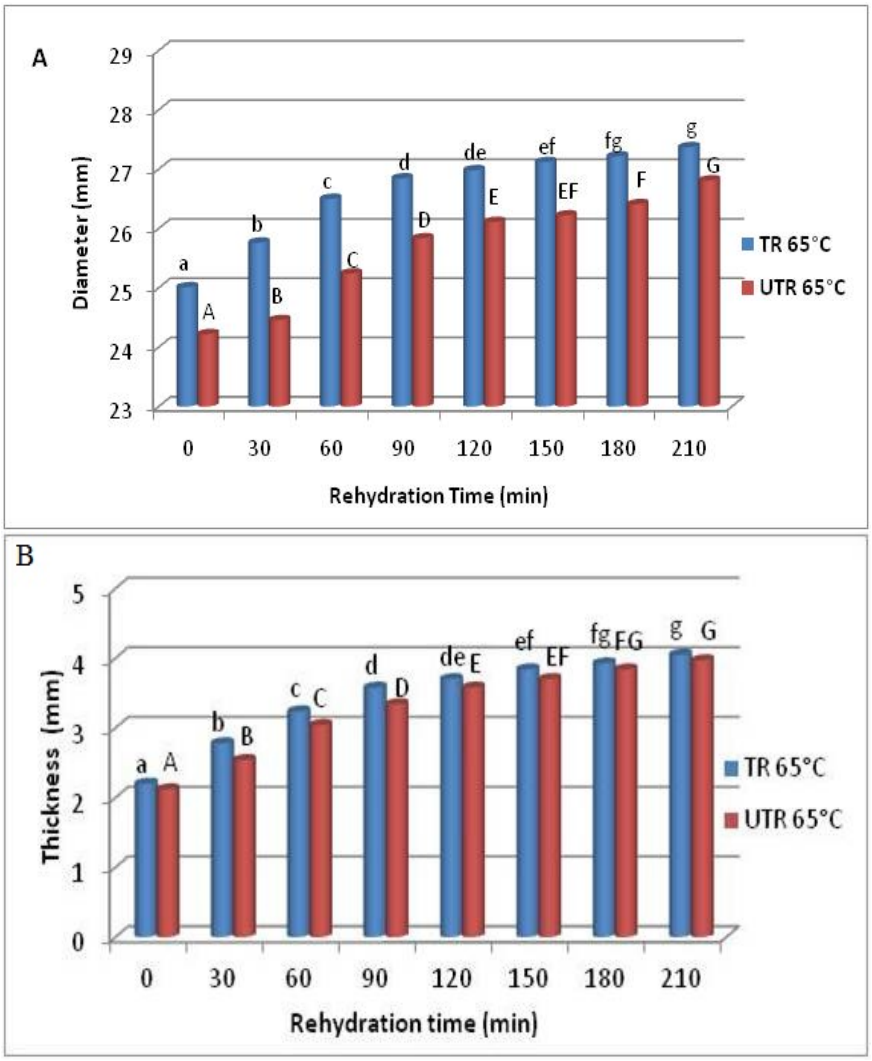

Figure 4. Diameter (A) and thickness (B) of untreated (UTR) and treated (TR) rehydrated samples dried at 65°C during rehydration at 70°C.
Mean values in treated samples with different lower letters are significantly different ($p<0.05$) during the rehydration time, and mean values in untreated samples column with different capital letters are significantly different ($p<0.05$) during the rehydration time.

The ability to be reconstituted of food products depends especially on the inner structure of the dehydrated samples. The rehydration temperature increament induces a structural damage, increasing that caused during the dehydration process. Moreover, increasing temperature results in low mechanical resistance and elasticity in the products. It is seen that, treated apples have homogenous structure which is protected by the pre-treatment and upon rehydration its functionally is restored. This maybe be attributed to the trehalose solution which replaces water in membrane and prevent the phase transition in dried apples. Moreover, trehalose is able to form glassy matrices, and direct the sugar

interaction with polar groups in phospholipids and proteins, thereby maintaining and stabilizing cellular structure (CROWE *et al.*, 1988; LEWICKI, 1998; BETORET *et al.*, 2015).
Several studied investigated the impact of pre-treatments and of drying/rehydration conditions on the dimension changes of dried fruit and vegetable during their rehydration. WINICZENKA *et al.* (2014) found that the temperature of drying influenced the relative increase of the volume of dried apples during rehydration at 20°C.
According to BILBAO-SÁINZ *et al.* (2005), apple cylinders dehydrated in microwave oven with vacuum impregnation showed that the recovered volume (%) of impregnated samples was higher than the recovered volume of non-impregnated ones. A justification to this result is that the major part of the initial gas (air) present in the pores is released by vacuum impregnation and entrance of the isotonic solution of the apple juice in the pores increases the mass of sample.

Table 3. Final diameter and thickness values of untreated (UTR) and treated (TR) rehydrated samples dried at 65°C during rehydration at 30° and 70°C.

Sample	Diameter (mm)	Thickness (mm)
65 UTR 30°C	27.82±0.05^{c}	4.17±0.04^{b}
65 UTR 70°C	26.81±0.05^{a}	3.97±0.04^{a}
65 TR 30°C	28.42±0.05^{d}	4.36±0.04^{c}
65 TR 70°C	27.37±0.05^{b}	4.05±0.04^{a}

3.3. Colour evaluation

Colour parameters are also important as quality indices for rehydrated food products and they should closely resemble the colour characteristics of fresh food material to increase consumer acceptability (COX *et al.*, 2012). The evaluation of rehydration conditions with the aim of minimizing the colour changes during drying/rehydration process is crucial from an economic perspective. The effects of pre-treatment and drying/rehydration temperatures on colour parameters of fresh and rehydrated apple slabs were presented in Table 4. Lightness (L*) and white index (WI) were reported. According to results, the colour values of rehydrated apple slabs were significantly different ($p<0.05$) in relation to fresh ones. It was observed a decrease in L* and WI values in all samples, indicating a reduction of lightness respect to fresh ones. This is an unexpected result since it is believed that an increase in water gain would normally lead to a higher luminosity (GOWEN *et al.*, 2006; MOREIRA *et al.*, 2008). Based on these results, it is argued that some modifications in the optical properties of the apples occurred during the rehydration. Oxidation processes or other chemical reactions such as Maillard reactions probably led to formation of browning agents (GOWEN *et al.*, 2006; MOREIRA *et al.*, 2008; LEMUS-MONDACA *et al.*, 2009; DENG *et al.*, 2017). Treated rehydrated samples had higher L* and WI values than untreated rehydrated ones at both rehydration temperatures (30° and 70°C), demonstrating that the pre-treatment preserves the colour stabilization of the final rehydrated apples. Furthermore, drying temperatures have a key role on colour attributes of dried products, as well as of rehydrated foodstuffs (LINK *et al.*, 2017). The higher drying temperature resulted in the best colour preservation in terms of lightness and white index at both rehydration temperatures. On the contrary, MOREIRA *et al.* (2008) and

ZURA-BRAVO *et al*. (2013) for rehydrated chestnut and apples, respectively, showed a reduction of L* values when the rehydration temperature increased.
As a consequence, the pre-treatment combined with higher drying temperatures (60° and 65°C) had significant effect on colour of rehydrated samples, while both used rehydration temperatures did not significantly influence the colour changes of rehydrated slabs.

Table 4. Colour parameters for untreated (UTR) and treated (TR) fresh and rehydrated samples (drying temperatures, 50°, 55°, 60° and 65°C) at rehydration temperatures 30° and 70°C.

Samples	L*	WI
Fresh Apples		
UTR Fresh	81.73±0.66^{f}	72.13±1.79^{g}
TR Fresh	84.79±2.89^{f}	76.91±1.02^{h}
Rehydrated Apples at 30°C		
UTR 50°C	53.26±1.97^{a}	45.30±2.00^{a}
TR 50°C	60.45±1.15^{bc}	59.48±1.31^{cde}
UTR 55°C	53.59±1.29^{a}	46.82±0.59^{a}
TR 55°C	65.98±0.77^{de}	58.31±0.90^{cd}
UTR 60°C	57.64±1.53^{ab}	45.68±0.89^{a}
TR 60°C	66.31±0.62^{de}	62.09±0.34^{ef}
UTR 65°C	59.15±2.79^{bc}	52.55±1.52^{b}
TR 65°C	66.81±0.25^{de}	62.88±1.60^{ef}
Rehydrated Apples at 70°C		
UTR 50°C	57.55±2.15^{ab}	56.25±1.93^{bc}
TR 50°C	65.51±1.71^{de}	62.57±0.31^{def}
UTR 55°C	59.35±0.61^{bc}	57.27±0.53^{c}
TR 55°C	65.39±0.55^{de}	64.74±1.61^{f}
UTR 60°C	62.77±1.59^{cd}	59.71±0.51^{cde}
TR 60°C	66.12±0.80^{de}	66.01±1.23^{f}
UTR 65°C	60.31±0.50^{bc}	58.13±1.38^{cd}
TR 65°C	68.13±0.18^{e}	65.75±1.83^{f}

Values are expressed as mean±standard deviation. All measurements are performed in triplicate. Values with different letters in a given column are significantly different ($p<0.05$).

3.4. DPPH radical scavenging activity

The knowledge of the content and stability of apple antioxidant components after rehydration treatments is essential to assess the nutritional values prior to its consumption (COX *et al.*, 2012). The radical scavenging activity for all analyzed samples was showed in Fig. 5 at the two rehydration temperatures (30° and 70°C). As expected, the lowest EC_{50} values (the highest antioxidant activity) were found as 16.06 and 18.66 mg/mL db in TR and UTR fresh samples, respectively.
Antioxidant activity of all untreated and treated rehydrated samples decreased after both rehydration processes at 30° and 70°C. For treated apples rehydrated at 30°C the DPPH activity was higher than those at 70°C. The pre-treatment combined with the lower drying

temperatures (50° and 55°C) and the lower rehydration temperature (30°C) can better protect the antioxidant activity of rehydrated apple slabs. The possible explanation of this trend is that during the drying and rehydration processes at higher temperatures modifications of the chemical structure of the main antioxidant compounds in apple (i.e., chlorogenic acid, quercetin, gallic acid, α- tocopherol) or interactions between antioxidant compounds and other apple constituents, such as proteins occurred (ÖNAL *et al.*, 2019). In addition, trehalose – dried and rehydrated foods showed a higher nutritional value with respect to foods processed by conventional system (COLAÇA and ROSER, 1994). In this case, lower rehydration temperature (30°C) had positive effect on the radical scavenging activity of rehydrated apples: higher water temperatures promoted significant loss of antioxidant compounds also into the water. Similar findings were stated by MOLDANADO *et al.* (2010), which reported that increases in temperature above 40°C resulted in higher loss of solid compounds.
In contrast to these results, COX *et al*. (2012) evaluated the influence of the rehydration temperatures (20°, 40°, 60°, 80° and 100°C) on the antioxidant activity of seaweed. They reported that the seaweed rehydrated at 80°C showed the highest antioxidant activity increment. Higher rehydration temperatures positively effect on the DPPH activity of seaweeds.
ZURA-BRAVO *et al.*, (2013) also found a higher antioxidant activity of rehydrated apple slices (*Granny Smith*) at 60°C rather than 20 and 40°C. This increment at higher rehydration temperature is attributed to the accumulation of melanoidins from Maillard reaction which have different antioxidant activity values. Hence, the higher rehydration temperature promoted the penetration of water, thus resulting in a high antioxidant activity (MIRANDA *et al.*, 2009; VEGA-GÁLVEZ *et al.*, 2009).

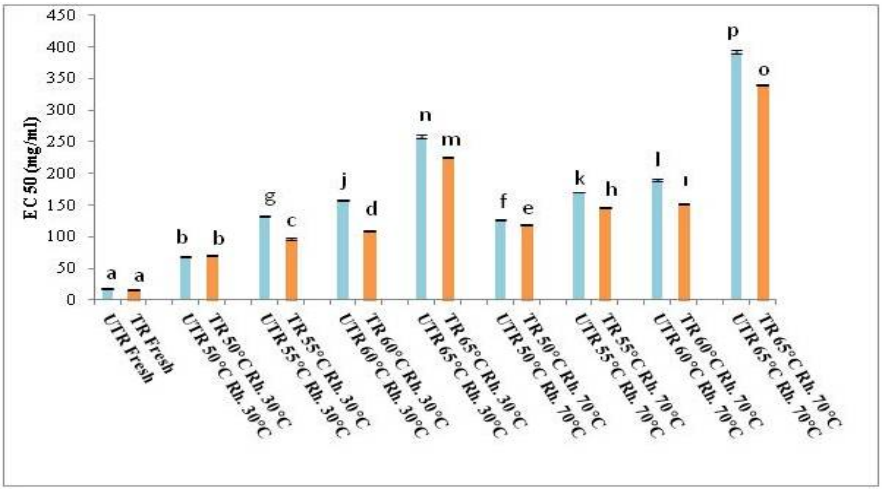

Figure 5. Antioxidant activity of both untreated (UTR) and treated (TR) fresh and rehydrated apples (drying temperature: 50°, 55°, 60° and 65°C) at 30°C and 70°C.

3.5. Effect of pre-treatment, drying/rehydration temperatures by PCA

The effect of pre-treatment and drying/rehydration temperatures on the qualitative traits of rehydrated apples were evaluated by PCA analysis. Covariance matrix showed that the eigenvalues accounted for 67.63% of the total variance in the dataset using two principal

components (PCs). PC1 explained 38.52% of the variance in the dataset, whereas PC2 explained an additional 29.11% of the variance. All loadings and scores were shown in the same PCA plot (Fig. 6).
WAC (R^2= 0.844), DHC (R^2=0.851), RA (R^2= 0.831), WHC (R^2= 0.590) were positively correlated to PC1; while L* (R^2= 0.807), WI (R^2=0.876), EC50 (R^2=0.585) indicated a positive correlation to PC2.
As shown in PCA plot, according to the rehydration temperature of 30°C, treated slabs dried at 50° and 55°C are more similar than those dried at 60° and 65°C. Furthermore, those dried at 60° and 65°C were more correlated with quality parameters in terms of WAC, DHC, RA and WHC along PC1, indicating that the drying temperature had significant impact on the ability to reconstitute the water content of apples during rehydration process. At the same rehydration temperature (30°C), untreated apples dried at 50° and 55°C were closer to each other than untreated apples dried at 60° and 65°C. As it is seen from Fig. 6, these latter (UTR dried at 60° and 65°C) were more correlated with quality parameters of rehydrated apples along PC2, i.e, colour parameters. It is clear that the higher drying temperatures (60° and 65°C) showed the better rehydration results in both UTR and TR apples.
With regard to the rehydration temperature of 70°C, treated dried samples at 50°, 55° and 60°C were similar. Scoring and loading plot enabled to differentiate the behaviour only for treated apples dried 65°C which were more correlated with PC2.
Untreated samples rehydrated at 70°C shifted from negative values to positive ones along PC2.
In conclusion, higher rehydration temperature (70°C) negatively affected quality parameters of rehydrated apples. The samples rehydrated at 30°C showed the better correlations with quality parameters.

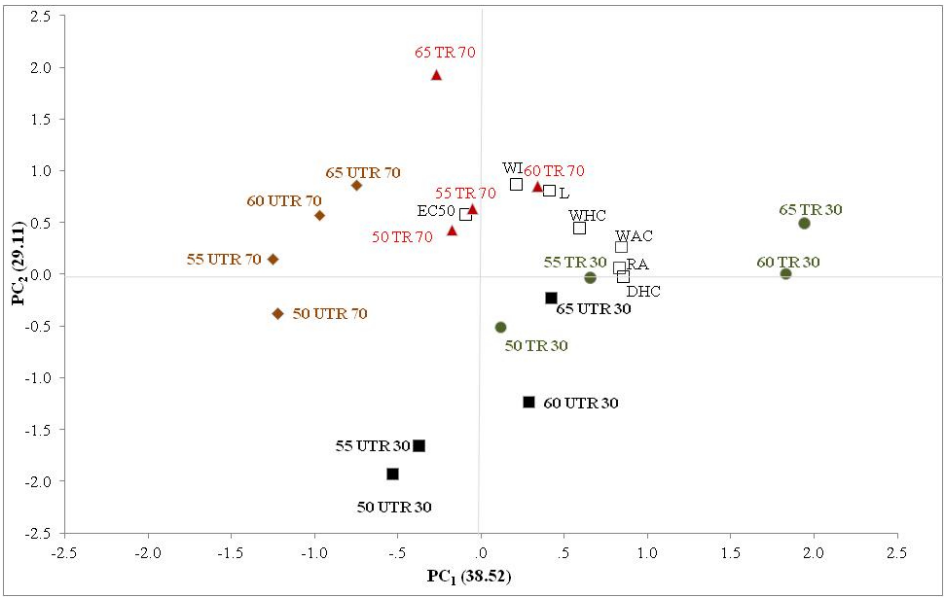

Figure 6. Two-dimensional principal component analysis of rehydration properties for untreated (UTR) and treated (TR) samples dried at 50°, 55°, 60° and 65°C and rehydrated at 30° and 70°C. (WI = white index; L= lightness; WAC = water absorption capacity; DHC = drying matter holding capacity; RA = rehydration ability; WHC = water holding capacity; EC50 = antioxidant activity).

4. CONCLUSIONS

The investigation of drying/rehydration process conditions and of an alternative dipping pre-treatment on the rehydration curves and qualitative traits of rehydrated 'Annurca' apple slabs, has shown valuable findings related to keeping quality of apples. Rehydration temperatures (30° and 70°C) did not significantly affect the weight gain of dried apples. On the other hand, lower rehydration temperature (30°C) with the pre-treatment had positive effect on the rehydration indices (WAC, DHC, RA and WHC). Higher increases in volume were observed in treated rehydrated samples in comparison with the untreated ones. The pre-treatment was able to preserve the colour properties at both rehydration temperatures, while the lower the drying temperature and the lower rehydration temperature, the higher antioxidant activity for both samples was measured. The results clearly highlighted that the rehydration process and quality of rehydrated apples are influenced by pre-treatment, drying and rehydration temperatures. The combination between dipping pre-treatment with trehalose and optimal drying temperature (65°C) at lower rehydration temperature (30°C) allowed to obtain the best overall reconstitution properties of the rehydrated apples in terms of the rehydration characteristics and structure. Thereby, it is recommended to combine the natural pre-treatment and those drying/rehydration process conditions to achieve the high quality attributes of dried/rehydrated apples to meet consumer expectation. These findings, combined with sensorial analysis with trained panel, will contribute to industrial applications and to literature information on the quality attributes of rehydrated apples. Thus, the understanding and characterisation of rehydration process make it a potential research area for designing of new and higher value - added products.

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PAPER

PHYSICOCHEMICAL, RHEOLOGICAL, AND SENSORY EVALUATION OF VOLUMINOUS BREADS ENRICHED BY *PURSLANE* (*PORTULACA OLERACEA L.*)

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ABSTRACT

Portulaca oleracea (purslane) can be used as a vegetable and herb for medical and food products. The aim of this study was to investigate the psychochemical, rheological, and sensory properties of voluminous breads enriched by different amounts of *purslane* powder (0, 5, 10, and 15%) were compared to a control group. The results showed that, with an increase in the concentration of *Purslane* in samples, water absorption capacity, stability under mixer, and softening level increased. Adding 15% of *purslane* powder decreased farinograph quality number significantly. Addition of *purslane* powder also improved resistance to extension and decreased extendibility, energy, and viscosity of the dough significantly. In terms of sensory properties, the sample with 15% *purslane* powder obtained the minimum score and other samples had acceptable conditions in terms of different sensory properties like taste, texture, color, odor, and general acceptability. In summary incorporation of *purslane* in voluminous bread is feasible and the optimum percentage of the purslane powder is 10% for the best acceptance in sensory evaluation.

Keywords: bread, *Portulaca oleracea*, *Purslane*, sensory evaluation, fortification, physichochemical properties

1. INTRODUCTION

Wheat products like flour and bread are good carries for adding nutrients needed by the consumers (EL KHOURY *et al*., 2018). In addition, bread is the staple food of about one half of the world population and a reliable source in terms of nutrition and inexpensive diet (GRAHAM, Welch, and Bouis, 2001). To improve nutritional support for low income families, it is essential to pay more attention to improving quality and diversity of available breads, minimize the wastes, and produce breads with strong sensory properties (GUYOT, 2012). Along with being a main source of energy, bread also supplies the dietary fibers, some minerals (iron (Fe), calcium (Ca), and vitamin B group and vitamin E (found in wheatgrass) (KAUR, 2011). Improvement of texture, volume, crust, and quality of bread is one of the advantages of using fat in bread formulation (EL-SOHAIMY *et al*., 2019). Another role that is filled is adding tastes and energy content of the product. As shown by the literature, fat tastes in breads are more desirable for the consumer than other tastes (HEENAN *et al*., 2008).

Portulaca oleracea (purslane) is a grassy annual plant in the family Oleracea with succulent stems, yellow or white small flowers, black seeds, and medicinal properties. The wild plant is a watery weed that prefers warm and arid condition and grows in a wide range of soils and climates. According to the traditional medicine, the plant has a cold and moist humor, styptic, and diuretic and decreases bile sack movement and bile flow in return (ASADI GHARNEH and REZA HASSANDOKHT, 2008; NAEEM and KHAN, 2013). *Purslane* is a well-known plant in traditional medicine with protective effect on the liver that is used for several therapeutic purposes. It preserves the liver against the damages caused by free radical invasion and lipid peroxidation in the endoplasmic grid of cells (ZAREI *et al*., 2015) (HADI *et al*., 2018). In addition, *purslane* is rich of antioxidant compounds and a good source for flavonoid, and carotenoid (M. ALAM *et al*., 2014). In addition, there has been no report of toxic effects of this plant. In addition to the said effects, *purslane* demonstrated anti-pain and anti-inflammatory effects. It is the richest plant source of W-3 (M. ALAM *et al*., 2014; UDDIN *et al*., 2014). Total fat content of the leaves range from 1.5 to 2.5 mg/g of fresh mass out of which around 60% and 40% is α-Linolenic acid (C18:3ω3) (LIU *et al*., 2000). Nowadays, researchers are looking for the ways to enrich food products, meet nutritional needs, and improve health in the consumers. So far, different additives have been added to bread formulation such as *purslane* seed flour (FATHNEJHAD KAZEMI *et al*., 2012), flaxseed flour (MERVAT *et al*., 2015), fenugreek flour (NASEHI *et al*., 2018), and garlic flour (BALESTRA *et al*., 2011). In addition, the importance of using medicinal plants in pharmaceutical and economic fields is quite clear (BHAT *et al*., 2013; DANESHZADEH *et al*., 2020; GUARIGUATA *et al*., 2014; WHITING *et al*., 2011).

Taking into account the high rate of linolenic fat acid in some of oil seeds like *purslane* seed, the high consumption rate of bread, and low consumption rate of essential fatty acids per capita, the present study is an attempt to survey the possibility of using *purslane* to enrich voluminous breads to improve physicochemical, rheological, and sensory qualities of the breads.

2. MATERIALS AND METHODS

2.1. *Purslane* powder preparation

Shahroud strand of *purslane* plant was collected from a vineyard in Shahroud, Semnan Province, Iran. The plats were washed with distilled water to remove dust, cleaned, and dried at room temperature (35±2°C) for several days till complete dryness and then powdered using a laboratory grinder (Pars Khazar, Rasht, Iran). The powder was sieved using a sieve with mesh size of 0.5 at most. The obtained powder was kept in a capped container in fridge (0-4°C).

2.2. Voluminous bread samples preparation

As the control group, voluminous bread formulation is listed in Table 1. To prepare the samples and according different levels of purslane powder to replace wheat flour (0 (control), 5, 10, and 15% w/w) was mixed with wheat flour, yeast suspension (2%) and other ingredients using an electrical mixer and incubated at 30°C for 30 minutes (Pars Khazar, Rasht, Iran).
Water content was determined using farinograph and the dough was mixed for 10min. To mix the dough, spiral mixer was used, and the dough was cut into 150 gr pieces. The baking process took 25-30 min at 180°C in an electrical convection oven (SM-705E Model, SINMAG, Jakarta, Indonesia). Afterwards, the breads were cooled down and packed in polyethylene packages for further examination (SHITTU *et al*., 2009).

Table 1. Voluminous bread formulation.

Ingredients	Amounts
Flour	1 kg
Sugar	10 g
Yeast	10 g
Vegetable oil	10 g
Salt	5 g
Water	Enough

2.3. Physicochemical and Rheological analysis

2.3.1 Analyzing wheat flour, purslane powder, and voluminous breads

Moisture, ash, total protein, fat, raw fiber, falling number, and moist gluten were determined according to AACC method (2000) under code numbers 44-15A, 08-01, 46-13, 30-10, 32-10, 81-56, 54-11, and 12-38, respectively (AACC, 2000).

2.3.2 Dough rheological analysis

To examine amylograph properties of dough samples, an amylograph device (Brabender, Germany) was used based on the standard instruction. Rheological properties of different samples were determined using farinograph and amylograph based on AACC method

(2000) No. 54-21 and 54-30. Then different parameters like water adsorption, dough development time (DDT), stability of dough, and mixture resistance index were plotted on farinograph diagrams and parameters like gelatinization and viscosity were extracted based on amylograph diagram (AACC, 2000).

2.3.3 Physicochemical analysis of voluminous bread

The bread samples were analyzed using AACC (2000). The moisture, raw protein content, fat content, ash content, and gluten content were determined through 44-15, 46-13, 30-25, 01-08, and 38-11 methods respectively (AACC, 2000).

2.4. Sensory evaluation

The quality of breads, fresh and cooled down in ambient temperature, was examined by 20 trained examiners (10 men and 10 women) were analyzed. Sensory specifications included tastes, texture, color, odor, and general acceptability. The samples were coded randomly and provided to the examiners in separate containers. They scored the samples based on a five-point scoring system (5= very good, 4 = good, 3= moderate, 2= bad, and 1= very bad) (GHANBARI and FARMANI, 2013; YASEEN *et al*., 2010).

2.5. Statistical analysis

All experiments were done with three replicates using ANOVA in SPSS 22 (Chicago, IL, USA). To compare mean score ($P<0.05$), Duncan's multiple range test was used. The independent variable was different levels of *purslane* powder and dependent variables were all the experiments on the treatments. Figures were developed in MS Excel.

3. RESULTS AND DISCUSSION

3.1. Physicochemical properties of wheat flour and purslane powder

Physicochemical properties of wheat flour and *purslane* are listed in Table 2. As listed, moisture of wheat flour is higher than that of *purslane* powder; while *purslane* powder has higher fat, protein, total ash, and fiber content compared to wheat. *Purslane* powder did not have gluten and alpha amylase activity.

3.2. Dough rheological analysis

3.2.1 Farinograph test on the dough

Results of water absorption (WA) in farinograph test (Table 3) showed that by adding *purslane* powder into the wheat flour, WA capacity decreases notably so that the control sample contained 57.96% water and the sample containing *purslane* powder (15%) only contained 55.1% water. Still, the decrease in WA rate in the samples with 5 and 10% of *purslane* powder was not significant ($p>0.05$). Since, *purslane* powder contains hydrophobe chemical compounds like fatty acids, the decrease in WA capacity by adding *purslane* powder is expectable.

As listed in Table 3, by increasing the level of *purslane* powder in dough sample, stability time in mixture decreases. Dough Stability (DS) time in the control sample was 4.98min and for the samples with 5, 10, and 15% *purslane* powder, this time was 4.63, 4.26, and 3.65 min respectively. The decrease in stability time by replacing wheat flower by *purslane* powder is rooted in dilution or degradation of gluten grid. Physical break of gluten grid can be a reason for the less stable dough as gluten proteins are responsible for viscoelastic grid and stability of dough and *purslane* powder does not contain gluten. (MACRITCHIE, 2010).

Table 2. Physico-chemical properties of wheat flour and *purslane* powder.

Parameters	Wheat flour	*Purslane* powder
Moisture (%)	13.83±0.59[a]	4.72±0.45[b]
Protein (%)	8.89±0.33[b]	16.39±0.24[a]
Fat (%)	1.49±0.14[b]	4.79±0.06[a]
Ash (%)	0.72±0.11[b]	18.12±0.08[a]
Crude fiber (%)	0.13±0.03[b]	4.78±0.05[a]
Wet gluten (%)	24.16±0.27	-
Felling number (Seconds)	**353**	-

*Different letters in the same row indicate significant differences ($P<0.05$).

The degree of loosening analysis showed that by adding high levels of *purslane* powder to the dough samples, looseness of the sample increased notably after 12 min (Table 3). This increase in looseness can be explained by the dilution of gluten proteins that weakens the dough. *Purslane* powder also contains unsolved fibers that weaken gluten functions. Dough weakening levels for the control and samples with 5, 10, and 15% *purslane* powder were 89.68, 91.29, 95.13, and 100.85 BU respectively.

Farinograph quality number (FQU) for different samples is listed in Table 3. Clearly, adding *purslane* powder to the dough sample (15%) caused a significant decrease in FQU ($p<0.05$) as adding the powder decreases stability of the dough. In general, there was no significant difference between FQU of the control and *purslane* samples 5% and 10% ($p>0.05$).

XU *et al*. (2014) showed that adding linseed flour to wheat flour increased WA, dough expansion time, and dough resistance (XU *et al*., 2014). GARDEN (1993) consistently found that mixing linseed and wheat flour decreased stability of dough significantly (Garden-ROBINSON, 1993).

KOCA and ANIL (2007) reported that the reason for the difference in the mixture of dough containing linseed was dilution of gluten protein by fiber and the reaction between fiber materials and gluten, which also affects the mixing process (KOCA and ANIL, 2007).

Farinograph results by KOCA *et al*. (2007) showed that by increasing linseed content, WA, dough expansion, and mixture resistant index increased; while DS decreased by adding different levels of linseed (KOCA and ANIL, 2007). These findings are consistent with the present study.

Table 3. Farinograph characteristics of dough samples.

Samples	Moisture absorption (%)	Dough stability time (min)	Degree of loosening (after 12 minutes fermentation) (B.U.)	Farinograph qualitative number
Control	57.96±0.42^{a}	4.98±0.32^{a}	89.68±2.42^{c}	58.16±2.37^{a}
5% of *purslane* powder	56.39±0.59^{b}	4.63±0.19^{ab}	91.29±2.01^{bc}	57.89±1.14^{a}
10% of *purslane* powder	55.61±0.32^{bc}	4.26±0.29^{b}	95.13±1.94 ^{b}	53.91±2.02^{a}
15% of *purslane* powder	55.11±0.48^{c}	3.65±0.24^{c}	100.85±2.06^{a}	47.96±1.68^{b}
P-value	**0.000**	**0.000**	**0.000**	**0.013**

*Different letters in the same column indicate significant differences (P<0.05).

3.2.2 Extensograph test on dough

Rheology tests with large deformation range, including one-side extension test using an *extensograph* device yielded information about viscoelastic behavior of dough and dilatancy of gluten grid (GILBERT, 2002). Since, extensograph results have direct relationship with gluten protein properties, changes in dough resistance to extension and extendibility of dough can be attributed to the interactions between fiber structure and gluten protein.

The results of the effects of different concentration of *purslane* powder on extension strength of the dough (Fig. 1a) showed that with a longer rest time of 45-135 min, extension strength of the samples increased significantly both in the control and experiment groups ($p<0.05$). The dough rest was in extensograph container and during resting and due to changes in glutens, the ingredients were revived and a uniform gluten grid was reestablished due to the changes in gluten (XU *et al*., 2014). Therefore, extension strength after the rest time is improved. At different rest times (45, 90, and 135min), adding *purslane* powder increases tensile strength of dough due to high fiber content of the powder. In general, the highest level of tensile strength happened in the samples with 15% *purslane* content and fermentation time of 135min (247.75 BU) and the lowest level was with the control sample with fermentation time of 45min (169.77 BU).

Extendibility levels of different dough samples are demonstrated in Fig. 1b. Clearly, in the samples with different concentrations of *purslane*, extendibility is notably less than the control samples ($p<0.05$). This can be due to the larger size of *purslane* powder particles compared to wheat flour that causes early rapture of gluten under extension. The second cause might be dilution of dough protein so that changes the ratio protein to starch (MACRITCHIE, 2010), the increase in tensile strength and decrease in its extendibility with different concentration of *purslane* powder is justifiable. In short, with different fermentation times, the highest extendibility was observed with the control sample and the lowest level was observed with the control sample with fermentation time of 45 min (14.73 cm).

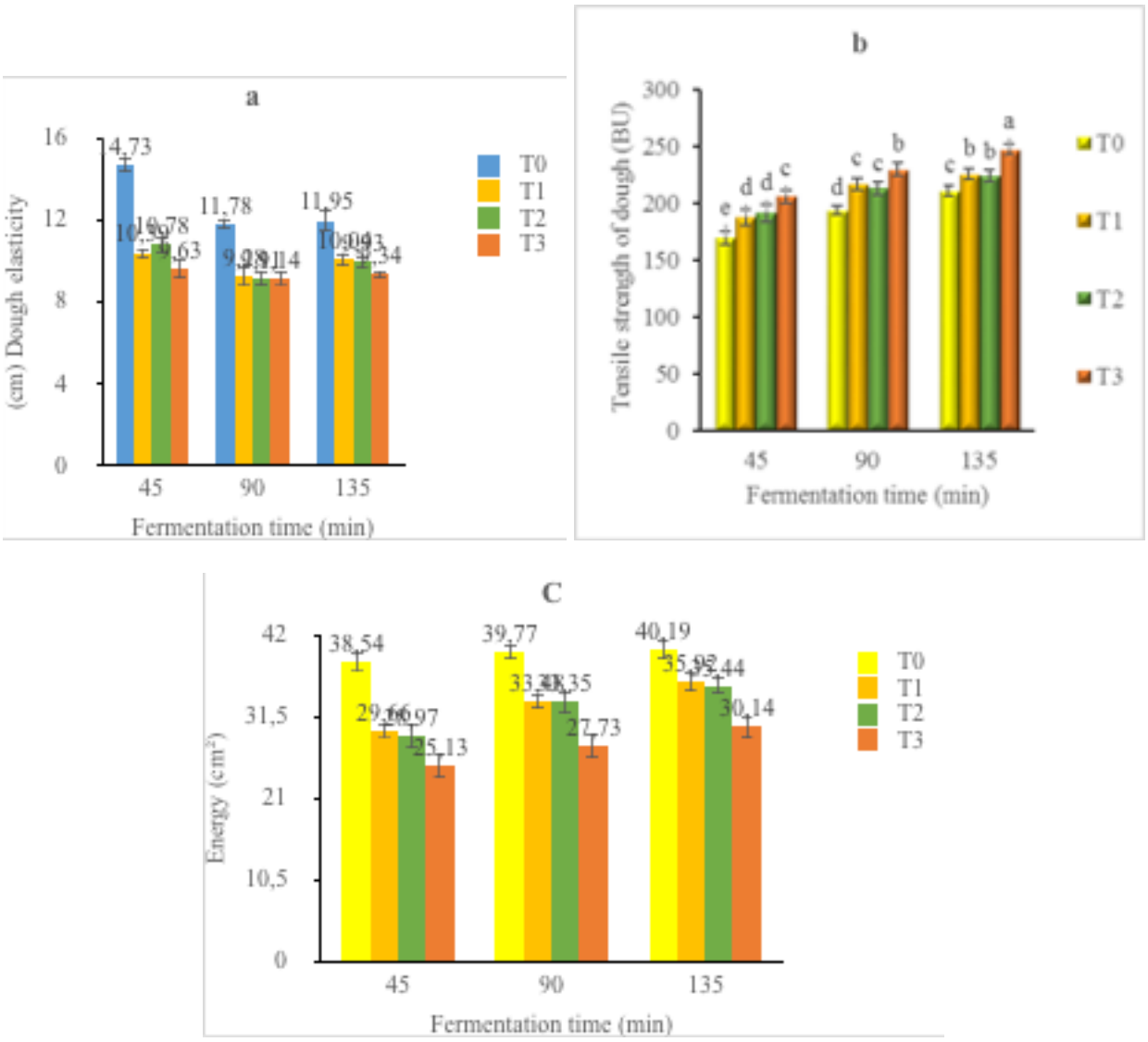

Figure 1. Comparison of mean values of (a) dough elasticity (cm), (b) tensile strength of dough (BU), (c) energy (cm2) of different dough samples during different fermentation times. Different letters on bars represent significant differences among means.

The area under diagram and dough energy indicates the energy or mechanical work needed for extending the dough until rapture. This is a reliable index of dough strength. For academic purposes, the curve height and the area under it are considered as strength index and the higher this index, the higher the strength of dough (GILBERT, 2002). The mean area under the diagram or tough energy of different dough samples containing different concentration of *purslane* powder with fermentation periods 45, 90, an d135min in extensograph is illustrated in Fig. 1c. The highest and lowest levels of dough energy with different fermentation time were obtained by the control and *purslane* powder (15%) respectively. There was no significant difference between the energy level of the samples with 5 and 10% of *purslane* powder regardless of fermentation time. Still, the increase in fermentation concentration time from 10 to 15% had a significant effect on the dough energy (considerable decrease). The sample with different levels of *purslane* powder

demonstrated a gradual increase in energy level of dough with an increase in fermentation time in the extensograph. MARIOTTI *et al*. (2006) reported rheological and baking performance specifications of bread with different levels of *Avena sativa* flour and showed that adding *Avena sativa* decreased WA and strength of breads (MARIOTTI *et al*., 2006).
STEPNIEWSKA *et al*. (2019) examined the quality of breads with rye flour and found that lower protein content, lower unsolved total pentosan content, higher solved pentosan content in water, flour granola, and solved content in water (pentosan in particular) had a significant effect on the hardiness of bread samples (STĘPNIEWSKA *et al*., 2019).
Marie and Ivan (2017), consistent with our findings, reported that replacing linseed fiber had a significant effect on the energy of extensogram curve (MARIE and IVAN, 2017).

3.2.3 Amylograph analysis

Table 4 lists the results about the effect of different levels of *purslane* powder on gelatinization of wheat flour-based dough. Clearly, despite the trivial increase in gelatinization temperature (GT) caused by the increase in the volume of *purslane* powder in the samples, there is no significant difference between the control and experiment samples in terms of GT ($p>0.05$).

Table 4. Amylograph results of dough samples.

Variables	Control	5%	10%	15%
Gelatinization temperature (°C)	58.37±0.08[a]	58.39±0.09[a]	58.46±0.05[a]	58.51±0.08[a]
Viscosity (BU)	1941.2±4.7[a]	1929±5.4 [b]	1917.5±2.9[c]	1906.2±4.1 [d]

*Different letters in the same column indicate significant differences ($P<0.05$).

Viscosity of the control and experiment samples is listed in Table 4. Clearly, the control sample has the highest viscosity (1941.2 BU) and adding *purslane* powder created a significant decrease in viscosity of the samples ($p<0.05$). That is, viscosity levels in the samples with 5, 10, and 15% of *purslane* powder were 1929.0, 1917.5, and 1906.2 BU respectively. The reason for this decrease in viscosity after adding *purslane* powder to the sample can be the decrease in gluten protein content. Therefore, with an increase in *purslane* concentration, the continuous grid of gluten is broken and viscosity declines (SALIM-UR-Rehman, 2006; YOUSIF *et al*., 2012).
SALIM-UR-REHMAN *et al*. (2006) showed that increasing the content of sorghum flour up to 30%, lowered the viscosity of dough (SALIM-UR-REHMAN, 2006). INDRANI *et al*. (2015) reported that adding ground black gram to dough sample increased viscosity of the dough samples notably. Their results are consistent with the results here (INDRANI *et al*., 2015). MLAKAR *et al*. (2009) showed that replacing amaranthus flour up to 10% did not have any effect on GT of wheat flour dough, while adding 20% amaranthus flour increased starch GT (MLAKAR *et al*., 2009). Moreover, a significant decrease in viscosity of the dough due to adding amaranthus flour was reported.

3.3. Bread samples analysis

3.3.1 Bread moisture content

Fig. 2a illustrates results of moisture assessment of the samples with different levels of *purslane*. Clearly, the lowest moisture level is with the control sample (33.53%) and the moisture increases significantly with the increase of *purslane* content to 5 and 10% ($p<0.05$). Still, the increase in *purslane* powder level from 10 to 15% decreases moisture content. The increase in moisture content with the lower levels of *purslane* can be explained by the high fiber content in *purslane*, which preserves moisture in the samples. Still, with further increase in *purslane* content, gluten grid is degraded and its capacity to store water decreases. The powder contains hydrophobic chemical compounds like fatty acids that decrease water content.
DEMINE *et al*. (2013) showed that adding quinoa flour to flour formulation creates a significant decrease in moisture content of bread samples (DEMIN *et al*., 2013). Still, the increase in quinoa flour did not have a significant effect on bread samples moisture. GOHAR *et al*. (2016) stated that replacing a part of wheat flour with quinoa flour decreased moisture content significantly (GEWEHR *et al*., 2016). As to the increase in moisture content after adding amaranthus flour to the formulations used by baking industries, INGLETT *et al*. (2015) studied cookies containing amaranthus flour. They showed that adding amaranthus flour increased moisture capacity in baking process comparing with other samples (INGLETT *et al*., 2015).
TEUTONIC and KNORR (1985) showed that amaranthus seeds have 3.54% lignin and this increases the capacity to store water (TEUTONICO and KNORR, 1985). In addition, ELGETI *et al*. (2014) used quinoa flour and replaced it with rice and corn flour up to 40-100% to obtain gluten free bread. The results showed that along with improving the volume of bread, quinoa flour created a softer inner texture, distributed air cell more evenly, had a positive effect on moisture content of the samples and delayed going stale (ELGETI *et al*., 2014). They argued that the increase in moisture content of the samples was due to adding fiber-rich flour (e.g. lignin) to the samples.

3.3.2 Breads protein content

Fig. 2b illustrates protein content of the samples. Clearly, the minimum protein content appears in the control sample (10.05%) and since *purslane* powder contains more protein than wheat flour, adding *purslane* powder to the formulation created a significant change on protein content ($p<0.05$) so that samples with 5%, 10%, and 15% *purslane* powder contained 11.82%, 12.48%, and 12.91% protein content respectively.
HOSSEIN and SALEM (2016) studied enrichment of gluten-free snacks using different levels of *purslane* and showed that adding *purslane* increased protein content of the products significantly (HUSSIEN and SALEM, 2016). ASMA and GINDY (2017) showed that adding *purslane* powder to bread samples increased protein content significantly (ASMA and GINDY, 2017). ALMASOUD and EMAN (2014) found that adding *purslane* significantly increased protein content of crackers (ALMASOUD and EMAN, 2014). These findings are consistent with our findings.

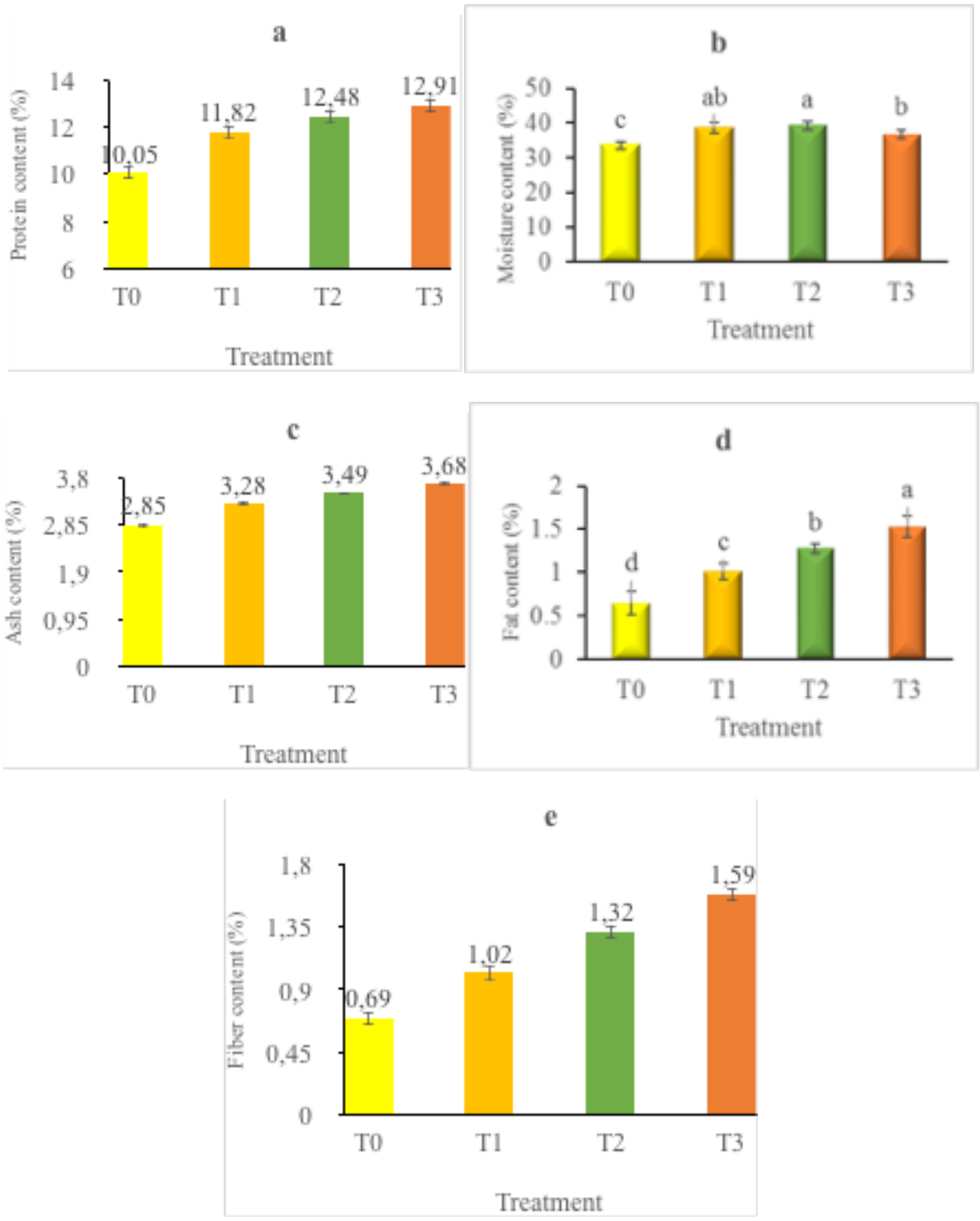

Figure 2. Comparison of (a) protein, (b) moisture, (c) ash, (d) fat and (e) fiber content (%) of different bread samples. Different letters on bars represent significant differences among means.

3.3.3 Breads fat content

Fat content results are demonstrated in Fig. 2c; clearly, the control sample has the lowest fat content (0.65%) and adding *purslane* powder makes a significant change in fat content ($p<0.05$). That is, samples with 5%, 10%, and 15% *purslane* powder contain 1.01, 1.25, and 1.53% fat respectively. Taking into account the fatty nature of *purslane*, the increase in fat content is expectable. DESTA and MOLLA (2020) suggest that the highest oil content was observed in seed (DESTA, 2020) in the present study, all parts of the plant have been used. HOSSEIN and SALEM (2016) argued that increasing *purslane* powder in gluten-free stack formulations increased fat content significantly (HUSSIEN and SALEM, 2016). ASMA and GINDY (2017) studied the increase in fat content of breads through increasing *purslane* level in the formulation (ASMA and GINDY, 2017).

3.3.4 Total ash content of breads

Fig. 2d illustrates total mean ash content of the control and experiment samples. Clearly, the lowest ash content is observed with the control sample (2.85%) and since *purslane* powder contains higher levels of mineral elements, it yields more ash than wheat flour (ALAM, Juraimi, Yusop, Hamid, and Hakim, 2014). By increasing the share of *purslane* in the formulation, a significant increase in ash content takes place ($p<0.05$). The samples with 5%, 10%, and 15% of *purslane* powder yielded total ash volumes of 3.28%, 3.49%, and 3.68% respectively.
A study by IGLESIAS-PUIG *et al*. (2015) on the breads produced with quinoa total flour showed that with an increase in quinoa flour, ash content increases, which is consistent with our findings (IGLESIAS-PUIG *et al*., 2015).
A study by HOSSEIN and SALEM (2016) reported similar results so that an increase on *purslane* powder content in gluten-free snacks increased ash content of the products significantly (HUSSIEN and SALEM, 2016). ASMA and GINDY (2017) showed that an increase of *purslane* powder in bread formulation significantly increased ash content of the samples, which is consistent with our results (ASMA and GINDY, 2017). As the ash increases, the amount of minerals in the raw material increases (UDDIN, 2012).

3.3.5 Bread fiber content

Raw fiber content in the control and experiment samples is illustrated in Fig. 2e. Clearly, the higher fiber content of *purslane* powder compared to wheat flour increases the fiber content significantly ($p<0.05$). With an increase in *purslane* content in the sample, the fiber content increases significantly. The control sample have 0.69% fiber and the samples with 5%, 10%, and 15% *purslane* powder have 1.02, 1.32, and 1.59% fiber content respectively. HOSSEIN and SALEM (2016) showed that an increase in *purslane* powder content in gluten-free snack increased fiber content significantly (HUSSIEN and SALEM, 2016). A study by ASMA and GINDY (2017) showed that using *purslane* powder in the sample increased fiber content (ASMA and GINDY, 2017). ALMASOUD and EMAN (2014) consistently reported that adding *purslane* formulation to cracker increased fiber content notably (ALMASOUD and EMAN, 2014).

3.3.6 Sensory evaluation of bread

Mean scores of taste, texture, color, odor, and general acceptability of the samples are illustrated in Fig. 3.

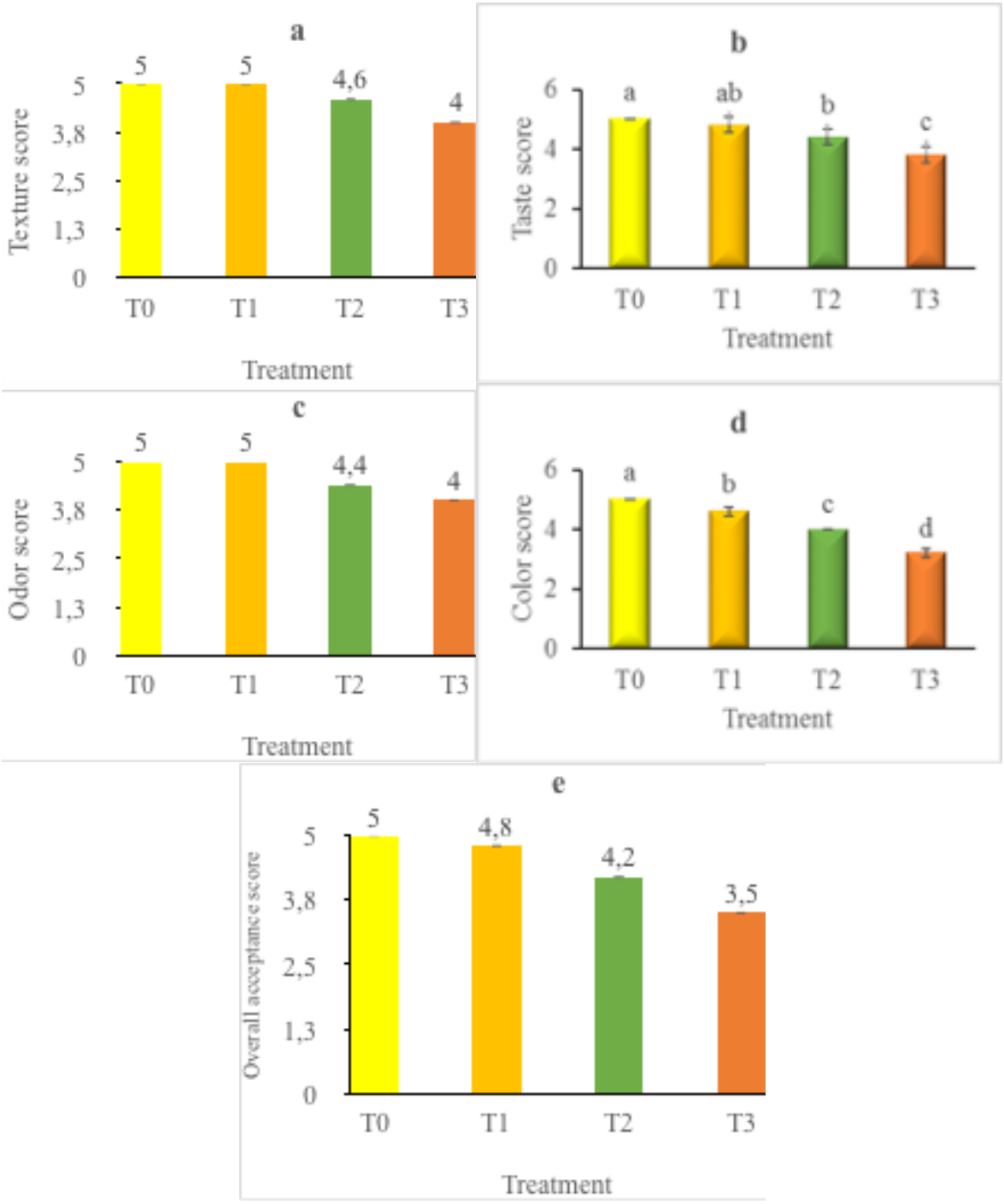

Figure 3. Comparison of (a) texture, (b) taste, (c) odor, (d) color and (e) overall acceptance score of different bread samples. Different letters on bars represent significant differences among means.

The control sample obtained the total score of taste, color, and general acceptability. In addition, the control and 5% samples obtained total score of texture and odor as well. Still, there was no significant difference between the control and 5% samples in terms of taste and general acceptability ($p>0.05$). Adding *purslane* powder to the bread formulation significantly lowered taste and general acceptability scores ($p<0.05$) so that the sample with 15% *purslane* content had the lowest score of color. Except for the 15% sample, the rest of the treatments were acceptable in terms of sensory indices. By increasing *purslane* content from 5% to 15%, texture, taste, and general acceptability scores declined ($p<0.05$) so that the 15% sample obtained the lowest scores of texture, odor, and general acceptability (FATHNEJHAD KAZEMI *et al*., 2012). Still, all the treatments were acceptable in terms of texture, odor, and general acceptability. High *purslane* content in the formulation decreased volume and moisture content of the breads and had a negative effect on the texture. The decrease in color score by adding *purslane* powder content can be explained by the dark color of *purslane* powder. HOSSEIN and SALEM (2016) showed that adding 5% of *purslane* powder had a significant effect on sensory acceptability of gluten-free snacks (HUSSIEN and SALEM, 2016). However, adding 10 and 15% of *purslane* powder resulted in a decrease in sensory score of products. However, all the enriched samples were acceptable in terms of sensory specifications. MERVAT *et al*. (2015) maintained that adding 10% of linseed flour with total fat content did not have a significant effect on sensory acceptability (MERVAT *et al*., 2015). GANORKAR and JAIN (2014) noted that dry crust, a decrease in tenderness, and feeling roughness in the mouth were the reasons for a decrease in general acceptability score after adding linseed to cookies formulation (GANORKAR and JAIN, 2014). These results show that increasing the additive content increases tenderness of the product due to the higher content of fatty acids content; however, color, odor, and general acceptability decrease, which is consistent with our results.

The reason for the noticeable decrease in the sensory score of the samples containing higher percentages of *Portulaca oleracea* was due to the black color of the *Portulaca oleracea* and its effect on the color of the bread samples. But in the MELILLI *et al*. (2020) study, the sensory score of 5% obtained the highest sensory score (MELILLI *et al*., 2020). It seems that this discrepancy is due to the difference of different varieties in different parts of the world and it is predicted that if the yellow varieties of portulaca are used, such a decrease will not be observed in fortified breads with a higher percentage of 10% *Portulaca oleracea*

4. CONCLUSION

hysicochemical, rheological, and sensory properties of voluminous wheat flour breads containing different levels of *purslane* powder were examined. An increase in *purslane* content in dough samples decreased DS against mixture and increased looseness level. Adding 15% of *purslane* powder decreased FQU significantly. In addition, despite the increase in extension strength and a significant decrease in dough extendibility, energy, and viscosity of the dough sample, the increase in GT was not notable. Moreover, increasing the content of *purslane* powder increased protein, fat, total ash, moisture, and fiber content of the samples compared to the control samples. In terms of sensory specifications, the samples with 15% *purslane* powder content obtained the lowest score and the rest of the samples obtained acceptable scores in terms of taste, texture, color, taste, and general acceptability. Using *purslane* in voluminous bread is feasible and the optimum formulation should contain 10% of *purslane* powder.

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PAPER

EXPLORING THE EFFECT OF APRICOT ADDITION ON NUTRITIONAL, ANTIOXIDANT, TEXTURAL AND SENSORY CHARACTERISTICS OF COOKIES APRICOT SUPPLEMENTED FUNCTIONAL COOKIES

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ABSTRACT

Current study was designed to explore the nutritional and antioxidant potential of Pakistani apricot's varieties (Marghulam, Halman, Kakas and Shakanda). The highest values of moisture, crude fat, crude protein, fiber and ash (7.95±0.05%, 2.29±0.07%, 3.22±0.06%, 3.82±0.91% and 4.26±0.08%) were found in Marhulam. The maximum value of total phenolic contents (TPC) 30.75±0.09mg/100gGAE and DPPH 40.64±0.04% was determined in Marghulam and Halman respectively. Cookies were prepared with incorporation of apricot powder (10%) and evaluated for compositional, mineral, TPC, antioxidant activity, physical parameters, color, texture and sensory attributes. Based on sensory evaluation, cookies supplemented with 10% Marghulam flour got the highest score.

Keywords: antioxidants, apricots, cookies, organolyptic characteristics

1. INTRODUCTION

Fruits and vegetables are at the base of the food pyramid and rich source of nutrients, bioactive compounds i.e. phytochemicals (flavonoids, phenolics, and carotenoids), minerals, vitamins and fibers (LIU 2013). Vitamins A, B and C along with magnesium, potassium, calcium and iron are present in fruits like papaya, mango, pineapple, lemon, guava, jackfruit. Globally, people consume fruits as a staple food. For example, banana is eaten by South Americans as the main course of their meal. Our food shortages can be minimized to a considerable extent by banana, jackfruit, guava and pineapple (YAHIA *et al*., 2019). Apricot (*Prunus armenia* L.) is one of the nutritious fruit and a member of the Rosaece family. "Apricot" is a derivative of the Latin term *Praecocia*, meaning "early maturing" or "precocious". China started its farming, three thousand years before Christ (KARACA *et al*., 2019).

The total area destined to apricot's farming in Pakistan accounts for 31256 hectares. Moreover, in Punjab, the apricot cultivation area is 42 hectares. Furthermore, the highest production of 220276 tons of apricot is in Baluchistan, with a total area of 28901 hectares (KOUSAR *et al*., 2019). Apricots are greatly valued both fresh and dried. A dried fruit delivers a sufficient amount of energy and nutrients, which is particularly important for the population of the mountainous Karakoram zone (CHEN *et al*., 2020). Apricot cultivation significantly contributes to the agricultural income of the Pakistani province of Gilgit-Baltistan, where the local varieties Marghulam, Kakas, Halman and Shakanda are mostly cultivated.

Apricot has a distinctive nutritive value amongst stone fruits. It has a high content of proteins (8%), crude fiber (11.50%), fat (2%), minerals (4%), sugars (>60%), vitamins (A, B complexes, C and K) and organic acids (malic and citric acid), as well as a substantial amount of flavonoids and phenolics (KAFKALETOU *et al*., 2019). Dried apricots such as Marghulam and Halman are rich source of nutrients, minerals and vitamin C. Dried apricots can also be used for the preparation of different value-added food products (KIRALAN *et al*., 2019).

Previous efforts were made to increase the nutritional and organoleptic characteristics of cookies through supplementation. UCHOA *et al*. (2009) supplemented apple and guava pomace (dehydrated fruit powders) at different level. The cookies prepared with 15% and 20% substitution showed the best results in sensory evaluation. Similarly, TAŃSKA *et al*. (2016) incorporated fruit pomace (rosehip, rowan, blackcurrant and elderberry) and studied the effect of antioxidants on the properties of shortbread cookies. Likewise, PASQUALONE *et al*. (2019) studied the acorn flour substitution effect on the physicochemical and sensory properties of biscuits. Various authors (CHENG and BHAT 2016; ERTAŞ and ASLAN 2020; KAPOOR and RANOTE 2016; LEPIONKA *et al*., 2019; YOUSAF *et al*., 2013; ZUCCO *et al*., 2011) supplemented legume, pulses, seeds, cereals and evaluated the effect of substitution on quality of cookies.

Functional food can be described as the food that can deliver consumers with a health benefit beyond their basic nutrients. Functional bakery products are gaining much importance to improve the health and wellbeing of consumers. Different functional beverages, functional bakery products, functional cereal-based food and dairy-based foods are available in the market (NINFALI *et al*., 2019). Among bakery products, cookies are full of carbohydrates and fats, and have low levels of fiber, vitamins and minerals, but are largely consumed all around the world. Cookies are considered the most common and consumable bakery items (CURUTCHET *et al*., 2019). To improve the nutritional profile of cookies, the present study is designed to develop potentially functional cookies by

supplementing them with dried powdered apricots After that, the quality testing of prepared cookies was carried out.

2. MATERIALS AND METHODS

The present study was carried out in the Food and Nutrition Laboratory, Government College Women University, Faisalabad, Pakistan. However, some of the product's analyses were carried out at the University of Agriculture, Faisalabad, Pakistan.

2.1. Raw materials

Apricots varieties Kakas, Marghulam, Halman and Shakanda were acquired from Mountain Agriculture Research Centre (MARC) located in Jaglot, in the Gilgit Baltistan province, Pakistan. The selected trees were seven-year-old and were subjected to the same agricultural practices. Three healthy plants of the same age of each variety were selected during the years 2018 and 2019. Apricots were harvested healthy and at full ripening stage (deep yellow color with red blush). All varieties were harvested on the same day to provide fruits with uniform maturity, color, size and firm texture. Particulates of herbicide, insecticide and dust present on fruit exterior were removed by washing. The commercial straight grade flour (SGF) and remaining ingredients for cookies formulation were purchased from the local market of Faisalabad, Pakistan. All reagents, chemicals and standards were bought from Sigma-Aldrich (Sigma-Aldrich Tokyo, Japan) and Merck (KGaA, Merck Darmstadt, Germany).

2.2. Preparation of apricot powder

Apricots were dehydrated by the sun-drying method. Initially, the apricots were washed, pitted, cut into small pieces (1.5 cm x 1.5 cm) and placed on wire mesh tray covered with cloth. The apricots were dried under direct sunlight with an overall maximum day time temperature (37°C) and a minimum night temperature (29°C) for 48 h until the final moisture (6.0%) was reached. Then the dried apricots were ground by using an electric grinder. The dry apricot powder was sieved, to ensure a homogeneous particle size, through a mesh sieve of 250 μm (Retsch Test Sieve, Germany). The powder was stored in sealed polyethylene bags at 4°C for further analyses.

2.3. Proximate and antioxidant analysis of apricot powder

Apricot powder of all varieties was analyzed individually for proximate composition and antioxidant potential. The proximate analyses (moisture, crude fat, crude protein, crude fiber, ash and NFE) were performed following the corresponding methods as stated in AOAC (2006). Folin-Ciocalteu's test was used to measure the total phenolic content and antioxidant activity (%) was assessed based on free radical scavenging action through 2, 2-diphenyl-l-picrylhydrazyl (DPPH) assay (IDRIS, 2019).

2.4. Mineral analysis of apricot powder

The mineral content of apricot powders was determined by the ashing method (AOAC, 2006). The acquired ash was digested with 5 mL of 6 M HCl in a water bath. Ca, Fe, Mn,

Cu, Zn and Mg of apricot powders were determined in an Atomic Absorption Spectrophotometer whereas Na and K were determined by Flame Photometer and P by using UV-Spectrophotometer.

2.5. β-carotene content of apricot powder

β-carotene was isolated through column chromatography by using aluminum oxide with an absorbent column length of 10 cm. β-carotene moves down through the absorbent column afore all other pigments and is collected till the chosen pigment/s moved through the column and eluent becomes colorless. The color intensity of the eluent with known volume was measured with a spectrophotometer at 452 nm while acetone in petroleum ether (3 % v/v) used as blank (GIRÓN *et al*., 2019).

2.6. Preparation of cookies

Cookies were prepared from composite blends by using the method of HAN *et al*. (2004). The standardized cookie recipe was followed that includes wheat flour 40 g, butter 26 g, sugar 18 g, egg 9, milk 5 mL and yeast 1.6 g. Apricot powder and wheat flour (10:30) ratio was set based on different experimental trails while remaining ingredients remained the same. All the ingredients were mixed properly to make the dough. Then, the dough was rolled into cookies sheets. The cookies were baked at a temperature of 220 °C for 8 min. After baking, the cookies were cooled at room temperature and stored in polyethylene bags until further analyses.

2.6.1. Proximate, mineral, antioxidant and β-carotene of apricot supplemented cookies (ASC)

Proximate composition (moisture, fat, protein, fiber, ash and NFE) of apricot supplemented cookies was determined according to the standard procedures of AOAC (2006). The mineral content of apricot supplemented cookies was also determined (AOAC, 2006). Total phenolic contents were determined through Folin-Ciocalteu's method and antioxidant activity (%) was assessed through 2, 2-diphenyl-l-picrylhydrazyl (DPPH) assay (IDRIS 2019). β-carotene was estimated by the method described by GIRÓN *et al*. (2019).

2.6.2. Physical analysis of apricot supplemented cookies

Thickness and diameter of apricot supplemented cookies were measured with vernier caliper at two different positions for each cookie and the average was calculated. The spread ratio was calculated using the formula: diameter of cookies/height of cookies. The bake loss of cookies was calculated by weighing five cookies before and after baking. The average difference in weight was noted and represented as percent bake loss (CHAUHAN *et al*., 2016).

2.6.3. Color of apricot supplemented cookies

The colorimeter was used to measure the color of cookies based on L, a*, b* value. L* values range (0-100) and represents black to white, a* values represent the red color, and b* values show yellow color (CHAUHAN *et al*., 2016).

2.6.4. Texture analysis of apricot supplemented cookies

The texture was determined by Texture profile analysis (TPA) by using a Texture Analyzer (Brookfield, Middleboro, USA). The probe was P/75 aluminum, 75 mm diameter and the test speed was 0.5 mm/s. Sample cookies were always placed with the top upward. Texture profile analysis includes several parameters i.e. hardness, springiness, cohesiveness and chewiness (GUPTA *et al*., 2011).

2.6.5. Sensory evaluation of apricot supplemented cookies

Sensory evaluation of cookies was performed by a group of ten male and female semi-trained panelists (20-35 years of age). The assessment of the overall sensory value of cookies was experienced by the sense of sight, taste, and touch. The 9 points hedonic scale rating test was used to assess the degree of acceptance. The panelists were offered with an assessment method, which described several sensory criteria and score choices with number rankings. When all the assessment forms were completed, the data was analyzed. The cookies were evaluated for different sensory attributes like taste, texture, color, appearance, and overall acceptability (GANORKAR and JAIN, 2014).

2.7. Statistical Analysis

The obtained data were evaluated by one-way analysis of variance (ANOVA) with the help of Statistix 8.1 (Florida, USA). The level of significance (α 0.05%) was determined through the analysis of variance (ANOVA) method under the principles explained by MONTGOMERY *et al*. (2008).

3. RESULTS AND DISCUSSION

3.1. Proximate composition of apricot powder

The proximate analysis of different apricot varieties is presented in Table 1. The highest moisture content was observed in V_1 (7.95%) followed by V_2 (7.39%) while the lowest moisture content was observed in V_4 (6.09%). The present results are contradictory to the outcomes of ASHRAF *et al*. (2018), they reported that the apricot powder had moisture content (6.85%). The difference might be due to different varieties used in both studies. Storage stability, processing and value of food are linked with its low moisture content (HUSSAIN *et al*., 2006). Due to the nutritional differences, Marghulam had the highest moisture content as compared to other apricot varieties. The highest value of fat content was reported in V_1 (2.29) followed by V_2 (2.15%). The present outcomes on fat contents are in line with the finding of HUSSAIN *et al*. (2010), they reported that the apricot powder contains 2.31% fat content. Similarly, another researcher ARSHAD *et al*. (2010), reported that the fat content of three different varieties of apricot Marghulam, Halman and Shakanda was 3.10%, 2.2% and 4.56%, respectively. The compositional differences may be due to genetic and environmental variation (HACıSEFEROĞULLARı *et al*., 2007; ÖZCAN and HACıSEFEROĞULLARı, 2007).
The ash content is considered as an indicator of minerals present in any commodity. The highest value of ash content was observed in V_1 (4.26%) followed by V_2 (4.21%). The results

of present study were according to the results of AKIN *et al*. (2008). The highest value of fiber content was observed in V_1 (3.82%) followed by V_2 (3.62%) and the lowest value was observed in V_4 (3.23%). ALI *et al*. (2014b) reported that the fiber content is 3.85% in apricot powder. Crude fiber is also a significant part of fruits and important in sustaining health through improving bowel movement and taking extra fat from the blood. Marghulam powder has the highest amount of fiber as compared to other apricot varieties. This compositional difference is common among varieties due to their differences in genotypes, geography and agricultural practices (ALI *et al*., 2014a).

Table 1. Proximate, antioxidant, β-carotene and mineral of apricot powder.

Apricot powder	V_1	V_2	V_3	V_4
Moisture (%)	7.95±0.05^a	7.39±0.04^b	6.75±0.03^c	6.09±0.02^d
Fat (%)	2.29±0.07^a	2.19±0.06^b	2.15±0.05^c	2.12±0.04^d
Protein (%)	3.22±0.06^a	3.18±0.05^b	3.15±0.04^c	3.12±0.03^d
Fiber (%)	3.82±0.09^a	3.62±0.08^b	3.42±0.07^c	3.23±0.06^d
Ash (%)	4.26±0.08^a	4.21±0.07^b	4.19±0.06^a	4.12±0.04^a
NFE (%)	78.50±0.09^d	79.50±0.10^c	80.02±0.11^b	80.06±0.012^d
DPPH (%)	32.77±0.07^b	40.64±0.06^a	30.54±0.05^c	28.43±0.03^d
TPC(mg/100gGAE)	30.75±0.09^a	29.64±0.04^b	28.54±0.02^c	27.43±0.03^d
ß-carotene (mg/100g)	46.12±1.00^a	40.07±0.02^b	35.41±0.07^c	30.74±0.04^d
Na (mg/100gdw)	22.05±1.30^a	18.71±0.60^b	15.79±0.23^c	14.30±0.60^d
K (mg/100gdw)	2240±38.00^a	1868±29.67^b	1730±30.00^c	1620±25.00^d
Ca (mg/100gdw)	111.30±3.20^a	105±2.40^b	98.40±2.28^c	76.71±0.72^d
Mg (mg/100gdw)	144.89±6.18^a	139.90±6.20^b	132.70±3.50^c	118. 90±4.30^d
Zin (mg/100gdw)	3.28±0.21^a	2.70±0.18^b	1.43±0.16^c	108±0.11^d
Fe (mg/100gdw)	9.96±0.50^a	6.34±0.41^b	4.04±0.31^c	3.90±0.24^d
Cu (mg/100gdw)	0.50±0.05^a	0.40±0.04^b	0.32±0.01^c	0.26±0.02^d
P (mg/100gdw)	250.80±7.24^a	247.90±5.30^b	212.40±3.48^c	190.60±3.10^d

Mean carrying same letters are not significantly different from each other.
Values are expressed as mean ±SD (n=3).
Whereas,
V_1: Marghulam, V_2: Halman, V_3: Shakanda, V_4: Kakas.

3.2. Total phenolic content (TPC) and antioxidant activity of apricot powder

The highest value of TPC was observed in V_1 (30.75 mg/100g GA) followed by V_2 (29.64 mg/100g GAE) and V_3 (28.54 mg/100g GAE) while the lowest TPC value was found in V_4 (27.43%) as presented in Table 1. The current outcomes of TPC are similar to the findings of (WANI *et al*., 2017), they reported that TPC contents of different varieties of apricot Harcot, Rival and Cuminis Haley were 25.44 mg/100g GAE, 32.86 mg/100g GAE and 24.87 mg/100g GAE, respectively.
DPPH value was highest in Halman while the maximum TPC content was found in Marghulam. The highest antioxidant value was observed in V_2 (40.64%) followed by V_1 (32.77%) whilst the lowest in V_4 (28.43 %). The current outcomes of DPPH are in coherence

with the results of WANI *et al*. (2015) who reported that DPPH of three varieties of apricot Harcot, Rival and Cuminis Haley was 21.68%, 34.16% and 44.62% respectively. The difference in phenolic content and antioxidant might be due to varietal, genetic and geographical difference.

3.3. Mineral content of apricot powder

The potassium concentration was found to be highest in V_1 (2240mg\100gdw) followed by V_2 (1868mg\100gdw) and V_3 (1730mg\100gdw). The highest sodium content was found in V_1 (22.05 mg\100gdw) followed by V_2 (18.71 mg\100gdw). The highest calcium content was present in V_1 (111.30 mg\100gdw) followed by V_2 (105.18 mg\100gdw) and V_3 (98.40mg\100gdw). The maximum iron content was in V_1 (9.96mg\100gdw) followed by V_2 (6.34 mg\100gdw) and V_3 (4.04mg\100gdw). The present results of different apricot varieties are following the outcomes of ALI *et al*. (2014b). Mineral composition showed that apricot varieties contain considerable amounts of minerals. The mineral composition showed that Marghulam variety has high mineral content followed by Halman Shakanda and Khakhas. These conclusions are in line with the previous study on Turkish Apricots (HACıSEFEROĞULLARı *et al*., 2007). However, differences in data might be due to the genotype, geographical origin, environment and agronomic practices (CHEN *et al*., 2020).

3.4. β-carotene content of apricot powder

The β-carotene were found to be highest in V_1 (46.12mg/100g) followed by V_2 (40.07mg/100g) and V_3 (35.41mg/100g). The present results of apricot cookies are consistent with the outcomes of Ali *et al*. (2014), who reported that Marghulam β-carotene (50.12±1.00 mg/100g) followed by Shakanda (46.07±0.88 mg/100g), Jahangir (40.54±0.78 mg/100g), Shirini (39.45±0.740 mg/100g), Yagoo (36.41±0.700 mg/100g) and Halman (30.61±0.50 mg/100g). Bioactive compounds *i.e.* phenolics and carotenoids are important quality index parameters of fruits and vegetables (GIRÓN *et al*. 2019).

3.5. Proximate composition of apricot supplemented cookies

The proximate composition of apricot supplemented cookies varied significantly among different treatments (Table 2). The highest moisture content was found in T_1 (7.85%) followed by T_2 (7.80%) and the lowest moisture content was found in T_4 (6.24%). The present results of different apricot cookies are in accordance with the results of MUNDEJA and HIRDYANI (2014), they reported 6.38% moisture content in apricot cookies. Similarly, another research work of PETER IKECHUKWU *et al*. (2017), found that the moisture content of whole wheat flour cookies was 7.70%. The reduction in crude fat content might be due to the lipolytic activity of the enzymes i.e., lipase and lipooxidase (VARSHNEY *et al*., 2008). The protein content reduction in different cookies might be due to the hydrolysis of the peptide bond with the help of protease enzyme that results in the splitting of protein molecules (WANI *et al*., 2015). The difference in fiber content may be due to the deprivation of hemicelluloses and other structural polysaccharides. The highest ash content was found in T_1 (1.36%) followed by T_2 (1.30%). Similar results were reported by other researchers (GAYAS *et al*., 2012; PRATYUSH *et al*., 2015).

3.6. Total phenolic content and antioxidant activity of apricot supplemented cookies

The total phenolic content and antioxidant activity varied significantly for treatments (Table 2). Total phenolic content and the antioxidant value decreased in apricot cookies as compared to apricot powder due to the processing loss during baking. The maximum TPC value was found in T_1 (25.69 mg/100g GAE) followed by T_2 (24.60 mg/100g GAE). The highest DPPH value was found in Halman and the highest TPC value was found in T_1. DPPH is a free radical compound that has been widely used to estimate the free radical-scavenging activity (AMAROWICZ *et al*., 2004). The highest value of DPPH was found in T_2 (38.52%) followed by T_1 (31.60%) and T_3 (28.39%) while the lowest DPPH value was found in T_4 (25.30%).

3.7. Mineral content of apricot supplemented cookies

The mineral contents of different apricot cookies also varied significantly ($p<0.05$) among all treatments. Potassium, sodium, calcium and iron concentration was found to be highest in T_1, these values for these minerals were 1940 mg\100g, 20.05 mg\100g, 108.29 mg\100g and 8.90 mg\100g on dry weight basis respectively. The present results of various apricot cookies are consistent with the outcomes of İNCEDAYI *et al*. (2016). Similarly, AKIN *et al*. (2008) determined the K, Mg, Ca and Zn contents of eleven apricot varieties similar to the present study as 1227-3455 mg/100 g, 110.4-284.4 mg/100 g, 87-240.5 mg/100 g and 1.38-4.24 mg/100 g based on dry matter, respectively. The data about mineral contents (mg/100 g) revealed K as the predominant element. These mineral compounds are present in any part of the plant and their concentration differs concerning crop, variety, growth condition, irrigation, genetics, temperature fluctuation, seasons, pre-harvest and post-harvest treatments. K, Ca and Mg are considered major minerals of the apricot fruit (DROGOUDI *et al*., 2008). For the human body, minerals are vital for a diversity of basic physiological and metabolic processes that include enzyme activation, muscle contraction, bone health, conduction of nerve impulse, transport of oxygen, antioxidant activity, acid and immune function of blood. An adult requires almost 350 mg Mg, 3000 mg K and 1000 mg Ca intake per day (WILLIAMS, 2005).

3.8. β-carotene content of apricot supplemented cookies

Maximum ß-carotene content was found in T_1 (43.79 mg\100g) followed by T_2 (36.99 mg\100g) and T_3 (32.88 mg\100g). The present results of various apricot cookies are consistent with the results of IGUAL *et al*. (2012). The carotenoids content is lesser than that stated for fresh apricot, which can be elucidated by the detail that drying and baking may lead to degradation of carotenoids due to oxidation and high temperature (DRAGOVIC-UZELAC *et al*., 2007). In addition to β-carotene, apricot fruit and its products contain smaller amounts of α-carotene, γ-carotene, zeaxanthin and lutein (FRASER and BRAMLEY 2004).

3.9. Physical analysis of apricot supplemented cookies

Physical properties (thickness, diameter, spread ratio, and bake loss) of cookies are presented in Table 3. Results revealed that there was a significant difference ($p<0.05$) among each treatment in terms of thickness, diameter, spread ratio and bake loss. The highest diameter was noted in T_1 (53.99 mm) followed by T_2 (53.77 mm), T_3(52.96 mm).

Table 2. Proximate, antioxidant, β-carotene and mineral of apricot supplemented cookies.

Apricot cookies	T_0	T_1	T_2	T_3	T_4
Moisture (%)	5.85±0.06^{e}	7.85±0.11^{a}	7.80±0.10^{b}	6.84±0.09^{c}	6.24±0.08^{d}
Fat (%)	7.68±0.01^{e}	9.77±0.05^{a}	9.26±0.04^{b}	8.27±0.03^{c}	8.07±0.02^{d}
Protein (%)	5.15±0.01^{e}	5.75±0.05a	5.48±0.04^{b}	5.38±0.03^{c}	5.24±0.02^{d}
Fiber (%)	0.72±0.01^{e}	1.45±0.05^{a}	1.33±0.04^{b}	1.19±0.03^{c}	1.14±0.02^{d}
Ash (%)	1.17±0.01^{e}	1.36±0.05^{a}	1.30±0.04^{b}	1.25±0.03^{c}	1.22±0.02^{d}
NFE (%)	79.04±0.09^{a}	77.35±0.08^{b}	75.41±0.07^{c}	73.68±0.06^{d}	63.78±0.05^{e}
DPPH (%)	20.6±0.09 ^{e}	31.60±0.04^{b}	38.52±0.02^{a}	28.39±0.03^{c}	25.30±0.02^{d}
TPC(mg/100gGAE)	15.69±0.12^{e}	25.69±0.06^{a}	24.60±0.04^{b}	23.37±0.15^{c}	22.26±0.03^{d}
ß-carotene (mg/100g)	15.61±0.30^{e}	43.99±0.05^{a}	36.99±0.03^{b}	32.88±0.02^{c}	27.50±0.04^{d}
Na (mg/100gdw)	8.40±0.40^{e}	20.05±1.28^{a}	16.71±0.58^{b}	12.79±0.20^{c}	10.30±0.57^{d}
K (mg/100gdw)	1250±20.00^{e}	1940±36.00^{a}	1762±27.60^{b}	1690±24.00^{c}	1599±20.00^{d}
Ca (mg/100gdw)	50.92±0.60^{e}	108.29±3.10^{a}	102.16±2.38^{b}	94.40±2.25^{c}	72.71±0.69^{d}
Mg (mg/100gdw)	110±4.27^{e}	140.89±6.15^{a}	136.88±6.20^{b}	128.68±3.48^{c}	116.90±4.27^{d}
Zn (mg/100gdw)	0.99±0.07^{e}	2.99±0.17^{a}	1.69±0.16^{b}	1.40±0.14^{c}	1.06±0.09^{d}
Fe (mg/100gdw)	1.99±0.20^{e}	8.90±0.48^{a}	5.32±0.38^{b}	3.02±0.29^{c}	2.90±0.22^{d}
Cu (mg/100gdw)	170±3.06^{e}	240.79±6.24^{a}	236.77±4.28^{b}	208.38±2.45^{c}	188.58±2.09^{d}
P (mg/100gdw)	0.18±0.01^{e}	0.48±0.04^{a}	0.38±0.03^{b}	0.28±0.02^{c}	0.24±0.02^{d}

Mean carrying same letters are not significantly different from each other.
Values are expressed as mean ±SD (n=3).
Whereas,
T_0 Cookies prepared with straight grade flour, T_1 Cookies prepared with straight grade flour and Marghulam (90:10), T_2 Cookies prepared with straight grade flour and Halman (90:10), T_3 Cookies prepared with straight grade flour and Shakanda (90:10), T_4 Cookies prepared with straight grade flour and Kakas (90:10).

Table 3. Physical analysis of apricot supplemented cookies.

Treatments	Weight (g)	Thickness (mm)	Diameter (mm)	Spread ratio	Bake loss (g/100g)
T_0	12.16±0.10^{e}	9.78±0.41^{a}	51.11±0.04^{e}	5.60±0.03^{e}	16.99±14.8a^{a}
T_1	15.47±0.20a	8.75±0.11^{b}	53.99±0.17^{a}	6.99±0.09^{a}	16.36±14.87^{b}
T_2	15.38±0.21b	8.48±0.09^{c}	53.77±0.15^{b}	6.70±0.06^{b}	14.99±14.87^{c}
T_3	14.35±018^{c}	8.35±0.07^{d}	52.96±0.12^{c}	6.55±0.05^{c}	14.50±14.87^{d}
T_4	13.66±0.15^{d}	8.20±0.06^{e}	52.09±0.09^{d}	6.40±0.04^{d}	14.25±14.87^{e}

Mean carrying same letters are not significantly different from each other.
Values are expressed as mean ±SD (n=3).
Whereas,
T_0 Cookies prepared with straight grade flour, T_1 Cookies prepared with straight grade flour and Marghulam (90:10), T_2 Cookies prepared with straight grade flour and Halman (90:10), T_3 Cookies prepared with straight grade flour and Shakanda (90:10), T_4 Cookies prepared with straight grade flour and Kakas (90:10).

The present results for diameter are coherent with the results of SHARIF *et al*. (2009) who reported that the diameter of the cookies increases as the concentration of defatted rice bran increases. The current findings are contradictory to the work of KAMALJIT *et al*.

(2010), they reported that the pea flour combination reduces the diameter of the cookie. The highest weight was observed in T_1 (15.47g) followed by T_2 (15.38) and T_3 (14.48g). The present results are coherent with the results of CHAUHAN *et al*. (2016). Baking loss of treatment cookies was decreased compared to control. This is due to the water holding capacity of apricot powder compared to wheat flour due to its high-protein content.

3.10. Color analysis of apricot supplemented cookies

The color values varied significantly ($p<0.05$) among the treatments (Table 4). L* a* and b* values were highest in T_1 65.58, 24.27 and 47.76 respectively. The current results are in line with the findings of İNCEDAYI *et al*. (2016). HEGEDÚ´ S *et al*. (2010) described hue angle values of 62.63-84.63, L∗ values of 60.15-72.43 and chroma values of 51.66-68.48 of fruit flesh of selected apricot cultivars. Particularly, L* values were similar to the results of KAMALJIT *et al*. (2010). Many scientists have reported that the L* value is a measure of a browning index in fruits and fruit products. The difference in color might be due to the moisture level of the apricot supplemented cookies or variation in processing conditions. Color is a key factor for assessing the baking performance of cookies, which not only provides adequacy to the raw materials but also provides information regarding baking excellence (PASHA *et al*., 2011). It is one of the demanding characteristics to determine the quality of a product. Variation of color in cookies during baking is a dynamic process in which color changes as the baking proceeds (MEPBA *et al*., 2007).

Table 4. Color of apricot supplemented cookies**.**

Treatments	L*	a*	b*
T_0	50.79±0.22^{e}	9.69±0.99^{e}	27.70±0.04^{e}
T_1	65.58±0.16^{a}	24.27±0.88^{a}	47.76±0.09^{a}
T_2	64.72^{c}±0.17^{b}	18.15±0.87^{b}	38.76±0.07^{b}
T_3	61.74±0.12^{c}	15.20±0.80^{c}	35.71^{d}±0.08^{c}
T_4	57.79±0.14^{d}	12.48±0.82^{d}	31.72±0.06^{d}

Mean carrying same letters are not significantly different from each other.
Values are expressed as mean ±SD (n=3).
Whereas,
T_0 Cookies prepared with straight grade flour, T_1 Cookies prepared with straight grade flour and Marghulam (90:10), T_2 Cookies prepared with straight grade flour and Halman (90:10), T_3 Cookies prepared with straight grade flour and Shakanda (90:10), T_4 Cookies prepared with straight grade flour and Kakas (90:10).

3.11. Texture profile of apricot supplemented cookies

The maximum hardness was found in T_1 (150.74N) followed by T_2 (145.66N) and T_3 (135.54N) (Table 5). Hardness is the textural property, which is one of the most important assessment parameters of baked food items. Maximum springiness was found in T_1 (74.55%) followed by T_2 (62.22%) and T_3 (57.66%). The lowest springiness was recorded in T_4 (50.66%). The current findings are comparable with PEREIRA *et al*. (2013), they reported that hardness, springiness, cohesiveness and chewiness of Maria type cookies was 158.74 N, 72.22%, 0.78 and 93.02 N, respectively. These differences might be due to varietal, genetic and geographical differences. These outcomes might be due to other reasons such

as protein denaturation, irregular structure, water holding capacity, moisture content, solubilization of proteins, protein coagulation and fat content.

Table 5. Texture analysis of apricot supplemented cookies.

Treatments	Hardness (N)	Springiness (%)	Cohesiveness	Chewiness(N)
T_0	120.55±20.78^{e}	40.22±8.79^{e}	0.40±0.01^{d}	35.63±6.53^{d}
T_1	150.74±37.79^{a}	74.55±11.79^{a}	0.70±0.09^{a}	90.02±23.71^{a}
T_2	145.66±23.80^{b}	62.22±10.22^{b}	0.60±0.07^{ab}	69.10±21.54^{abc}
T_3	135.54±14.87^{c}	57.66±7.92^{c}	0.55±0.06^{c}	51.64±21.80^{bc}
T_4	130.88±20.80^{d}	50.66±6.22^{d}	0.50±0.04^{cd}	39.47±15.20^{c}

Mean carrying same letters are not significantly different from each other.
Values are expressed as mean ±SD (n=3).
Whereas,
T_0 Cookies prepared with straight grade flour, T_1 Cookies prepared with straight grade flour and Marghulam (90:10), T_2 Cookies prepared with straight grade flour and Halman (90:10), T_3 Cookies prepared with straight grade flour and Shakanda (90:10), T_4 Cookies prepared with straight grade flour and Kakas (90:10).

3.12. Sensory evaluation of apricot supplemented cookies

Sensory evaluation is important for quality assessment and commonly done by the panelist's judgment. Sensory evaluation of apricot supplemented cookies depicted significant effect ($p<0.05$) of apricot powder on different sensory parameters (color, flavor, taste, texture and overall acceptability). After control, T_1 got a maximum score for all the sensory parameters (Fig. 1). The color acts as an indicator of the final baked items and is linked to differences in taste and smell. The decreasing tendency in texture scores was probably owing to humidity from the atmosphere (SHARIF *et al*., 2009).

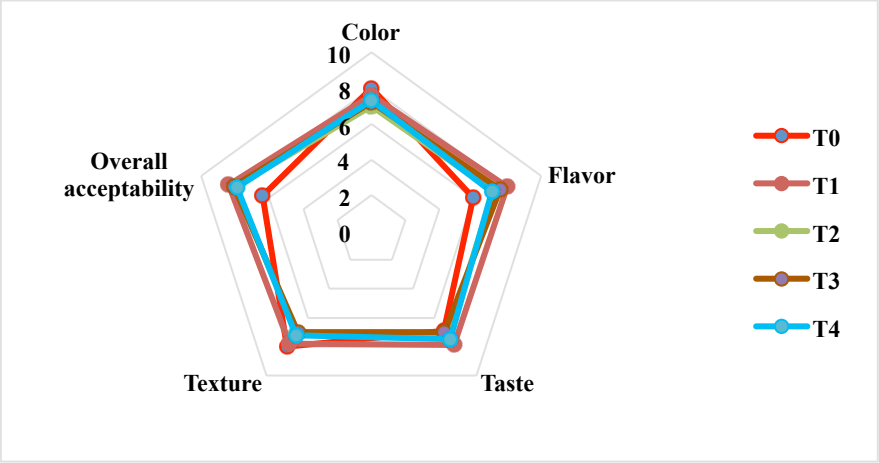

Figure 1. Sensory evaluation of apricot supplemented cookies.
T_0 Cookies prepared with straight grade flour, T_1 Cookies prepared with straight grade flour and Marghulam (90:10), T_2 Cookies prepared with straight grade flour and Halman (90:10), T_3 Cookies prepared with straight grade flour and Shakanda (90:10), T_4 Cookies prepared with straight grade flour and Kakas (90:10)

4. CONCLUSIONS

Apricot is one of the nutritious fruit, which is consumed either fresh or dried all around the globe. A blend of straight grade flour and apricot powder could make excellent baked product with improved economic worth. It not only improves the overall acceptability but also improves the nutritive value of the products without adding much to the cost of the product. This shows the effective utilization of apricot powder in baked goods. The result of the study revealed that the Marghulam variety has high antioxidant activity as compared to other varieties. The cookies made out of straight grade flour supplemented with 10% Marghulam apricot powder were satisfactory in terms of functional, nutritional and organoleptic characteristics as compared to control and other treatments. The results of the present study could be beneficial for the development of functional cookies.

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PAPER

CHANGES IN PHYSICO-CHEMICAL TRAITS AND ENZYMES OXIDATIVE SYSTEM DURING COLD STORAGE OF '*FORMOSA*' PAPAYA FRESH CUT FRUITS GROWN IN THE MEDITERRANEAN AREA (SICILY)

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ABSTRACT

In this study, the effects of cold storage (5±0.5°C and relative humidity of 90±1%) on the quality of fresh papaya slices packed in a passive atmosphere with a semi-permeable film were evaluated. Physico-chemical traits such as total soluble solids, reducing sugar, pH increased during storage as well as the polyphenols, carotenoid content and antioxidant activity that reaching the highest values at end of trials. Changes in colorimetric parameters resulted in a significant decrease after 4 days of hue angle values, which then remained constant. The cutting process enhanced the antioxidant enzymes activity such as superoxide dismutase, catalase and ascorbate peroxidase. The analysis of the main components showed physical-chemical, qualitative, and enzymatic changes in papaya samples during cold storage, showing a shift from negative to positive values along the PC1 and indicating a qualitative decay of sliced papaya.

Keywords: papaya, minimally processing, enzymes, color, antioxidant, packaging

1. INTRODUCTION

Papaya (*Carica papaya* L.) is the fifth most widely produced tropical fruit worldwide after mango, banana, pineapple, and avocado, with 13.0 million tons per year (LIU *et al*., 2019). Papaya is a perennial herbaceous plant recently spread in Spain and Italy (FARINA *et al*., 2020a), where, in recent years, papaya has adapted to the Mediterranean climate under sheltered structures (FARINA *et al*., 2020b) with good quality results (FARINA *et al*., 2020a), like other new crops (NIRO *et al*., 2017). Its cultivation is supported by a constant demand for freshly cut papaya, especially in Europe, mainly to young consumers and "baby boomers", who eat it as a snack (JAMES *et al*., 2010). Papaya is a type of climacteric fruit whose maturation after harvest is accompanied by tissue softening and microbial growth (GONZALEZ-AGUILAR *et al*., 2009). The proximity to European markets makes it possible to harvest the climacteric fruits close to their ripening stage, allowing for excellent organoleptic attributes (GENTILE *et al*., 2019) that reduce the number of kilometers of food and greenhouse gas emissions. In this context, the search for methods that use simple operations and equipment to improve the shelf life of minimally processed papaya is of interest to farmers and consumers (JAYATHUNGE *et al*., 2014). Several studies have demonstrated an increase in the reactive oxygen species (ROS) level after the cutting process in fresh fruit such as carrot (JACOBO-VELÁZQUEZ *et al*., 2011), *Zizania latifolia* (LIU *et al*., 2012), and pitaya (LI *et al*., 2017). ROS have harmful effects in various cellular compartments at high levels, but these compounds may also act as signaling molecules in ripening and senescence processes in response to cutting stress (JACOBO-VELÁZQUEZ *et al*., 2011). However, the balance between ROS generation and scavenging is closely modulate by antioxidant defense system characterized by enzymatic and non-enzymatic components in fresh-cut fruit (HODGES *et al*., 2008). Different enzymes control the intracellular metabolism of ROS, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT), which modulate the ROS level. The cutting process induces an increase of ROS above threshold levels, with damages to cell membranes due to lipid peroxidation with a high lipoxygenase activity and malondialdehyde content (KARAKURT and HUBER, 2003; SOUZA *et al*., 2015; WU *et al*., 2019). Furthermore, in fresh cut fruit have been observed a high enzymatic browning due to loss of cellular compartmentalization that allows to the polyphenol oxidases (PPOs) come in contact with phenolic compounds causing fruit color changes (WU *et al*., 2019). The aim of this research was to study the effect of cold storage (5°C and RH of 90±1%) and passive atmosphere packaging on the shelf-life of minimally processed papaya fruit grown in Mediterranean climate under greenhouse, by monitoring the variations in physico-chemical, microbiological and nutraceutical traits. In addition, in order to assess the physiological stress induced by the cutting and slicing process, the enzymatic oxidative system and the markers of oxidative damage have also been studied.

2. MATERIALS AND METHODS

2.1. Fruit samples and experimental design

'*Formosa*' papaya fruits were picked in a commercial orchard in Palermo, Italy (33S 0333746 m.E, 4217131.00 m.N). The fruits were harvested at stage 3 (one or more orange-colored stripes in skin; pulp almost completely orange in color, except near skin, still hard but contains less latex), using the skin color of the fruit as a maturity index (BASULTO *et*

al., 2009). The subsample of 25 fruits (5 per tree) was selected. Fruits were stored before the evaluation under a controlled temperature (18°C, 90% RH) and analyzed when papayas reached the stage 4 (BASULTO *et al*., 2009) after 3 days. 9 subsamples of the fruit were submitted to physical-chemical determination whereas 6 subsamples of the fruit were washed with cold tap water and peeled with the help of a stainless-steel knife; the seeds were removed, and the fruits were cut into small pieces approximately 2.5 cm thick. About 120 g of the cut samples were placed inside sanitized plastic trays (142 x 95 x 50 mm) and wrapped with a film Cryovac Sealappeal PSF. The characteristics of the film are as follows: thickness 25 μm; transmission rate of CO_2 800 $cm^3 m^{-2} day^{-1}$ at 23°C and 0% RH; transmission rate of O_2 72 $cm^3 m^{-2} day^{-1}$ at 4°C and 0% RH; moisture transmission vapor 47 $gm^{-2} day^{-1}$ at 38°C and 100% RH. Three replicates were prepared for each sampling time (9 packages) that were stored for 12 days at 5°C and RH of 90±1%. Samples were analyzed every four days and for each biological replicate were realized two technical replicates. The chosen time of storage adopted in this study emerged from preliminary assays performed to determine the probable shelf life resulted in early mold formation on the 12th day at 5ºC (data is not shown).

2.2. Determination of physico-chemical traits

2.2.1 Skin color (raw fruit)

For the skin cover color index (CC) evaluation (at harvest and consumption points), we used the fruit analysis system (FAS) procedure in agreement with FARINA *et al*. (2011).

2.2.2 Flesh color

Chromaticity values L* (Lightness), a* (green to red), and b* (blue to yellow) of flesh color fruit was determined with a colorimeter (Minolta C2500. Konica, Ramsey, NY), Chroma (Chr) and hue angle (Hue) were also calculated (FARINA *et al*., (2020c).

2.3. Physico-chemicals analyses

A digital scale (Gibertini, Italia) was used for determining the fresh weight (FW) and the size code was determined (CBI, 2018). Flesh firmness (FF) was measured using a digital penetrometer TR5325 (Turoni, Forlì, Italy) with a cylindrical needle (8 mm diameter) and values expressed in Newtons (N). Flesh juice was used to detect the total soluble solids content (TSS) with an optical digital refractometer (Atago, Japan), titratable acidity (TA) using a compact titrator (Crisom, Spain) and expressed in g citric acid/100 g fresh fruit and pH with a digital pH-meter (Model 2001, Crison, Barcelona, Spain). Reducing sugars were evaluated through a volumetric Fehling assay as described previously by ADILETTA *et al*. (2018).

2.4. Carotenoids, polyphenols content and antioxidant activity

Spectrophotometric detection of carotenoids content, xanthophylls plus carotenes (CAR), was determined in agreement with PETRICCIONE *et al*. (2015) and results were expressed as $\mu g\ 100\ g^{-1}$ FW applying Wellburn equations (WELLBURN, 1994). The total phenolic compounds (TP) were assessed as reported by MAGRI *et al*. (2020). The free radical

scavenging activity was gauged by 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to CINQUANTA *et al*. (2013).

2.5. Evaluation of antioxidant enzymatic system

Total soluble proteins were obtained from fresh papaya (1:3 w/v) blended in an extraction buffer prepared in agreement with MAGRI *et al*. (2020) and determined by the Bradford assay. Catalase activity (EC 1.11.1.6) (CAT) was estimated in according to the method described by ADILETTA *et al*. (2019) using 100 μL of crude enzyme extract. The activity was expressed in μmol of H_2O_2 g^{-1} FW. Superoxide dismutase activity (EC 1.15.1.1) (SOD) was evaluated with the method of nitroblue tetrazolium (NBT) reduction inhibition in agreement with ADILETTA *et al*. (2018a) using 50 μL of crude enzyme extract. SOD activity was expressed as U mg^{-1} FW, considering that one SOD unit corresponds to the amount of enzyme that, in the assay conditions, inhibits 50% the NBT reduction. Guaiacol peroxidase activity (EC 1.11.1.7) (GPX) was determined according to PETRICCIONE *et al*. (2015) and expressed as nmol g^{-1} FW. Ascorbate peroxidase activity (EC 1.11.1.7) was assessed as reported by ADILETTA *et al*. (2018b). The activity was expressed as μmol g^{-1} FW. Hydrogen peroxide content was estimated with the method reported by GOFFI *et al*. (2020) and expressed as nmol g^{-1} FW.

2.6. Oxidative damage markers and enzymatic browning

Polyphenoloxidase activity (EC.1.10.3.1) (PPO) was established with the method described by ADILETTA *et al*. (2018b) and PPO assay was carried out with 10 μL of crude enzyme extract. PPO activity was expressed as μmol g^{-1} FW. Malondialdehyde content (MDA) was determined as described by ADILETTA *et al*. (2018b) and expressed as nmol g^{-1} FW. Lipoxygenase activity (EC 1.13.11.12) (LOX) was determined in according to ADILETTA *et al*. (2018b) and expressed in nmol $m^{-1}g^{-1}$ FW, as the specific rate of molar change of hydroperoxides.

2.7. Microbiological analysis

Total aerobic bacteria (TAB) and yeast and mold count (YM) were conducted using a method designated by YOUSUF and SRIVASTAVA (2015). Results were reported as log colony forming units per gram.

2.8. Statistical analysis

Analysis of variance (ANOVA) and Duncan's test at a 5% level were used to compare the differences between samples analysed during storage. Principal components analysis (PCA) was realized to reduce the multidimensionality of dataset generating new principal components that account for most of the total variation. All statistical analyses were realized by SPSS software package, Version 20.0 (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Fresh fruits

The examined fruits were classified in H size (1101 - 1500 g) in according to CBI Size Codes A–J used for marketing channels (CODEX STAN, 2001). Consumers tend to prefer smaller papayas, particularly in northwestern Europe; because they suit individual consumption better and consider the fresh-cut papaya more convenient than the whole fruit (which should be peeled, deseeded and sliced before consumption) and they find the large size of some cultivars off-putting (RIVERA-LÓPEZ *et al*., 2005). Fruit with <55% yellow skin was the best to slicing and deseeding while those with <25% yellow skin showed no soft and edible flesh. In this study, papaya fruit were harvested at mature green stage 3 (BASULTO *et al*., 2009) and was characterized by some orange-colored stripes in skin; pulp almost completely orange in color, except near the skin, but still hard for consumption. In this stage, the color of the skin changes from dark green to light green and one or more yellow streak begins to develop from the base upwards. The cover color analysis revealed 33.3% of skin cover color at picking; afterwards fruits were let to ripe at room temperature reaching 59.6 % of yellow skin color after 4 days. Our results agree with YAHIA (2011), who suggested to select whole fruit with 55-80% of yellow skin which ensures > 50% of edible flesh recovery for production of fresh-cut papaya because fully ripe fruit were easily bruised and difficult to handle YAHIA (2011). Nonetheless, after these storage conditions the fruit reached good physico-chemical characteristics for fresh-cut processing (Table. 1). L*, chroma and hue values were comparable with those reported by GAYOSSO-GARCÍA *et al*. (2011) in the flesh of raw papaya fruit and by FARINA *et al*. (2020b) in fresh-cut coated fruit; similar TSS, TA (ZUHAIR *et al*., 2013) and pH values were observed by ZUHAIR *et al*. (2016). Finally, flesh firmness values were in agreement with other studies carried out on papaya fruit at the same ripening stage grown in tropical (FARINA *et al*., 2020a) and Mediterranean area (FARINA *et al*., 2020b).

3.2. Physico-chemical traits in fresh-cut fruits

Formosa papaya has fruit with a large size and orange flesh, for these features are generally valued as minimally processed fruits. In papaya fruit, flesh colour changes can be attributed to different regulation of carotenoid biosynthesis, a secondary metabolic pathway that yield metabolites also destined to other important fruit qualitative features (YAHIA, 2011; SHEN *et al*., 2019). Furthermore, the increase in metabolic activities also causes flesh colour changes during fresh-cut processing (RIVERA-LÓPEZ *et al*., 2005). The intensity and uniformity colour of flesh fruit affect its quality and consumer's choice and preference (RIVERA-LÓPEZ *et al*., 2005). Samples showed a significant decrease in L*after 4 days of cold storage, followed by a constant trend up to 12 days (Fig. 1A). Furthermore, the results showed a significant increase in redness a* (from 46.3 to 52.3) and decrease in yellowness b* (from 56.2 to 48.6) after 12 days of storage (Figs. 1B and C). WAGHMARE and ANNAPURE (2013) observed a similar trend in passive atmosphere packaging, and JAYATHUNGE *et al*. (2014), using micro-perforated polyvinyl chloride containers. As a result, changes in colorimetric parameters resulted in a significant decrease after 4 days of hue angle values, which then remained constant (Fig. 1D), while no significant differences were registered in chroma values during storage conditions (data not shown). In other word, red colour increased its intensity in all samples during the storage period; this was due to the degradation of chlorophylls or unmasking of preformed pigments during fruit

development. The colour changes ranged from yellow to reddish orange and were associated with ripening progress, as well as at the onset of browning. TSS in fresh-cut papaya tended to increase during cold storage with significant differences during storage time (Table 1). The highest TSS (8.51%) value in papaya samples was found on day 12. WAGHMARE and ANNAPURE (2013) reported a similar trend in TSS, explaining it by the solubilisation and synthesis of carbohydrates in fresh-cut papaya packed in polypropylene film with and without modified atmosphere and stored for 25 days at 5°C. Moreover, the low temperature helped to maintain a low level of respiration rates stopping the decrease in TSS content (RIVERA-LÓPEZ *et al*., 2005). Reducing sugar slightly increased in fresh-cut papaya during storage showing a high positive correlation (R^2=0.969 $p < 0.01$) with TSS (Table 2). The pH values increased significantly during storage, ranging from 5.51 to 5.64 after 12 days of cold storage.

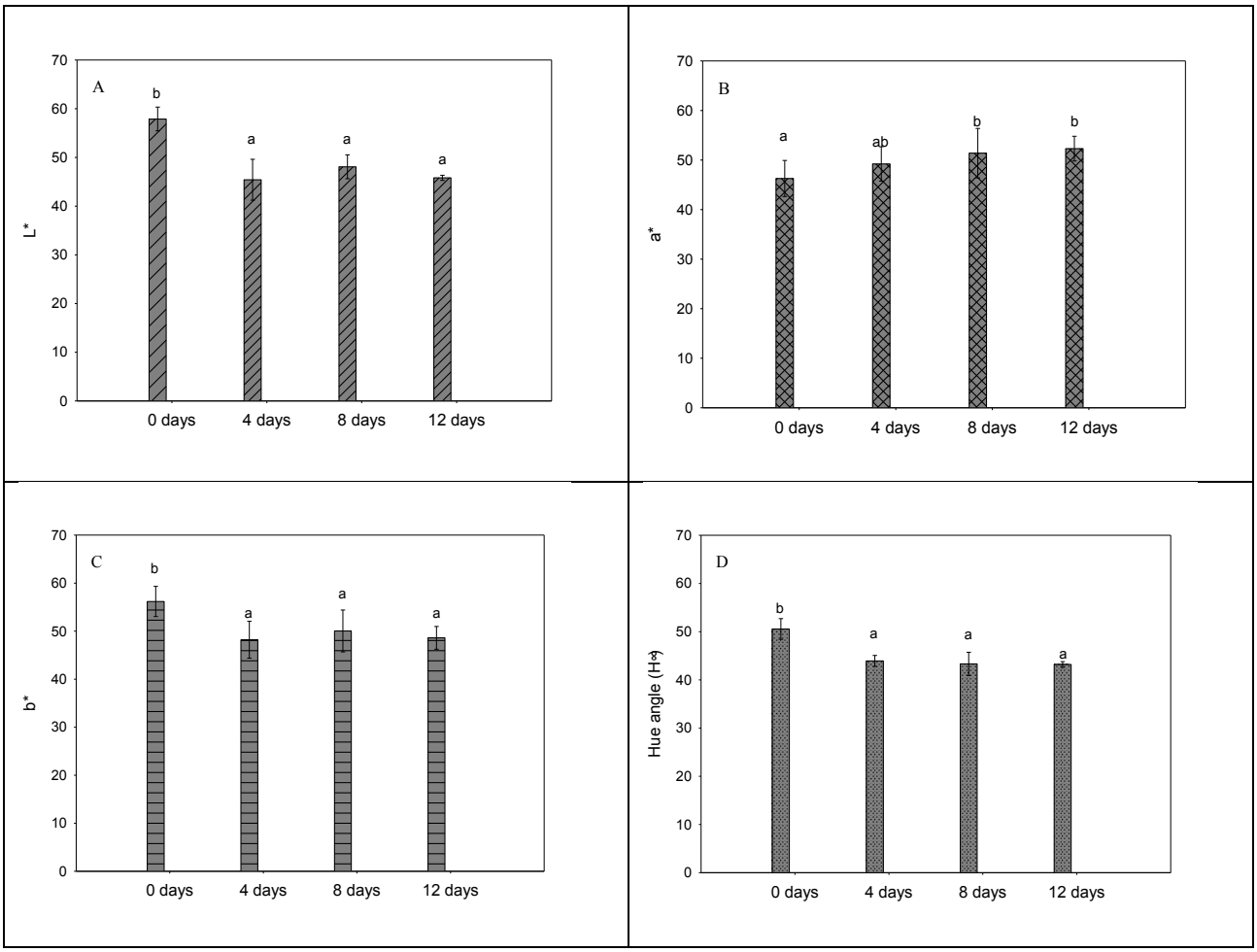

Figure 1. Evaluation of colorimetric traits, lightness L*(A), redness index a* (B), yellowness index b* (C) and Hue angle H° (D) in cut '*Formosa*' papaya fruit packaged in passive atmosphere stored at 5°C for 12 days. Means ± standard deviations followed by the same letter do not differ significantly at P = 0.05 (Duncan Test).

Table 1. Pomological traits of the *Formosa* papaya cultivar after three days of ripening at room temperature (18°C - RH 90%). Values represented as mean ±SD (n=9). Fruit weight (FW), Solid soluble content (TSS), Titratable acidity (TA), Firmness (F), Juiciness (J), skin cover color (CC), Lightness(L*), Chroma (Chr), hue angle (Hue).

FW (g)	TSS (Brix°)	TA (g/L)	F (N)	J (ml/100 g)	CC (%)	L*	Chr	Hue
1380±20	8.03±0.9	0.8±0.21	33.42±3.8	46.21±2.6	59.6%	62.85±7.9	60.96±7	1.02±0.3

Table 2. Changes in total soluble solid (TSS), reducing sugar (RS) and pH in cut 'Formosa' papaya fruit packaged in passive atmosphere stored at 5°C for 12 days.

Storage Time	TSS	RS	pH
Time 0	8.03±0.02 a	2.23±0.11 a	5.51±0.01 a
4 days	8.02±0.02 a	2.29±0,22 b	5.56±0.01 ab
8 days	8.09±0.01 b	2.42±0.13 c	5.59±0.01 bc
12 days	8.51±0.01 c	2.79±0.41 d	5.64±0.01 c

Means followed by the same letter do not differ significantly at P = 0.05 (Duncan Test).

3.2.1 Microbial growth in cut fruit

Fresh-cut fruit is more prone to the rapid growth of spoilage microorganisms as well as the pathogens of public health significance. Peeling process eases cross-contamination and the transfer of microflora from peel to the flesh fruit that represents an optimal substrate for microbial growth. Cold storage of fresh-cut papaya leads to an increase in TAB and YM values. TAB increased in all stored samples ranging from 1.8 (0 day) to 5.6 log CFU/g (12 days) (data not shown). These values were lower than the critical limit for total microbial loads of vegetables (8.0 log CFU/g) (JACXSENS *et al*., 2002). YM values increased from 1.3 to 5.2 log CFU/g, overcoming the critical limits of 5 log CFU/g for yeasts (JACXSENS *et al*., 2003) after 12 days of storage. Increased trend in microbial counts of TAB and YM throughout the storage of fresh-cut papaya were also reported by Gonzalez-Aguilar *et al*. (2009) and WAGHMARE and ANNAPURE (2013), correlated to the packaging systems, storage temperatures and different cut types of fresh-cut fruits.

3.2.2 Free radical scavenging power components in fresh cut fruits

Papaya is a tropical fruit with a high concentration of bioactive compounds such as polyphenols, vitamins and carotenoids, whose interactions contribute to the overall antioxidant activity of this fruit. Carotenoids are a group of fat-soluble molecules responsible to yellow-red color of fruits and vegetables. Ripening, cold storage and postharvest treatments can influence carotenoids content in fresh-cut fruit (SUPAPVANICH *et al*., 2020). The samples tested showed an increase in the carotenoids content during storage time, ranging from 570±12 (0 days) to 1600±92 μg 100 g FW^{-1} (12 days) (Fig. 2A). Our results were in agreement with FAJAR FALAH *et al*. (2015) who evaluated fresh-cut '*Bangkok*' papaya at different storage temperatures suggesting that this climacteric fruit continues to ripe during storage. In addition, it can also be assumed that carotenoids are more extractable due to changes in cell structure during storage time. TP

content significantly increased during 12 days of cold storage in fresh-cut '*Formosa*' papaya ranged from 19.1±1.8 (0 day) to 54.3±4 (12 days) mg GAE 100 g^{-1} FW (Fig. 2B). Wounding stress caused by cutting process might contribute to an increase of secondary metabolites such as phenolic compounds. Several studies have evaluated TP content in different papaya fruit highlighting that several factors such as fruit ripening, agronomic practices and post-harvest storage conditions affect the content of these bioactive compounds (ALI *et al*., 2014; ZUHAIR *et al*., 2013). Throughout the 12 days of cold storage, the DPPH radical-scavenging activity significantly increased ($p<0.05$) in stored samples (Fig. 2C). Bioactive compounds can act as antioxidants and our results indicated that high antioxidant activity in fresh-cut papaya fruit was related to the increase of polyphenols ($R^2 = 0.840$; $p<0.01$) content due to ripening process occurring during storage.

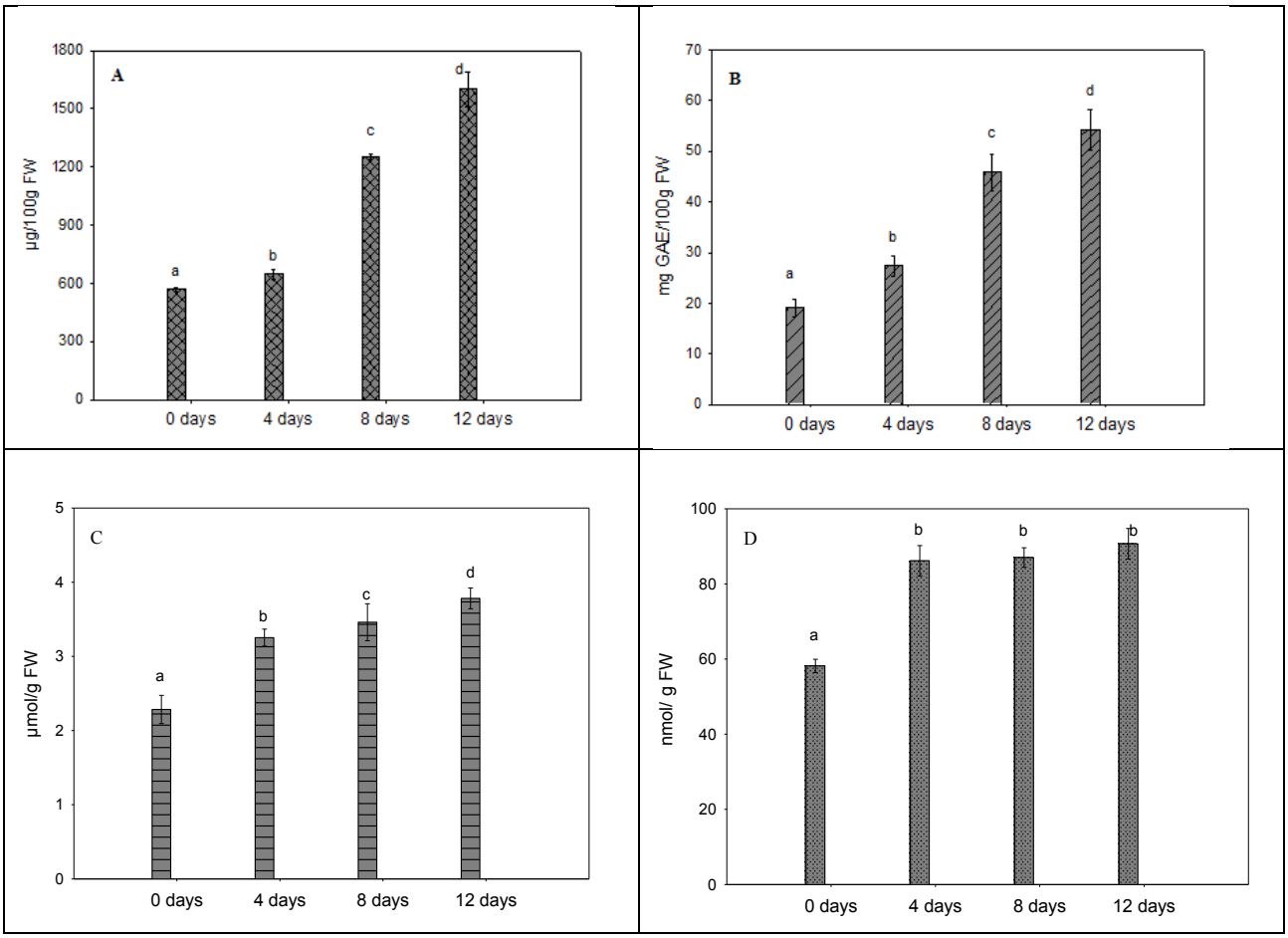

Figure 2. Carotenoids content (A; μg 100 g^{-1} FW), polyphenols content (B; mg GAE 100 g^{-1} FW), antioxidant activity (DPPH assay) (C; μmol TE g^{-1}FW), malondialdehyde content (D; nmol g^{-1}FW) in cut '*Formosa*' papaya fruit packaged in passive atmosphere stored at 5°C for 12 days.
Means followed by the same letter do not differ significantly at P = 0.05 (Duncan Test).

3.2.3 Antioxidant system in cut fruits

The stress by cutting process induces a physiological disorder with an alteration in cellular homeostasis increasing the ROS production in damaged cells. In fresh-cut fruit, the imbalance between production and accumulation of ROS could be due to enhancing the respiration rate and activation of amine- and NADPH-oxidases (MITTLER, 2002). H_2O_2 level increased rapidly during the first 4 days from cutting process and this trend continued up to 12 days of storage. H_2O_2 acts as a second messenger for the induction of several defense genes in crops in response to wounding (Table 3). Our results highlighted marked changes in enzymatic oxidative system due to the exposure to wounding stress in fresh-cut fruit. The **s**uperoxide dismutase (SOD) activity of fresh-cut papaya showed a slow increase through storage time, with minimal significant changes from 15.4±2 U mg^{-1} FW (0 day) to 20.2±4 U mg^{-1} FW (12 days) (Table 3). During the first 8 days of storage, the ascorbate peroxidase (APX) activity had no significant changes until 8 days, with an average value of 0.87±0.12 μmol g^{-1} FW, while its activity significant increased at the end of storage (12 days) (Table 2). A significant increase was registered in catalase (CAT) activity that during storage time up to 14.9±2 μmol g^{-1} FW (Table 3). Our results suggest that an increase of activities of antioxidant enzymes such as SOD, CAT, and APX, could improve the ability of the fresh-cut fruit to dismutate superoxide radicals and to eliminate hydrogen peroxide. In papaya, these enzymes prevent oxidative damage and reduce the susceptibility to chilling injury at low-temperature storage (HANIF *et al*., 2020). In cut fruit, antioxidant enzymes can modulate ROS levels and CAT and APX activities increased when ROS reached toxic levels. At low and moderate levels, ROS can act as signaling molecules and in cut fruit such as pitaya and strawberry mediating wounding-induced phenolic accumulation (LI *et al*., 2017; JACOBO-VELAZQUEZ *et al*., 2015).

3.2.4 Oxidative damage in cut fruits

The cut fruit showed a high perishability due to the peeling and cutting processes that caused cell disruption and membrane damage with decompartmentation of cellular structures, cellular functions and quality loss (PAL *et al*., 2004; JACOBO-VELAZQUEZ *et al*., 2015). In cut tissues, several enzymes come into physical contact with their substrates afterwards the cellular damages due to the cutting process (KARAKURT and HUBER, 2003). Browning is the result of enzymatic oxidation of phenolic compounds in lightly processed fruit (JACOBO-VELAZQUEZ *et al*., 2015). Polyphenoloxidase (PPO) and GPX are the main intracellular oxidative enzymes involved in enzymatic oxidation in stored fresh-cut fruit. PPO and GPX activities increased significantly during storage in the fresh-cut papaya up to 2.5- and 3.1-fold, respectively at the end of the experiment (Table 3). This suggests that the browning of fresh-cut papaya is primarily due to phenolic compounds oxidation caused by PPO and GPX activities. In the cut papaya, fruit lipoxygenase (LOX) activity significantly increased throughout cold storage with values ranging from 12.4±1 nmol g^{-1}FW (0 days) to 36.2±3 nmol g^{-1}FW (12 days) (Table 3). Instead, MDA content increased rapidly (47 %) during the first 4 days and then was stable around 88.9±5 nmol g^{-1} FW up to the end of storage (Fig. 2D). As suggested by KARAKURT and HUBER (2003), LOX activity is involved in peroxidative lipid metabolism with a possible relationship with tissue softening in fresh-cut and whole 'Sunrise Solo' papaya during cold storage. Several studies have demonstrated that LOXs are ripening-related enzymes in papaya fruit (FARINA *et al*., 2011). Our results confirm that in fresh-cut papaya the membrane lipid peroxidation occurred during cold storage. MDA content is useful to

evaluate the cell oxidative damage during storage and the effectiveness of post-harvest treatments in several fresh-cut fruit samples (GONZALEZ-AGUILAR *et al*., 2009; SOUZA *et al*., 2015; JACOBO-VELÁZQUEZ *et al*., 2011).

Table 3. Evaluation of **s**uperoxide dismutase (SOD; U mg^{-1} FW), catalase (CAT; μmol g^{-1} FW), ascorbate peroxidase (APX; μmol g^{-1} FW), guaiacol peroxidase (GPX; nmol g^{-1} FW), polyphenoloxidase (PPO; μmol g^{-1} FW), lipoxygenase (LOX; nmol g^{-1} FW) activity and H_2O_2 content (nmol g^{-1} FW) in cut '*Formosa*' papaya fruit packaged in passive atmosphere stored at 5°C for 12 days.

Storage Time	SOD	CAT	APX	GPX	PPO	LOX	H_2O_2
Time 0	15.4±2a	6.1±0.9a	0.8±0.1a	39.9±3a	0.4±0.05a	13±1.2a	0.02±0.01a
4 days	16.8±3ab	8.9±1b	0.9±0.1a	82.7±2b	0.4±0.1b	20±2.1b	0.06±0.01b
8 days	19.6±3ab	12.9±1c	0.9±0.1a	97.6±2c	0.6±0.1c	31±2.9c	0.11±0.03c
12 days	20.2±4c	14.9±1d	1.3±0.2b	124.8±7d	1.1±0.12d	39±3.8d	0.11±0.02c

Means followed by the same letter do not differ significantly at P = 0.05 (Duncan Test).

3.3. PCA in cut fruit

A dimensional map with loadings and scores plot obtained by PCA analysis is shown in Fig. 3.

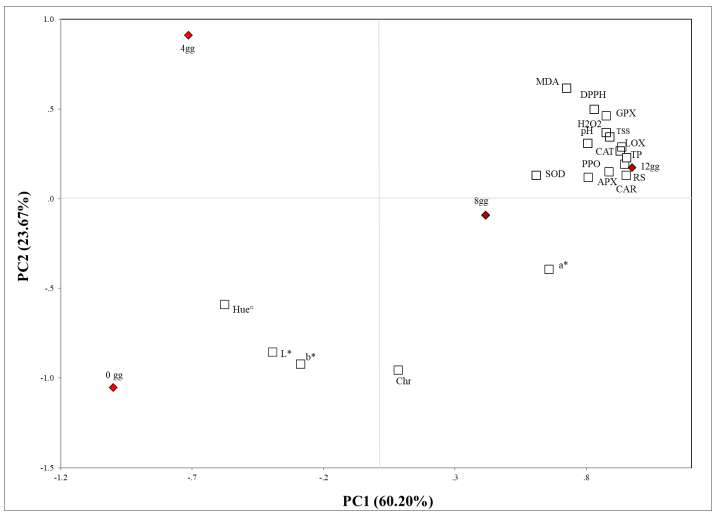

Figure 3 Principal component analysis of the physico-chemical, nutraceutical, and enzymatic traits in cut '*Formosa*' papaya fruit packaged in passive atmosphere stored at 5°C for 12 days. (TSS: total soluble solid; pH; L*: lightness; a*: redness; b*: yellowness; HUE: Hue angle; Chr: chroma; TP: total polyphenol content; CAR: carotenoid content; DPPH: antioxidant activity; SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase; PPO: polyphenoloxidase; GPX: guaiacol peroxidase; LOX: lipoxygenase; MDA: malondialdehyde content; H_2O_2: hydrogen peroxide content.

The first two principal components (PCs) explained 60.20% and 23.67% of the total variance, respectively. TSS, pH, a*, CAT, GPX, SOD, APX, PPO, LOX, H_2O_2 content, TP and CAR were positively correlated to PC1. PC2 showed a positive correlation with MDA and DPPH and a negative correlation with L*, b* and HUE. A shift along the first two PCs of score values highlighted physico-chemical, qualitative and enzymatic changes in fresh-cut papaya samples during cold storage.
At the beginning of cold storage, fresh-cut samples were situated in III quadrant after four days a shift from negative to positive ones along PC2 was registered. As cold storage progressed, samples displayed a shift from negative values to positive ones along PC1 showing qualitative decay of the fresh-cut papaya. The quality of fresh-cut papaya fruit, which includes physico-chemical, microbiological and nutritional traits, was preserved for 8 days of cold storage, while oxidative damages and qualitative decay were observed as the storage period progressed (12 days).

4. CONCLUSIONS

The semi-permeable packaging and cold storage (5±0.5°C) have extended the post-harvest period up to 12 days of minimally processed 'Formosa' papaya, preserving its microbiological and qualitative decay. Physico-chemical and nutritional traits changed as storage progressed due to the physiological processes associated with the ripening stage in papaya fruit. Cold storage leads to an increase in total aerobic bacteria, with lower values to the critical limit for total microbial loads of vegetables (8.0 log CFU/g) while the yeast and mold count slightly exceeded (5.2) the critical limit of 5 log CFU/g after 12 days of storage. Cutting process improved the enzymatic antioxidant system to modulate the ROS level. Enzymatic browning markers highlighted that a progressing oxidative damage occurred during cold storage. The balance between scavenging and production of ROS by the enzymatic antioxidant system allows regulating of ROS content that could be important signaling molecules in mediating the wound-induced bioactive compounds accumulated in fresh-cut papaya fruit during storage.

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PAPER

TREND OF POLYCHLORINATED DIBENZO-P-DIOXINS AND DIBENZOFURANS (PCDD/PCDFS) IN BEEHIVE MATRICES

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ABSTRACT

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDFs) are well-known persistent organic pollutants (POPs) with highly toxic potential. These compounds are released in the environment as a complex mixture of various congeners which shown significant physico-chemical differences, as well as different environmental fates. PCDD/PCDF mixtures change spatially and temporally in the environment and biota, complicating the risk assessment and regulatory control for human and animal exposure. Considering the well-known role of honeybees as bioindicators for pesticides, heavy metals and other chemicals, the present study has been developed to assess the use of honeybees and honeybee products in biomonitoring projects about PCDD/PCDFs. Three Dadant-Blatt type beehives, located since March 2017 in the headquarter of Ducati Motor Holding S.p.A. (Borgo Panigale, Bologna, Italy) have been used as monitoring stations. Honeybees, honey and beeswax have been sampled and analyzed for PCDD/PCDFs detection in June and in September of the same year. Among the analyzed

matrices, beeswax has shown the highest WHO-TEQ values, probably due to its lipidic nature capable of accumulating fat-soluble, non-volatile, persistent organic pollutants. Hexaclorodibenzo-p-dioxin (HxCDD), usually measured in vegetables and fruits, has been detected only in honey samples. Maximum levels of PCDD/PCDFs are settled by Commission Regulation (EC) No 1259/2011 of 2 December 2011, but only on animal-derived products. Considering the role of dietary-model adopted by the consumers on toxic substances dietary intake and associated exposure risks, limits on botanical derived products are needed. But more controls about bee-products are advisable also in order to reduce the exposure risk for bees and for protecting biodiversity.

Keywords: POPs, PCDD/PCDFs, honeybees, bio-indicators, environment, health

1. INTRODUCTION

Various attributes make the honeybee (*Apis mellifera*) the "ideal bioindicator" (TONG *et al*., 1975; STÖCKER, 1980; WALLWORK-BARBER, 1982; RAES *et al*., 1992; LEITA *et al*., 1996; ZHELYAZKOVA, 2012).
Due to the intense forager activity and the high sensitivity of bees toward toxic substances, the hives can give informations about environmental pollution via health-status and high mortality of bees or via the residues detection in honey, pollen, propolis, beeswax, royal-jelly, larvae and bees (CONTI and BOTRÈ, 2001; CRANE, 1984; BOGDANOV, 2006; CHAUZAT *et al*., 2011; PERUGINI *et al*., 2017).
As far as the biomonitoring of environmental pollution is concerned, honeybees have been used in a lot of investigations to evaluate different type of contaminants (KIRKHAM and COREY, 1977; BROMENSHENK *et al*., 1985; TONELLI *et al*., 1990; FRANCO *et al*., 1997; FRANCO *et al*., 1998; CELLI and MACCAGNANI, 2003; BALAYIANNIS and BALAYIANNIS, 2008; PORRINI *et al*., 2014). However, only few studies have tried to evaluate the possible application of honeybees as bioindicators for dioxin and furan detection (PORRINI *et al*., 2014; ÖZKÖK *et al*., 2018). Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDFs) are well-known persistent organic pollutants (POPs), with highly toxic potential (SEMANAINEN *et al*., 2002; Birnbaum *et al*., 2003). These compounds are released in the environment as a complex mixture of various congeners, produced primarily as by-products of chemical manufacturing activities and during the combustion of municipal and chemical waste (HUTZINGER *et al*., 1985; MENESES, 2004). Atmospheric transport and deposition processes lead to the dispersion of these compounds into soils, plant surfaces, bodies of water and sediments (VAN DEN BERG *et al*., 1994; LOHMANN and JONES, 1998). Due to the significant differences in physico-chemical properties (solubilities, volatilities, rates of degradation/metabolism, exc.) of each congener, the complex mixtures of PCDD/PCDFs change spatially and temporally in the environment and in animal tissues (SCHRENK *et al*., 1991; WEGIEL *et al*., 2018; ZHENG *et al*., 2008). Due to their lipophilic properties, PCDD/PCDFs may concentrate in fatty tissues and bioaccumulate through the food chain (TRAAG *et al*., 2006).
Considering the well-known role of honeybees as bioindicators, the present study has been developed to evaluate the distribution of PCDD/PCDFs in the hive. Due to their wide use, honey and beeswax contamination could also represent an important safety concern. Nevertheless, this investigation has the main purpose of improving data about the possible application of honeybees as bioindicators for monitoring environmental pollution and human exposure risks.

2. MATERIALS AND METHODS

2.1. Beehives location

Monitoring station in the headquarter of Ducati Motor Holding S.p.A. (Borgo Panigale, Bologna, Italy), was placed in an important Italian industrial area, at less than 7 Km from Bologna Central Station. Bologna city represents one of the most populated cities in Italy (2783 p/Km^2) and its province extends for about 3702 Km^2 representing in Italy, the most productive industrial area for metalworking and engine sector. The monitoring station, consisting of three beehives (BH1, BH2, BH3), Dadant-Blatt type with 12 frames have been

located in the headquarter of Ducati Motor Holding S.p.A. (Borgo Panigale, Bologna, Italy) at the end of March 2017. The hives, homogeneous in colony strength (colonies bring up on 10 frames) were placed into a wooden gazebo open frontally and laterally, with roof on top, at the distance of 40 cm between them. As suggested by PORRINI *et al*, (2014), all the beehives have been provided with cages for dead bees sampling (under-basket type) and were periodically monitored.

2.2. Sampling

Honeybees, honey and beeswax were sampled from each beehive in June and in September 2017.
Bees have been collected alive in airtight container directly from the combs, taking care to not involve the queen. Honey and wax have been collected as two honeycomb centrifuged for honey extraction.
Stored at −20°C for 24 hours, each sample (50g) was homogenized with liquid nitrogen by a crushing mill (IKA, Wilmington, NC), then analyzed for PCDD/PCDFs detection. The analyzed congeners have been reported in Table 1.

2.3. Chemical analysis

Determination of PCDD/Fs were performed following analytical methods based on international norms for dioxin analysis, such as EPA 1613 (EPA, 1994), and following the requirements of European Directives related to this subject.
Extraction of the fat fraction, including the compounds of interest, was performed in Soxhlet apparatus with solvents (hexane/dichlormethane or hexane/diethyl ether). Beeswax samples were directly dissolved in 20 ml of hexane. Sample extracts were purified in a 4 cm diameter multilayer column, containing (top to bottom) Na2SO4 , 44% H2SO4/silica, 22 %H2SO4/silica, NaOH/silica and AgNO3/silica. PCDD/Fs were eluted with hexane. The purified extracts were fractionated in SPE pre-packed carbon tubes (Supelclean Envi-Carb), from SUPELCO (Bellefonte, PA, USA). The obtained PCDD/F fractions were evaporated to 15 μl under nitrogen stream and corresponding PCDD/F 13C syringe standards (1,2,3,4-TeCDD and 1,2,3,7,8,9-HxCDD) were added.
Samples were analysed in a 6890N gas chromatograph (Agilent, Santa Clara, CA, USA), coupled to an Autospec Ultima high resolution mass spectrometer (Micromass, Manchester, UK), operating in electronic impact ionization mode and at 10,000 resolving power. For the PCDD/F analysis, samples were injected (2 μl) on splitless mode (1 min) into the injector at 280 ºC. The chromatograph was fitted with a RTX-5MS column (60 m x 0.25 mm i.d., 0.25 μm) from Restek (Bellefonte, PA, USA). Carrier gas was helium at 250 kPa constant pressure mode. The temperature program was 150 ºC (held for 1 min), increased at 30 min-1 to 200 ºC, increased at 3 ºC min-1 to 235 ºC (held for 10 min) and increased at 6 min-1 to 300 ºC (held 17 min). Monitored masses were those proposed by EPA 1613 method (EPA, 1994). Samples were quantified according to the isotopic dilution method, with the use of 13C12-labelled PCDD/F as internal standards. Among 200 PCDDs and 70 PCDFs, 17 congeners considered dangerous from a toxicological point of view (Council Regulation (EU) 1259/2011), have been evaluated for the present investigation (Table 1).

Table 1. LOQ, LOD and WHO-TEFs (Van den Berg *et al*., 2006) of researched PCDD/PCDF congeners.

	LOQ (pg/g)	LOD (pg/g)	WHO 2005 TEFs
2, 3, 7, 8 - Tetrachlorodibenzo-p-dioxin (TCDD)	0.04	0.02	1
1, 2, 3, 7, 8 - Pentachlorodibenzo-p-dioxin (PeCDD)	0.05	0.025	1
1, 2, 3, 4, 7, 8 - Hexachlorodibenzo-p-dioxin (HxCDD)	0.10	0.05	0.1
1, 2, 3, 6, 7, 8 - Hexachlorodibenzo-p-dioxin (HxCDD)	0.10	0.05	0.1
1, 2, 3, 7, 8, 9 - Hexachlorodibenzo-p-dioxin (HxCDD)	0.10	0.05	0.1
1, 2, 3, 4, 6, 7, 8 - Heptachlorodibenzo-p-dioxin (HpCDD)	0.25	0.13	0.01
1, 2, 3, 4, 6, 7, 8, 9 - Octachlorodibenzo-p-dioxin (OCDD)	0.50	0.25	0.0003
2, 3, 7, 8 - Tetrachlorodibenzofuran (TCDF)	0.04	0.02	0.1
1, 2, 3, 7, 8 - Pentachlorodibenzofuran (PeCDF)	0.05	0.025	0.03
2, 3, 4, 7, 8 - Pentachlorodibenzofuran (PeCDF)	0.05	0.025	0.3
1, 2, 3, 4, 7, 8 - Hexachlorodibenzofuran (HxCDF)	0.10	0.05	0.1
1, 2, 3, 6, 7, 8 - Hexachlorodibenzofuran (HxCDF)	0.10	0.05	0.1
2, 3, 4, 6, 7, 8 - Hexachlorodibenzofuran (HxCDF)	0.10	0.05	0.1
1, 2, 3, 7, 8, 9 - Hexachlorodibenzofuran (HxCDF)	0.10	0.05	0.1
1, 2, 3, 4, 6, 7, 8 - Heptachlorodibenzofuran (HpCDF)	0.25	0.13	0.01
1, 2, 3, 4, 7, 8, 9 - Heptachlorodibenzofuran (HpCDF)	0.25	0.13	0.01
1, 2, 3, 4, 6, 7, 8, 9 - Octachlorodibenzofuran (OCDF)	0.50	0.25	0.0003

2.4. WHO-TEF and WHO-TEQ

The concepts of toxic equivalency factor (TEF) and total toxic equivalent (TEQ) have been developed and introduced by the World Health Organization (WHO) to facilitate risk assessment and regulatory control of exposure to PCDD/PCDFs mixtures. The WHO-TEF estimates the toxic potential of each congener comparing its affinity for a cytosolic receptor protein (aryl hydrocarbon receptor – AhR) with the highest affinity associated to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Table 1).
The WHO-TEQ is operationally defined by the sum of each compound concentration multiplied by its TEF value and represents an evaluation of the total 2,3,7,8-TCDD–like activity of the PCDD/PCDFs mixture, as well as of their total potential toxicity (VAN DEN BERG *et al*., 1998, VAN DEN BERG *et al*., 2006; VAN DEN BERG *et al*., 2013).
Two different methods can be used for WHO-TEQ evaluation. Usually, it is calculated as *lower-bound* for environmental matrices, considering the undetectable concentrations equal to zero. Instead, for high-lipid-content food products it is calculated with the upper-bound method, considering the undetectable concentrations equal to the detection limit of each congener (LOD) (Commission Regulation (EU) No 589/2014). For the present study the WHO-TEQs were calculated on honeybees, honey and beeswax PCDD/PCDFs concentrations with both lower-bound and upper-bound methods (Table 2).

3. RESULTS AND DISCUSSION

Ingestion of contaminated food is the principal way of human exposure to PCDD/PCDFs, accounting for 90% if compared to other ways such as inhalation and dermal contact (SWEETMAN *et al*., 2000). This concern about the human health impact and continuous

encouragement from scientific committees to monitor food samples across Europe, have led to numerous international and local studies on the concentration of dioxins in particular food items or on the estimation of the daily intake from food (European Commission, Brusseles, June 2002; FOCANT *et al*. 2002; KARL *et al*., 2002). Seafood represents the most contaminated foodstuff and the congeners most frequently detected in all type of analyzed foodstuff were OCDD and HpCDD, as well as PeCDD. Regarding dietary intake evaluation on human health it was carry out combining data on consumption habits with the different concentrations of PCDD/PCDFs expressed in WHO-TEQ found in food samples (BORDAJANDI *et al*., 2004).

Honey and other bee-products are included in nutritional habits of a lot of country and currently are widely used also as dietary supplements for health purposes. However these product were never been taking into account for human dietary exposure calculation and then never analysed for PCDD/PCDFs evaluation. This study has been performed for improving this lack o data profile on food PCDD/PCDFs concentration and to evaluate a possible application of honeybee as bio-indicators for PCDD/PCDFs monitoring in the environment. Results are showed in Tables 2 and 3.

Octachlorodibenzo-p-dioxin (OCDD), as well as being reported in other studies regarding PCDD/PCDFs, is the congener most frequently detected during the present investigation (BORDAJANDI *et al*., 2004; DOMINGO *et al*., 1999). It has been quantified in all the analysed matrices (Figure 1 and Figure 2). Aside for one honeybee sample collected in September (BH1), that reported 0.07 pg/g of 2, 3, 7, 8 – tetraclorodibenzofuran (TCDF), OCDD was the only congener detected in honeybee samples. This trend is confirmed also for honey samples. TCDF has been quantified only in one sample of honey collected in September from BH1 (0.07 pg/g), while interesting concentrations of OCDD have been detected in all honey samples collected in June and in September (Figs. 1 and 2).

Detectable concentrations of 1, 2, 3, 4, 7, 8 - hexaclorodibenzo-p-dioxin and 1, 2, 3, 6, 7, 8 - hexaclorodibenzo-p-dioxin (HxCDD), usually measured in vegetables and fruits (DOMINGO *et al*., 1999), have been detected, in June and in September, only in honey samples (Figs. 1 and 2). Honey is a natural product that honeybees make from blossom's nectar or from secretions coming from living parts of plants (ÖZKÖK *et al*., 2017), but ÖZKÖK *et al*. (2018) monitoring PCDD/PCDFs in honeybee pollen (honey component) had encountered different results. 2, 3, 7, 8 – TCDF, 1, 2, 3, 7, 8 – PeCDD, 2, 3, 4, 7, 8 – PeCDF, showed the higher concentrations with both analytical methods employed for the study. However, mentioned studies confirm with present data, that maximum levels should be established also for cereals, vegetables and bee-products, in which not negligible concentrations have been reported (ÖZKÖK *et al*., 2018). Moreover, vegetables represent the most frequent consumed food for a healthy diet and dietary-model adopted by the consumers should be considered important for assessing daily pollutants intake for humans (DOMINGO *et al*., 1999; SCHECTER *et al*., 2006).

The most toxic PCDD/PCDF congeners are 2,3,7,8-substituted tetra-, penta-, and hexachloro compounds that, in addition to OCDD, have the greatest tendency to bioaccumulate (COHEN *et al*., 2002; BOCIO and DOMINGO, 2005). Nevertheless, the highest concentrations registered during the present investigation and associated to heptaclorodibenzo-p-dioxin (HpCDD) and octachlorodibenzo-p-dioxin (OCDD), have been detected in beeswax samples collected in June (Fig. 1).

Table 2. Concentrations detected in June 2017.

Analyzed congeners	Bees					Honey					WAX				
	BH1* (pg/g)	BH2* (pg/g)	BH3* (pg/g)	Average* (pg/g)	SD	BH1* (pg/g)	BH2* (pg/g)	BH3* (pg/g)	Average* (pg/g)	SD	BH1* (pg/g)	BH2* (pg/g)	BH3* (pg/g)	Average* (pg/g)	SD
2,3,7,8 - Tetraclorodibenzo-p-diossina (TCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8 - Pentaclorodibenzo-p-diossina (PeCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,7,8 - Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	0,11	0,11	0,15	0,12	0,02	<LOD	<LOD	<LOD	ND	ND
1,2,3,6,7,8 - Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	0,12	0,12	0,08	0,07	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8,9 - Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,6,7,8 - Eptaclorodibenzo-p-diossina (EpCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	2,01	1,39	1,73	1,71	0,31
Octaclorodibenzo-p-diossina (OCDD)	0,52	0,56	<LOD	0,36	0,31	0,54	0,52	0,51	0,52	0,02	12,05	9,08	9,97	10,37	1,52
2,3,7,8 - Tetraclorodibenzofurano (TCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,09	0,07	0,08	0,08	0,01
1,2,3,7,8 - Pentaclorodibenzofurano (PeCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,07	0,07	0,08	0,07	0,01
2,3,4,7,8 - Pentaclorodibenzofurano (PeCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,7,8 - Esaclorodibenzofurano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND

1,2,3,6,7,8 - Esaclorodibenzo furano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
2,3,4,6,7,8 - Esaclorodibenzo furano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8,9 - Esaclorodibenzo furano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,6,7,8 - Eptaclorodibenzo furano (EpCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,41	1,20	0,86	0,82	0,40
1,2,3,4,7,8,9 - Eptaclorodibenzo furano (EpCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,25	<LOD	<LOD	0,08	ND
Octaclorodibenzofu rano (OCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,84	0,66	0,88	0,79	0,12

Table 3. Concentrations detected in September 2017.

	Bees					Honey					WAX				
Analyzed congeners	**BH1* (pg/g)**	**BH2* (pg/g)**	**BH3* (pg/g)**	**Average* (pg/g)**	**SD**	**BH1* (pg/g)**	**BH2* (pg/g)**	**BH3* (pg/g)**	**Average* (pg/g)**	**SD**	**BH1* (pg/g)**	**BH2* (pg/g)**	**BH3* (pg/g)**	**Average* (pg/g)**	**SD**
2,3,7,8 - Tetraclorodibenzo-p-diossina (TCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8 - Pentaclorodibenzo-p-diossina (PeCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,7,8 – Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	0,11	0,16	0,15	0,14	0,03	<LOD	<LOD	<LOD	ND	ND
1,2,3,6,7,8 – Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	0,11	0,18	0,12	0,14	0,04	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8,9 - Esaclorodibenzo-p-diossina (ExCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,6,7,8 - Eptaclorodibenzo-p-diossina (EpCDD)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,58	1,36	1,36	1,10	0,45
Octaclorodibenzo-p-diossina (OCDD)	0,77	0,90	0,79	0,82	0,07	0,52	0,59	0,52	0,54	0,04	5,68	8,42	10,04	8,05	2,20
2,3,7,8 - Tetraclorodibenzofurano (TCDF)	<LOD	<LOD	0,07	0,07	0,00	<LOD	<LOD	0,07	0,07	0,00	0,14	0,15	0,16	0,15	0,01

1,2,3,7,8 - Pentaclorodibenzofurano (PeCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,05	0,08	0,05	0,06	0,02
2,3,4,7,8 - Pentaclorodibenzofurano (PeCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,09	0,08	0,05	0,07	0,02
1,2,3,4,7,8 - Esaclorodibenzofurano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	0,12	0,10	0,10	0,11	0,01
1,2,3,6,7,8 - Esaclorodibenzofurano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
2,3,4,6,7,8 - Esaclorodibenzofurano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,7,8,9 - Esaclorodibenzofurano (ExCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,6,7,8 - Eptaclorodibenzofurano (EpCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
1,2,3,4,7,8,9 - Eptaclorodibenzofurano (EpCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND
Octaclorodibenzofurano (OCDF)	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	<LOD	ND	ND	<LOD	<LOD	1,31	1,31	0,00

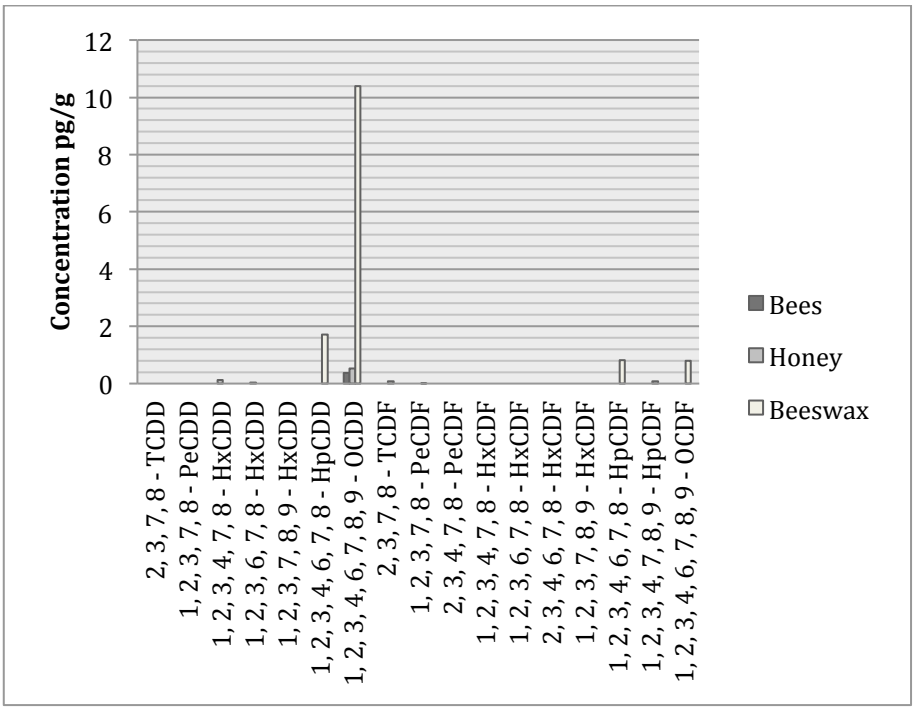

Figure 1. Average concentrations of PCDD/PCDFs detected in hive matrices in June 2017.

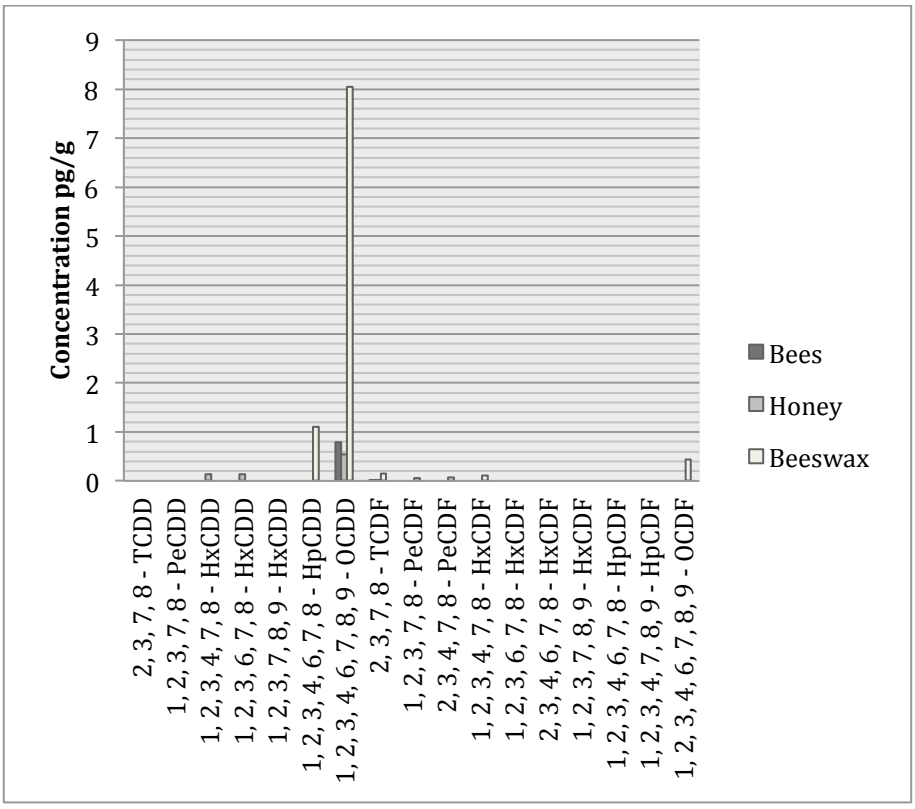

Figure 2. Average concentrations of PCDD/PCDFs detected in hive matrices in September 2017.

Among the analysed matrices, beeswax has shown the highest number of detectable congeners. HpCDD, OCDD, TCDF, pentachlorodibenzofuran (PeCDF), heptachlorodibenzofuran (HpCDF) and octachlorodibenzofuran (OCDF) have all been detected in beeswax (Figs. 1 and 2). A larger number of congeners have been quantified in beeswax samples collected in September as compared to those collected in June (Fig. 2) and indeed, the highest WHO-TEQ values calculated for the present investigation have been associated to them (Table 4).

Table 4. Average WHO-TEQ *lower-* and *upper-bound* values, calculated on the average PCDD/PCDF concentrations measured in honeybee, honey.

	June 2017			September 2017		
	Honeybees	**Honey**	**Beeswax**	**Honeybees**	**Honey**	**Beeswax**
WHO-TEQ *lower bound*	0.0001 pg/WHO-TEQ/g	0.0088 pg/WHO-TEQ/g	0.0382 pg/WHO-TEQ/g	0.0025 pg/WHO-TEQ/g	0.0164 pg/WHO-TEQ/g	0.0630 pg/WHO-TEQ/g
WHO-TEQ *upper bound*	0.1884 pg/WHO-TEQ/g	0.1913 pg/WHO-TEQ/g	0.2159 pg/WHO-TEQ/g	0.1894 pg/WHO-TEQ/g	0.1971 pg/WHO-TEQ/g	0.2181 pg/WHO-TEQ/g

Higher values of WHO-TEQ lower- and upper-bound have been registered in September then in June for all the analyzed matrices (Table 2). The highest amounts of WHO-TEQ lower- and upper-bound have been associated to the beeswax samples in June and in September (Table 2). Beeswax composition, consisting in a mixture of fatty acids, fatty alcohols, paraffinic hydrocarbons and free fatty acids, is capable of accumulating of fat soluble, non-volatile and persistent pollutants (TULLOCH, 1980; JOHNSON *et al*., 2010; SERRA-BONVEHÍ and ORANTES-BERMEJO, 2010; RAVOET *et al*., 2015; PERUGINI *et al*., 2017). However, the mechanisms of beeswax contamination have not been well investigated yet. Beeswax is made by young worker bees who have never been out of the hive and its contamination could be the result of chemicals transmigration between different matrices, as well as the result of degradation/metabolism processes allowed by the bees consuming contaminated pollen and nectar. Currently, regarding PCDD/PCDFs, a possible bioaccumulation phenomenon in the hive cannot be excluded. Similarly to animal's fat-tissues, beeswax is the main reservoir for PCDD/PCDFs mixtures that change in the "hive tissues" during the exposure time according to specific degradation/metabolism processes.
Although further studies would be advisable, honeybees, honey and beeswax data suggest the possible use of them as indicators for PCDD/PCDFs distribution in the environment (bees), in vegetable foodstuffs (honey) and in animal fat-tissues (beeswax).

4. CONCLUSIONS

"Honeybees monitoring stations" have been confirmed as an effective and inexpensive method for controlling the levels of PCDD/PCDFs, as well as other pollutants, in the environment.
Nevertheless, honey and beeswax contamination also represents an important concern for beekeeping practices and for honeybee products consumer health.

Honey is an important food product in many countries and beeswax finds important applications in food, cosmetic and pharmaceutical industries, representing possible sources of exposure for humans (PERUGINI *et al*., 2017). Considering the beekeeping common practice to recycle not controlled beeswax for wax foundation sheets production, it can become a source of subsequent recirculation of pollutants in the hive, with serious risks for honeybee health and for biodiversity protection (MULLIN *et al*., 2010; WU *et al*., 2011; WU *et al*., 2012).
Based on WHO-TEF and WHO-TEQ concepts, the Commission Regulation (EC) No 1259/2011 of 2 December 2011 set PCDD/PCDFs maximum levels modifying those established by the Commission Regulation (EC) No 1881/2006 of 19 December 2006. These limits are settled mainly for animal-derived products and expressed as pg WHO-PCDD/F-TEQ/g, but currently, no maximum levels are applied to cereals, fruit and vegetables, or to honey and other honeybee products.
According to many studies this lack should be revised in order to guarantee risk assessment and regulatory control of exposure to PCDD/PCDFs mixtures, taking into account real nutritional habit in different countries. Cereals, vegetables and bee-products have long been considered with a "low-impact" for humans daily intake, but recent studies (ÖZKÖK *et al*., 2018) have demonstrated that interesting concentrations can also be found in honeybee pollen (honey component) as well in cereals and vegetables (BORDAJANDI *et al*., 2004; DOMINGO *et al*., 1999; FOCANT *et al*., 2002; KARL *et al*., 2002; SCHECTER *et al*., 2006).

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PAPER

SCREENING OF EXTRA VIRGIN OLIVE OIL-IN-BITTER ORANGE JUICE (O/W) NANO-EMULSIONS STABILIZED WITH DIFFERENT FOOD-GRADE SURFACTANTS: A MODEL SYSTEM FOR NATURAL DAILY USE SALAD DRESSING

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ABSTRACT

Olive oil-in-bitter orange juice (O/W) nano-emulsions were prepared by phase inversion composition method at 25°C. The emulsions were formulated with extra virgin olive oil as an organic phase, bitter orange juice (pH 2.57) as an aqueous phase and binary combinations of polyoxyethylene sorbitan monooleate, sorbitan monooleate, sucrose monopalmitate and sunflower lecithin as surfactants. Visual appearance, transparency, microstructure and particle size distribution of the nano-emulsions were influenced by surfactant blend composition and concentration. The current study may promote usage of bitter orange for flavoring and acidifying salads via increasing consumer awareness, and to promote being fit with daily diet routines.

Keywords: bitter orange, nano-emulsion, olive oil, salad dressing, surfactant

1. INTRODUCTION

Because of the long-term kinetic stability of nano-emulsions against physical effects such as temperature changes and dilution, their popularity and usage demands increase day by day (ANTON *et al.*, 2008). Their good resistance to gravity separation, coalescence and flocculation makes them incomparable and they are regarded as achieving 'approaches thermodynamic stability' (TADROS *et al.*, 2004). When the current food grade nano-emulsion studies in the literature are examined, most of them are studied under ideal conditions (that is, low density essential oils such as orange oil, lemon oil and etc. as an organic phase and buffer solutions with neutral pH as an aqueous phase) by using high energy methods and the characteristic properties of the emulsions such as particle size, physical properties and stability are examined (CHOI *et al.*, 2011; RAO and MCCLEMENTS, 2011; QIAN and MCCLEMENTS, 2011; RAO and MCCLEMENTS, 2012a; KALTSA *et al.*, 2013). So, nano-emulsion studies related to real food systems is lacking.

The phase inversion composition (PIC) method, which is one of the low energy emulsification methods, uses chemical energy liberated by the phase transition occurring while the emulsification procedure at constant temperature (SOLANS and SOLÉ, 2012). PIC procedure includes the introducing of an aqueous phase into an agitating organic phase. That is, the surfactant and oil constituents which making up the organic phase are uniformly blended together to get homogeneous solution. The aqueous phase is then introduced into the continuously stirring organic phase at a controlled injection rate (i.e., injection amount per time) (KOMAIKO and MCCLEMENTS, 2016). Considering the PIC method in detail to understand how the phase transitions are led by variation in composition during emulsification process itself at constant temperature, SOLANS and SOLÉ (2012) reported that when the aqueous phase is initially introduced into the organic phase, water-in-oil (w/o) emulsion is produced. As more aqueous phase is introduced, a liquid crystalline phase may be presented which can be so viscous to inhibit further stirring. As extra aqueous phase is introduced, a multiple emulsion (oil-in-water-in-oil, o/w/o) is fabricated, and the viscosity of the system diminishes. As the volume fraction of aqueous phase further increases, a transitional phase inversion happens (o/w/o to o/w), and the small oil droplets available within the aqueous phase of the o/w/o emulsions are liberated (KOMAIKO and MCCLEMENTS, 2016). Although studies have shown that low energy methods are generally more effective in producing smaller droplet sizes than high energy methods; the low energy methods are still not common in the food industry and also, the factors affecting the performance of low energy methods are still not fully understood (SABERI *et al.*, 2013).

Olive oil, as an organic phase of the current study, is high nutritious and beneficial to human health with its unique fatty acid composition, as well as high stability to lipid oxidation. However, finely dispersed olive oil droplets within emulsions have been examined usually over enzymatic reactions and, studies relating finely dispersed oil droplets within real food-grade emulsion systems are lacking (POLYCHNIATOU and TZIA, 2014). The bitter orange (*Citrus aurantium*), whose juice is the aqueous phase of the current study, is a fruit known with strong natural sour flavor due to the presence of beneficial flavonoids, mostly naringin, and neohespiridin, and due to its own tissue acidity (PETERSON *et al.*, 2006). Bitter orange has received great interest recently for its use in the prevention of major health challenge of 21st century: obesity. *p*-sinephrine, found in unripe bitter orange (*Citrus aurantium*) fruit or shell extract, is commonly used to control weight, weight loss and strengthen stamina in sports performance products (STOHS *et al.*, 2012; STOHS *et al.*, 2011). American Food and Drug Administration on 11 April 2004

restricted the use of anti-obesity products containing ephedrine since the possible health problems related to them such as vascular occlusion, hypertension, stroke, psychiatric problems. Thus, bitter orange has become a safe alternative to ephedrine, and the importance of the use of bitter orange in dietary products has increased (TOKGOZ and GÖLCÜKLÜ, 2009). Bitter orange juice can be alternative to lemon juice for flavoring and acidifying salads (KARABIYIKLI *et al.*, 2014), which may promote being fit with easy daily diet routines. Since bitter orange is produced large quantities in the Mediterranean region, the use of bitter orange could be expanded by increasing consumer awareness. Hovewer, there has been no detailed research and data on the use of bitter orange juice in food systems, especially no studies have been done on olive oil-in-bitter orange juice emulsions at nano-scale. Therefore, current study has emerged from the intention of expanding usage of bitter orange for flavoring and acidifying salads via increasing consumer awareness, and to promote being fit with daily diet routines. Olive oil-in-bitter orange juice (O/W) nano-emulsions were prepared by phase inversion composition (PIC) method with binary combinations different surfactants. Visual appearance, transparency, emulsion stability index, microstructure and particle size distribution of emulsions were investigated.

2. MATERIALS AND METHODS

2.1. Materials

Bitter oranges (*Citrus aurantium* L.) were harvested at optimum maturity in February 2017, from Mersin, Çukurova region, in Turkey. Polyoxyethylene sorbitan monooleate (Tween 80) (Hydrophilic–lipophilic balance (HLB) =15.0), Sorbitan monooleate (Span 20) (HLB=8.6), Sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Chemical Company. Sucrose Monopalmitate (SMP) (HLB=18.5) and Sunflower Lecithin (SL) were kindly gifted from Compass Foods Company (Singapore). Since HLB of SL was not reported in literature, also in product specifications, it was assumed to have similar HLB of sunflower and soybean lecithin,i.e., HLB=8.0 (CHEN *et al.*, 2015). Commercially available Turkish extra virgin olive oil (EVOO) Tariş was obtained from the local supermarkets of Gaziantep/Turkey and used without further purification. Ultrapure water from a Milli-Q Plus system was used for dilution of bitter orange juice concentrates.

2.2. Preparation of nano-emulsions by low energy method

Oil-in-water (O/W) nano-emulsions with 90 % aqueous phase and 10 % organic phase (surfactant blend + olive oil) were prepared by a low energy method of Phase Inversion Composition (PIC). Four food-grade surfactants, namely, two types of low molecular weight surfactants (Polyoxyethylene sorbitan monooleate, Tween80, and Sorbitan monooleate, Span20), one sucrose ester (Sucrose monopalmitate, SMP) and one lipid based surfactant (Sunflower lecithin, SL) were selected to stabilize the nano-emulsions as binary combinations of Tween80/Span20, Tween80/SL, Tween80/SMP and SMP/SL. Extra virgin olive oil (EVOO) was used as an organic phase. Bitter orange juice was centrifugated at 5000 rpm for 60 min at three times, filtered using 20 micron filter paper, which was used as aqueous phase.

Prescreening tests: To define adequate surfactant blend (S_A/S_B) weight ratios (w/w) and oil to surfactant blend (O/S, where S stand for S_A+S_B) weight ratios (w/w), nano-emulsions

were prepared at many O/S weight ratios with changing surfactant blend (S_A/S_B) weight ratios (w/w). Due to the PIC method, first surfactant blends of Tween80/Span20, Tween80/SL, Tween80/SMP and SMP/SL were prepared by blending with weight ratios of (S_A/S_B) 90/10, 85/15, 80/20, 75/25 at ambient temperature. A certain amount of EVOO, was evenly mixed with surfactant blends at O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 under a stirring rate of 500 rpm via vortex (Velp Scientifica, Europe). The final mass of organic phase was 0.4 grams. Finally, fixed amount of bitter orange juice as an aqueous phase (3.6 grams) was continuously added to the organic phase by a disposable plastic syringe (5mL) at the injection rate of 1mL per minute and, stirring rate of 2400 rpm was maintained in the meantime. These nano-emulsions observed after 24 hour storage at 25 °C to evaluate visual physical stability (no creaming or phase separation after one night stand). In the light of prescreening tests, to test the effect of surfactant blend composition and O/S weight ratio on transparency, microstructure and particle size distribution of nano-emulsions, nano-emulsions were prepared at many O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 with constant surfactant blend (S_A/S_B) weight ratio of 90/10.

2.3. Visual appearance

Visual appearances of nano-emulsions after 24 hour storage at 25°C were captured by Nikon 5300 camera.

2.4. Transparency analysis

Transparency of extra virgin olive oil-in-bitter orange juice was determined by recording the absorbance at 600 nm, using a UV-VIS spectrophotometer (SP-3000nano, OPTIMA, Tokyo, Japan). The transparency was calculated by the following equation:

$$T = 1/10^{A} \quad (1)$$

where T is the transparency and A is the value of absorbance at 600 nm. A high T value would represent a transparent appearance (HA *et al.*, 2015). Transparency results are given as a percentage.

2.5. Emulsion stability index

Emulsion stability index (ESI) of the emulsions was determined by turbidimetrically (WANG *et al.*, 2008). An aliquot (50 μL) of freshly prepared emulsion was taken from the bottom of emulsion, immediately (0 min) and 10 min after preparation and, diluted (1:10, v/v) in 0.1% (w/v) SDS solution. The absorbance of the diluted emulsion at 0 and 10 min was measured at 500 nm by UV-VIS spectrophotometer (SP-3000nano, OPTIMA, Tokyo, Japan). ESI values were calculated by the following equation:

$$\text{ESI (min)} = \frac{Ao}{Ao - A_{10}} \times 10 \quad (2)$$

where A_0 and A_{10} are the absorbance of the diluted emulsion at 0 and 10 min, respectively. These measurements were performed in triplicate.

2.6. Raman microscopy

Droplet images for the emulsions were captured under Raman microscope at room temperature. 50 μm of freshly made nano-emulsion was placed on a 1.2 mm thick glass slide without a coverslip, photomicrographs (20X magnification) were taken using A Renishaw InVia Raman Microscope (Renishaw Plc., U.K.) equipped with a Leica ×20 objective lens.

2.7. Particle size distribution

The particle size distribution (PSD) of the emulsions was measured using a laser light scattering instrument (Partica LA-950, Horiba Ltd., Japan). The device could detect particle sizes ranging from 10 nm to 3 mm. To avoid multiple scattering effects, emulsions were diluted with distilled water. Particle size measurements were reported as Sauter, or surface mean diameters D_{32} ($D_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$) or volume mean diameters D_{43} ($D_{43} = \sum n_i d_i^4 / \sum n_i d_i^3$), where n_i is the number of particles with diameter d_i. The refractive indices of the dispersed and continuous phases used in the calculations of the PSD were 1.135 and 1.330, respectively. To determine the width of the distribution of particle sizes, 'span' was calculated from the following formula:

$$\text{Span} = [d(v, 90) - d(v, 10)] \,/\, d(v, 50) \qquad (3)$$

In this formula, $d(v, 10)$, $d(v, 50)$, and $d(v, 90)$ are diameters at 10, 50, and 90% cumulative volume, respectively. In other words, $[d(v, 90) - d(v, 10)]$ is the range of the data and $d(v,50)$ is the median diameter (MAHDI JAFARI *et al.*, 2006).

2.8. Statistical analyses

All measurements were performed on freshly prepared samples, and were reported as means and standard deviations (n=2). This study is designated as a 4 (number of surfactant blend compositions) x 4 (number of oil to surfactant blend weight ratios) factorial structure (4x4). Experimental data were subjected to two-way analysis of variance and the means were compared by Tukey multiple range test at $p < 0.05$ significance level using SPSS version 22 (SPSS Inc., Chicago, IL, USA). Pearson's correlation test was used to determine point- biserial correlation among experimental parameters. Sigma Plot (Sigma Plot 10.0 Windows version, SPSS Inc.) is used for graph preparation.

3. RESULTS AND DISCUSSION

Initial evaluation of freshly prepared nano-emulsions was made by prescreening tests to define adequate surfactant blend (S_A/S_B) weight ratios and oil to surfactant blend weight ratios for obtaining clear, translucent nano-emulsions granted with good physical stability. In prescreening tests, nano-emulsions were prepared at many O/S weight ratios with changing surfactant blend (S_A/S_B) weight ratios were observed after 24 hour storage at 25 °C to evaluate visual physical stability against gravitational seperation. Table 1 shows results of the prescreening tests. Table 1 clearly indicates that all nano-emulsion formulations prepared with O/S weight ratios of 10/90, 15/85 along with 90/10 surfactant mixing ratio (S_A/S_B= 90/10) resulted in clear, translucent nano-emulsions with

good physical stability without creaming or phase separation after one night stand at 25°C . In the light of prescreening tests and to make system less complex, to test the effect of surfactant blend composition and O/S weight ratio on transparency, microstructure and particle size distribution of nano-emulsions, nano-emulsions were prepared at many O/S weight ratios of 10/90, 15/85, 20/80, and 25/75 with constant surfactant blend (S_A/S_B) weight ratio of 90/10.

For many industrial applications optical transparency of emulsion-based delivery system is quite important (QIAN and MCCLEMENTS, 2011; ULUATA *et al.*, 2016), so relationship between O/S weight ratio and transparency of nano-emulsions with respect to surfactant blend composition were represented in Fig. 1.

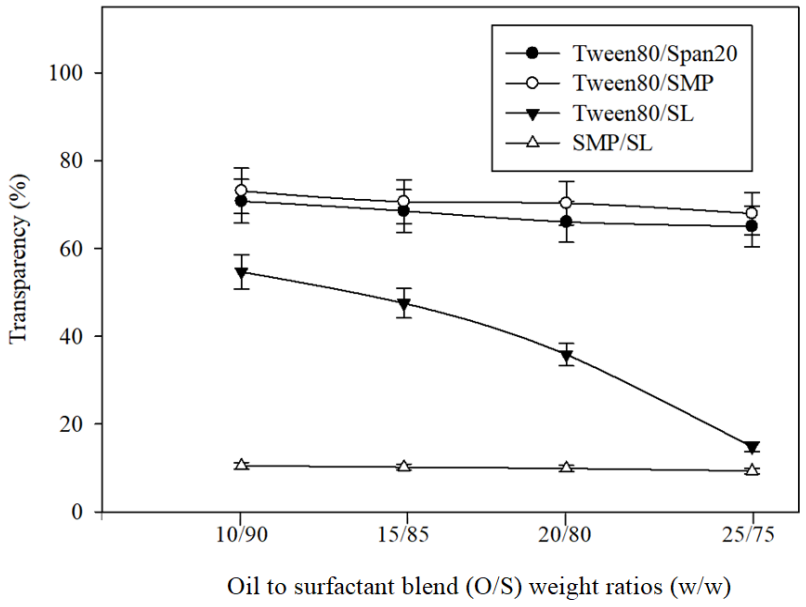

Figure 1. Change in the transparency of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) as a function of O/S weight ratio (O/S= 10/90, 15/85, 20/80 and 25/75). (Bars indicate the standard deviations).

Statistical analyses revealed that transparency of nano-emulsions was significantly (p<0.05) affected by both main factors (Surfactant blend composition and O/S weight ratio) and two way interaction of these factors (Surfactant blend composition and O/S weight ratio) (Table 2). For all surfactant combinations, nano-emulsions prepared at 10/90 O/S weight ratio exhibited the highest transparency. As the surfactant concentration decreased with increasing O/S weight ratio from 10/90 to 25/75, emulsions became less translucent even milky. These findings have good agreement with photographs of nano-emulsions (Table 1). Nano-emulsions prepared with binary combinations of Tween80/Span20 and Tween80/SMP showed the highest transparency, over than 65 % (Fig. 1) and, similar decreasing trend was observed as the surfactant concentration decreased. Most pronounced change in transparency was seen in emulsions stabilized with Tween80/SL. The change can be clearly observed as transformation from transparent appearance to the milky one with the increase in the O/S weight ratio (Table 1).

Transparency of emulsions prepared with SMP/SL showed the lowest transparency with almost no change as O/S weight ratio change. Pearson's test showed significant negative correlation between surfactant blend composition and transparency, (r= -0.885; p<0.01) (Table 3). Statistical analyses also revealed that D_{32} and D_{43} of nano-emulsions were significantly (p<0.05) influenced by both main factors (Surfactant blend composition and O/S weight ratio) and two way interaction of these factors (Surfactant blend composition and O/S weight ratio) (Table 2). There was positive correlation between surfactant blend composition and D_{32} (r= 0.807; p<0.01) and D_{43} (r= 0.731; p<0.01) of emulsions, respectively, due to the Pearson's test (Table 3). Surfactant concentration has a direct effect on the particle size, which in turn affects turbidity of the emulsion. As the surfactant concentration increase, generally particle size of the emulsion droplets decreases (Table 4). Small emulsion droplets are skilled to scatter light less efficiently compared to large ones (QIAN and MCCLEMENTS, 2011) and, when looking under a white light source, nano-emulsions with small droplets appear transparent with a reddish tinge (MASON *et al.*, 2006), which accounts for the higher transparency of the nano-emulsion with small droplet sizes. At higher droplet sizes, nano-emulsions appear milky due to the strong multiple scattering of light (MASON *et al.*, 2006). So, nano-emulsion can be seen transparent or semi-transparent due to their small particle diameters (SOLANS *et al.*, 2005; PEY *et al.*, 2006) and even milky up to 500 nm (PORRAS *et al.*, 2008), which supports our findings in Table 1. In literature, several authors reported increased turbidity as the particle size of the emulsion increases (QIAN and MCCLEMENTS, 2011; SABERI *et al.*, 2013; ULUATA *et al.*, 2016).

Effects of O/S weight ratio on emulsion stability index (ESI) were expressed as the change in absorbance during 10 min interval and shown in Fig. 2. As shown from Fig. 2, generally, ESI of nano-emulsions stabilized with Tween80/Span20, Tween80/SL, and SMP/SL decreased as the surfactant concentration increased with decreasing O/S weight ratio. However, for the emulsions prepared with Tween80/SMP did not show a definite trend with change in O/S weight ratio. This is probably caused by change in emulsion turbidity, either raise or reduces, with rising particle size compared to initial particle size (MCCLEMENTS, 2007). Moreover, MCCLEMENTS (2007) states that there is no simple mathematical relation between particle size and turbidity, particularly in region where the particle radius is almost equivalent to the wavelength of light, which may accounted for fluctuations in ESI. Moreover, statistical analyses also revealed that only O/S weight ratio had statistically significant (p < 0.05) effect on ESI of nano-emulsions (Table 2). Pearson's test showed significant negative correlation between O/S weight ratio and ESI, (r= -0.523; p < 0.01) (Table 3).

Microscopic photographs of emulsions were taken using Raman microscope with 20X magnification. Fig. 3 represented the microstructures of extra virgin olive oil-in-bitter orange juice nano-emulsions formulated with different compositions. Fig. 3 indicated that surfactant blend composition and O/S weight ratio did have appreciable effect on microstructure. Emulsions prepared with Tween80/Span20, Tween80/SMP, and Tween80/SL showed similar spherical droplet structure. The particle size of these nano-emulsions gets smaller and more distributed as the surfactant concentration increases with decreasing O/S weight ratio, shift from O/S of 25/75 to 10/90. Emulsions prepared with SMP/SL showed completely different structure compared to emulsions prepared blends of Tween80/Span20, Tween80/SMP, and Tween80/SL.

Table 1. Photographs of bitter orange juice/surfactant blend (S_A+S_B)/ olive oil (EVOO) % organic phase (surfactant blend + olive oil). O/S = 10/90, 15/85, 20/80 and 25/75. S_A/S_B = 90/10, 85/15, 80/20 and 75/25.

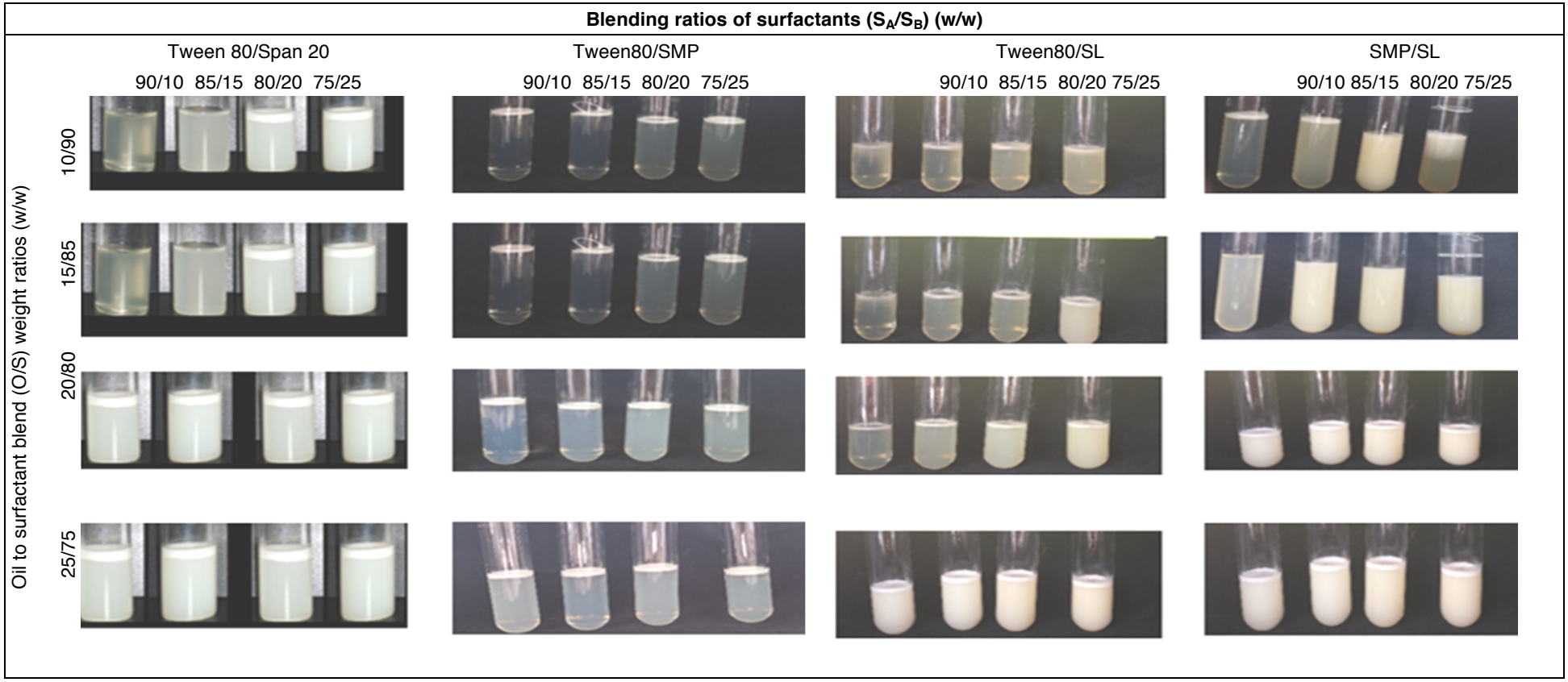

Table 2. Mean squares from two-way analysis of variance results of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) and O/S weight ratios (O/S=10/90, 15/85, 20/80 and 25/75).

Source of variation	DF	Transparency (%)	ESI (min)	Area-based droplet size (D_{32})	Volume-based droplet size (D_{43})	Span (Dimensionless)
Main effects						
Surfactant blend composition	3	6464.561*	173.272	3655430.031*	8988824.365*	83.534
O/S weight ratio (w/w)	3	249.759*	281.385*	568763.948*	1590930.281*	85.789
Two-way interaction						
Surfactant blend composition*O/S weight ratio	9	126.457*	72.934	239800.642*	1345705.087*	29.211
Error		14.161	64.791	4648.781	14157.406	64.739

*Significant at (p<0.05).

Table 3. Pearson's correlation results of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10) and O/S weight ratios (O/S=10/90, 15/85, 20/80 and 25/75).

	Surfactant blend composition	O/S weight ratio (w/w)	Transparency (%)	ESI (min)	Area-based droplet size (D_{32})	Volume-based droplet size (D_{43})	Span (Dimensionless)
Surfactant blend composition	1						
O/S weight ratio (w/w)	0.000	1					
Transparency (%)	-0.885**	-0.185	1				
ESI (min)	-0.094	-0.523**	0.419*	1			
Area-based droplet size (D_{32})	0.807**	0.328	-0.926**	-0.426*	1		
Volume-based droplet size (D_{43})	0.731**	0.322	-0.875**	-0.404*	0.945**	1	
Span (Dimensionless)	-0.142	-0.346	0.171	0.336	-0.262	-0.190	1

**Correlation is significant at the 0.01 level (2-tailed)
*Correlation is significant at the 0.05 level (2-tailed).

Clustered, nonhomogeneous and, irregular shaped droplets were seen in emulsions prepared with SMP/SL, as the surfactant concentration decreases from O/S weight ratios of 15/85 to 25/75, flocculation of emulsion particles increases, which may resulted from the lack of minimum surfactant concentration to hold the emulsion particles apart from each other.

If one compares all emulsion in terms of effect of O/S weight ratio over particle size without considering their surfactant blend compositions, as expected, an increase in O/S weight ratio, i.e., decreasing surfactant concentration from 10/90 to 25/75 resulted an increase in the particle size, D_{32} and D_{43} (Table 4). That is, smallest droplets were seen at 10/90 O/S weight ratio, while the highest droplets were seen at 25/75 O/S weight ratio. This probably caused by as the particle size decreases surface area increases, so it increases the extent of surfactant to hydrate the droplets. That is, increased adsorption of surfactant to oil-water interface thus more surfactant available to stabilize newly formed droplets which in turn facilitates formation of smaller droplets (CHOI *et al.*, 2011; SABERI *et al.*, 2013).

Table 4. Effect of O/S weight ratio on the emulsion particle size of extra virgin olive oil-in-bitter orange juice nano-emulsions prepared with different surfactant blend compositions (Constant S_A/S_B= 90/10). (O/S=10/90, 15/85, 20/80 and 25/75).

O/S	D_{32} (nm±SD)	D_{43}(nm±SD)	Span (dimensionless)
	Tween80/Span20		
10/90	130±9.19a,A	1203±85.07a,A	23.84±1.69a,A
15/85	126±8.92a,B	143±10.13a,A	0.93±0.07 a,A
20/80	134±9.48a,C	155±10.93a,B	0.96±0.07 a,A
25/75	140±9.89a,D	157±11.09a,C	0.86±0.06 a,A
	Tween80/SMP		
10/90	255±18.03b,A	311±22.01a,A	1.03±0.07 a,A
15/85	260±18.36b,B	348±24.60a,A	0.92±0.07 a,A
20/80	262±18.55b,C	356±25.19a,B	1.30±0.09 a,A
25/75	314±22.17b,D	373±26.38a,C	1.06±0.07 a,A
	Tween80/SL		
10/90	163±11.54c,A	231±16.33b,A	1.40±0.10 a,A
15/85	242±17.11c,B	569±40.23b,A	1.34±0.09 a,A
20/80	655±47.02c,C	2018±142.70b,B	23.97±1.66 a,A
25/75	1730±122.33c,D	3079±217.73b,C	1.58±0.11 a,A
	SMP/SL		
10/90	1171±82.77d,A	1350±95.47c,A	0.97±0.07 a,A
15/85	1642±116.10d,B	2574±181.97c,A	1.48±0.10 a,A
20/80	1690±119.52d,C	3146±222.47c,B	1.69±0.12 a,A
25/75	2022±142.99d,D	3317±234.56c,C	1.79±0.13 a,A

Mean values ± Standard deviation (n=2). Values in the same column followed by different lowercase letters (surfactant blend composition effect) indicate statistical difference at $p<0.05$ significance level among emulsions with change in surfactant blend composition at constant O/S weight ratio due to the Tukey multiple range test. Values in the same column followed by different uppercase letters (O/S weight ratio effect) indicate statistical difference at $p<0.05$ significance level among emulsions with change in O/S weight

ratios at constant surfactant blend composition due to the Tukey multiple range test. Each column is evaluated within itself.

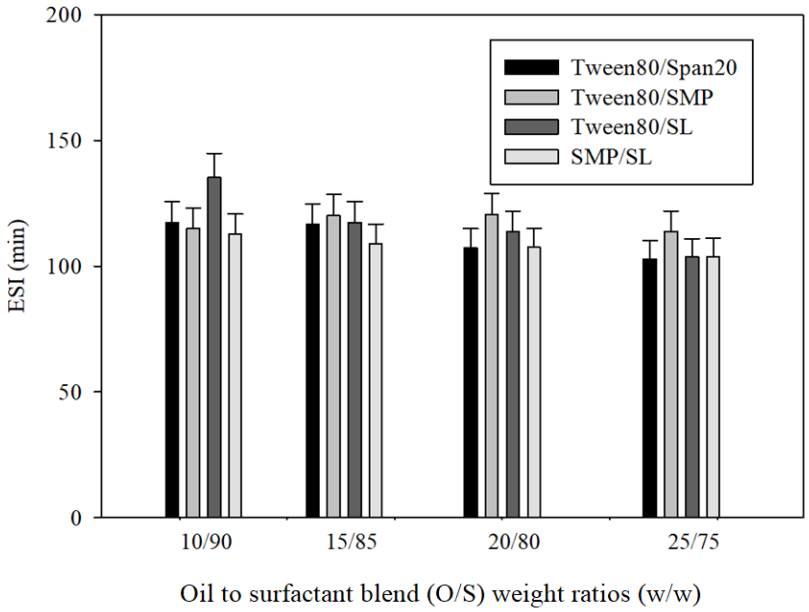

Figure 2. Change in ESI of extra virgin olive oil-in-bitter orange juice nano-emulsions formulated with different surfactant blend compositions (Constant S_A/S_B= 90/10 as a function of O/S weight ratio (O/S=10/90, 15/85, 20/80 and 25/75). (Bars indicate the standard deviations).

In literature several authors observed decrease in particle size of droplets with increase in surfactant concentration up to the certain levels (YUAN *et al.*, 2008; CHOI *et al.*, 2011; SABERI *et al.*, 2013; ULUATA *et al.*, 2016). The results presented Figs. 4 to 7 and given in Table 4, indicate that emulsion distribution getting narrower and span getting smaller as the surfactant concentration increased by reduction O/S weight ratio, which supports findings of (LOVELYN and ATTAMA, 2011), that is, the smaller the Span value, the narrower the particle size distribution. Statistical analyses showed that neither O/S weight ratio nor surfactant blend composition had statistically significant (p>0.05) effect on Span of nano-emulsions (Table 2).

These results can be explained by the different hydrophilicity of the surfactants. The hydrophilicity of surfactants is usually measured by their hydrophilic-lipophilic balance (HLB). Lipophilic surfactant has HLB number below 9.0, hydrophilic surfactant has HLB number above 11.0 and, theoretically, low HLB (3–6) surfactants are utilized in the preperation of W/O nano-emulsions while high HLB (8-18) surfactants are utilized in the preperation of O/W nano-emulsions (DAVIES, 1957). The HLB of a surfactant blend can be calculated by the summation of the product of HLB and weight percentage of each surfactant (CHEN *et al.*, 2015). HLB values of Tween80, Span 20, SMP and SL were 15.0, 8.6, 18.5 and 8.0, respectively. HLB values of the binary combinations of Tween80/Span20, Tween80/SMP, Tween 80/SL and SMP/SL used in the current study were calculated as 14.36, 15.35, 14.30, and 17.45 respectively. Contrary to theoretically expected, in theory it is supposed to have the lowest particle sizes in the o/w nano-emulsions composed of high

hydrophilic surfactants (high HLB value) due to increased packing and stabilizing effect of the surfactant blend (TAN and NAKAJIMA, 2005), SMP/SL (HLB=17.45) nano-emulsions had the highest particle sizes. Also, even though the Tween80/Span20 (HLB=14.36) and Tween 80/SL (HLB=14.30) nano-emulsions had nearly same HLB value, Tween80/Span20 (HLB=14.36) emulsions result with the highest transparency (Table 1, Fig. 1) and relatively low particle diameters (Table 4) at O/S weight ratios of 15/85 and 10/90, while Tween 80/SL nano-emulsions was semi-transparent (Table 1) and were only in nano-scale at O/S weight ratios of 15/85 and 10/90 (Table 4). As stated by YALÇINÖZ and ERÇELEBİ (2018), although the HLB dictionary provides theoretically relevant information about the required system, it is essential to make experimental screening to test the actual suitability of the surfactants in the system.

These results can also be caused by the different physicochemical properties of the surfactants. Tweens are nonionic surfactants with a polyoxyethylene head group and a single hydrocarbon tail (MAHDI *et al.*, 2011). The hydrophobic tail of Tween 80 is curled resulting in maximum curvature and packing parameter to facilitate the preperation of nano-emulsions (KOMAIKO and MCCLEMENTS, 2016). Tween 80 is the most soluble surfactant among Tween series (MAHDI *et al.*, 2011). In the current study, all nano-emulsion contained equal amounts of Tween 80, so it was assumed that Tween 80 has the same effect to all nano-emulsions regardless of differences in co-surfactant composition. These results suggest that Tween80 was good worked with Span20, followed by SMP. MCCLEMENTS and RAO (2011) reported that Tweens and Spans are very conventional in food use due to their low toxicity, not being irritant, and facility to fit both high-energy and low-energy emulsification methods. Spans and Tweens work well for producing stable emulsions, even though the individual surfactants alone do not produce stable multiple emulsion systems (LU and RHODES, 2000), which supports our findings. In literature there are several studies using Spans and Tweens solely or as binary combinations (YUAN *et al.*, 2008; LEONG *et al.*, 2009; SILVA *et al.*, 2012; KUMAR DEY *et al.*, 2012; ABBAS *et al.*, 2013; PESHKOVSKY *et al.*, 2013).

Sucrose monopalmitate (SMP) is a hydrophilic, water soluble, nonionic surfactant, which has a polar head group (sucrose) and a nonpolar tail group (palmitate) (CHOI *et al.*, 2011). Recent studies revealed that SMP is not an effective surfactant to stabilize O/W nano-emulsions and emulsions under acidic pH when used solely (RAO and MCCLEMENTS, 2011; RAO and MCCLEMENTS, 2012b). In the current study, Tween80/SMP nano-emulsions showed good transparency (Table 1, Fig. 1) and fine droplets (Table 4) even under acidic pH (at pH 2.57). These results were also in good accordance with the studies of RAO and MCCLEMENTS (2012b) stating that acid stability of SMP stabilized nano-emulsions can be enhanced by blending SMP and Tween 80. Including, nowadays, surfactants of sugar esters are very popular in food due to their good taste and aroma profile, low toxicity, and high biodegradability compared to petrochemical-based surfactants (RAO and MCCLEMENTS, 2011).

Lecithin is a lipid mixture with phospholipids, which contains two nonpolar hydrocarbon chains and a zwitterionic polar head group with positive and negative charges deriving from amine and phosphate groups, respectively (CHEN *et al.*, 2015). Lecithin is very common emulsifying agent in food due to its low toxicity, biocompatibility, and generally recognized-as-safe regulatory status (CHEN *et al.*, 2015). Sunflower lecithin could take place of soybean lecithin due to being a non-GMO product (CABEZAS *et al.*, 2016). However, in the current study, particle sizes of nano-emulsions stabilized with Tween80/SL were only in nano-scale at 10/90 and 15/85 O/S weight ratio (Table 4).

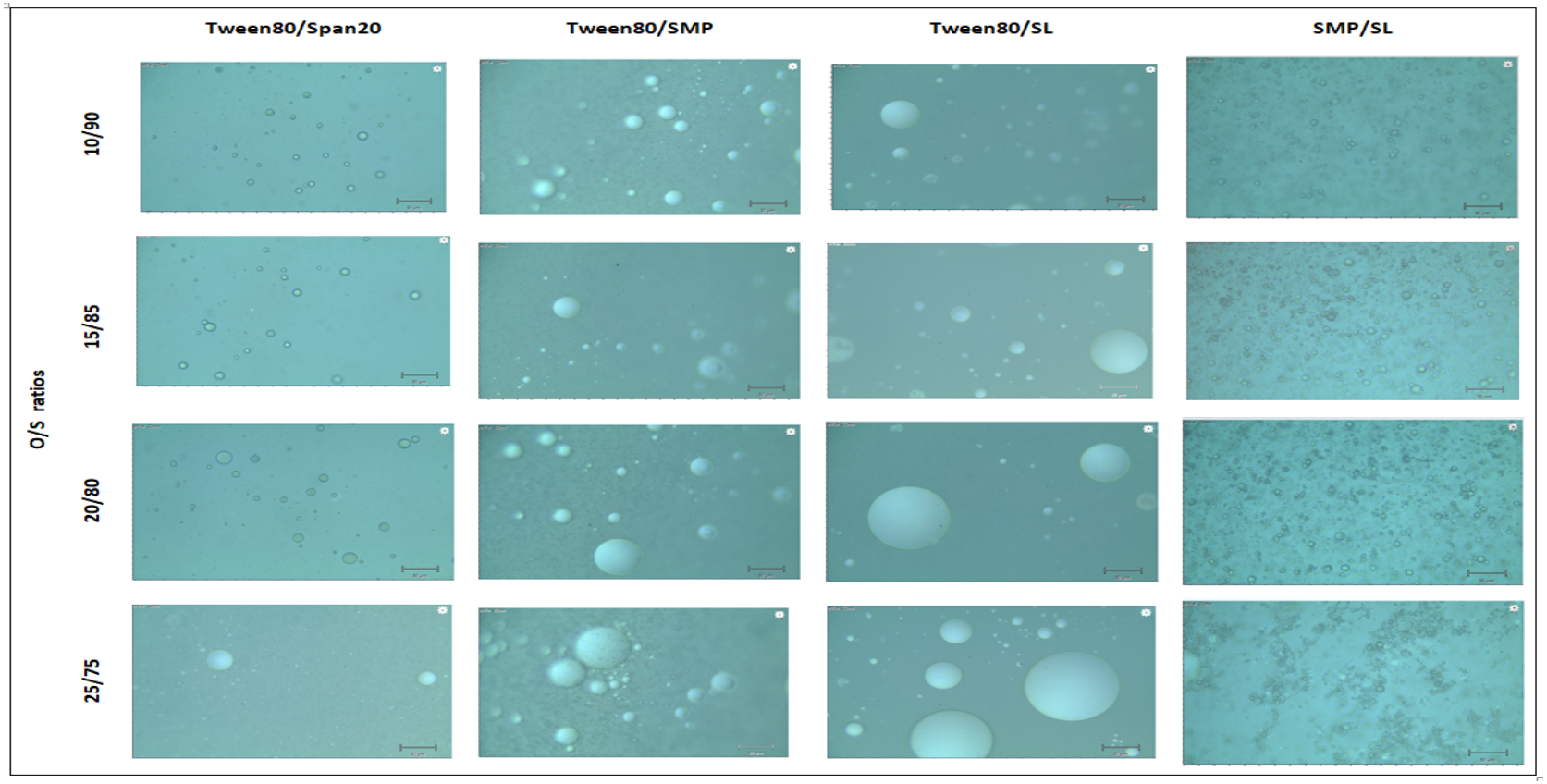

Figure 3. Micrographs of freshly prepared bitter orange juice/Surfactant blend (S_A+S_B)/ olive oil (EVOO) nano-emulsions at 25 °C, which were nano-emulsions with 90 % aqueous phase (at pH 2.57), i.e., 90% aqueous phase and 10 % organic phase (surfactant blend + olive oil). O/S weight ratios = 10/90, 15/85, 20/80 and 25/75. S_A/S_B = 90/10. The scale bars indicate 50 μm.

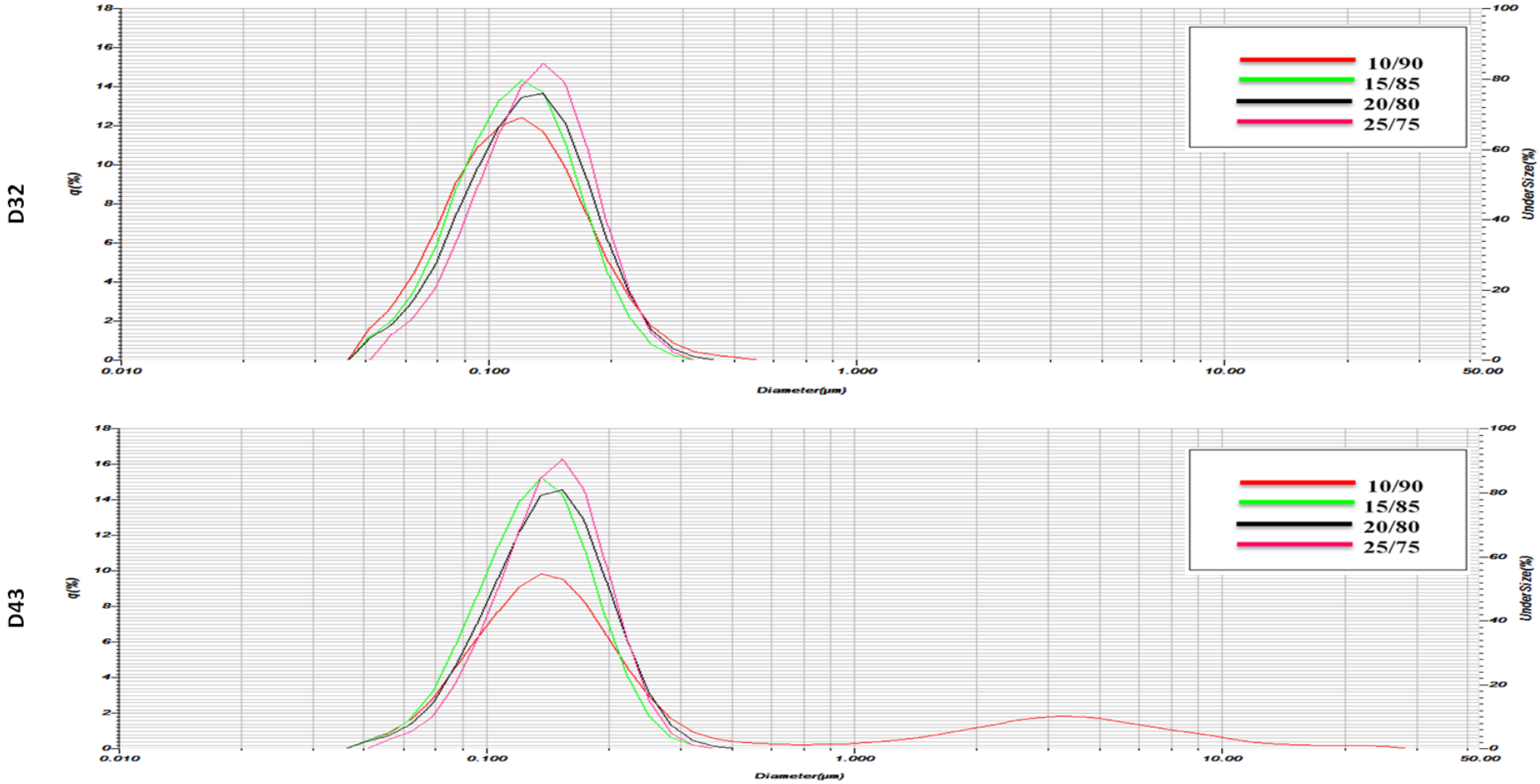

Figure 4. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/Span20 with constant surfactant mixing ratio (Tween80/Span20 = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

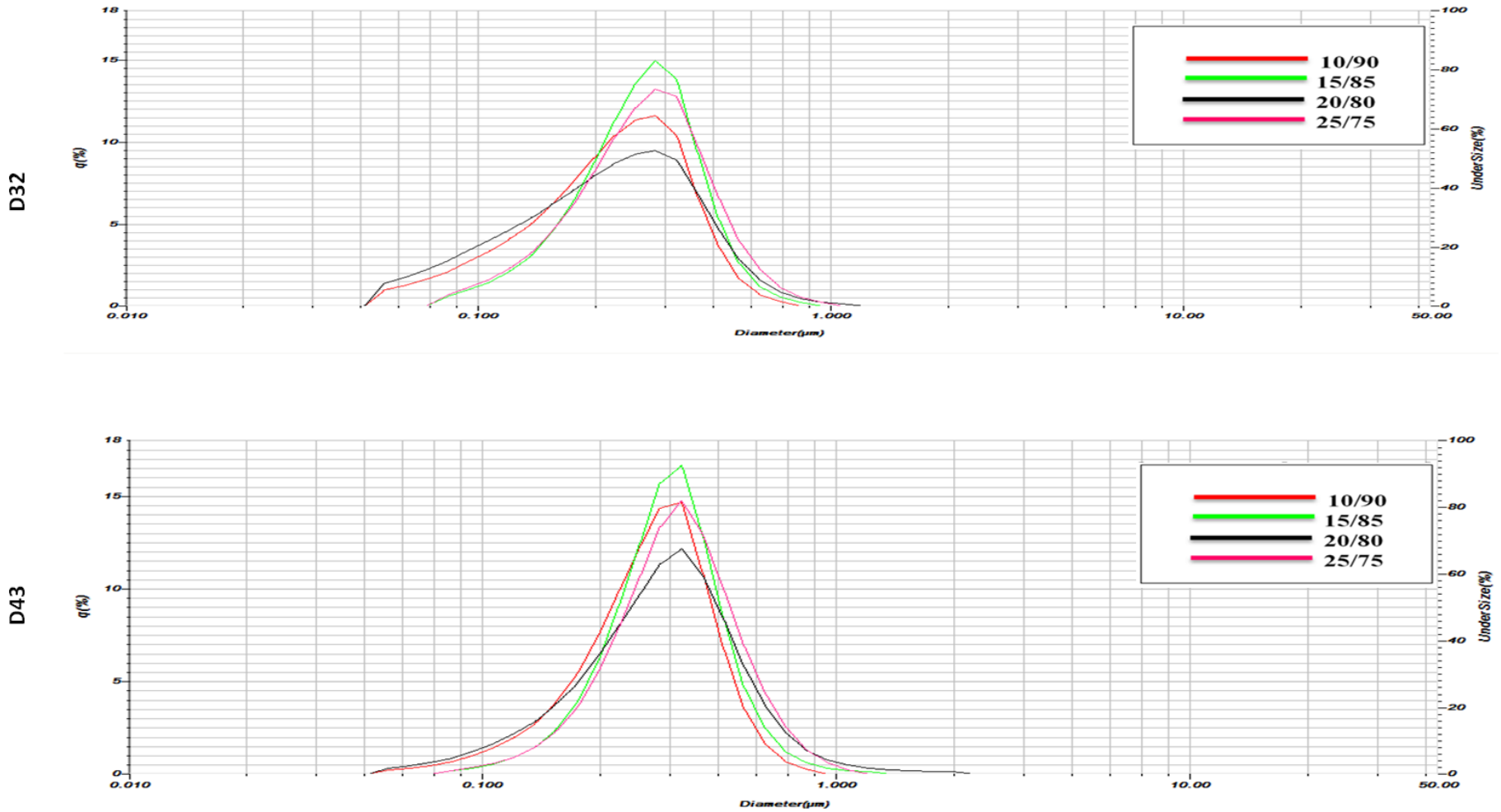

Figure 5. Particle size distribution of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/SMP with constant surfactant mixing ratio (Tween80/SMP = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

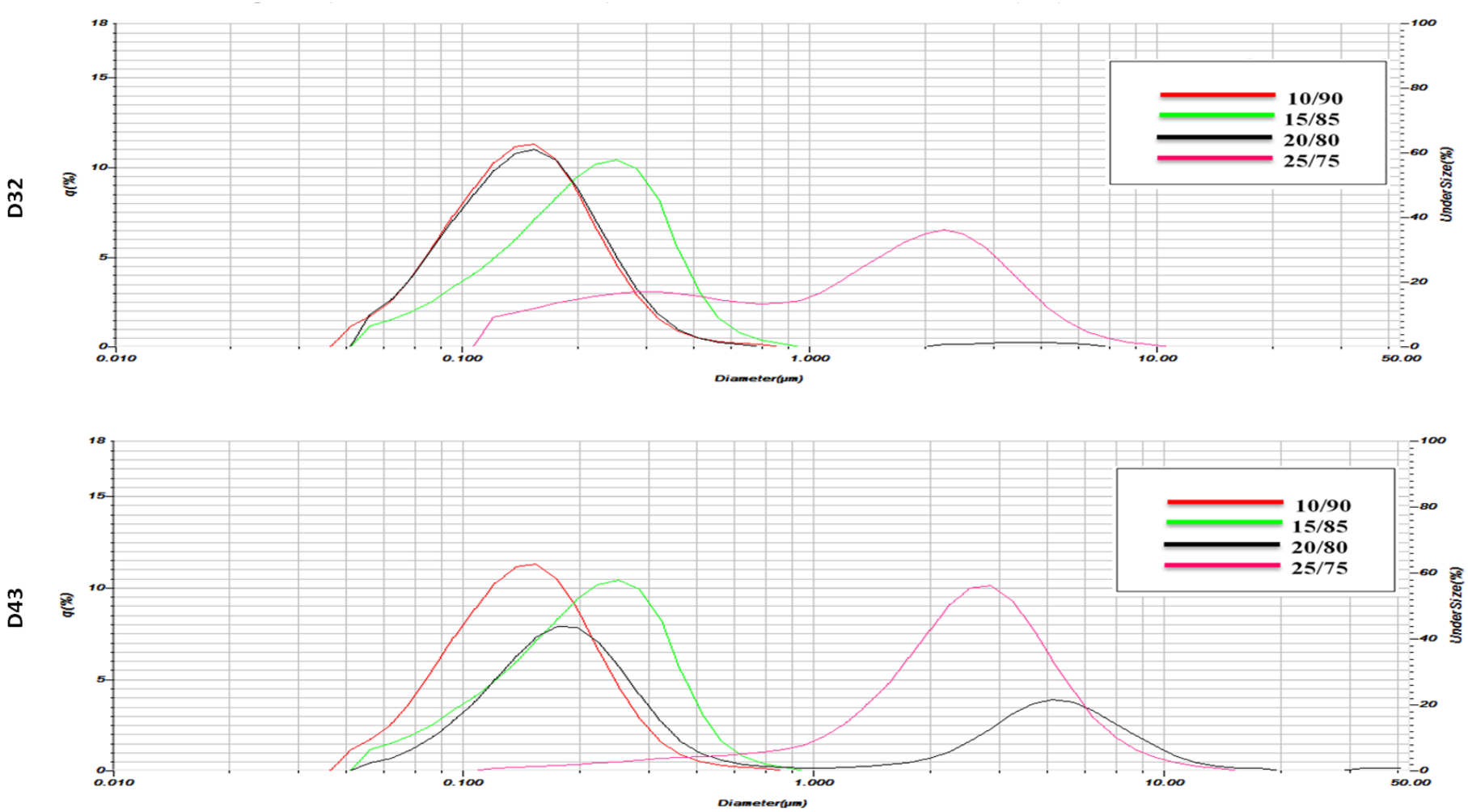

Figure 6. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with Tween80/SL with constant surfactant mixing ratio (Tween80/SL = 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

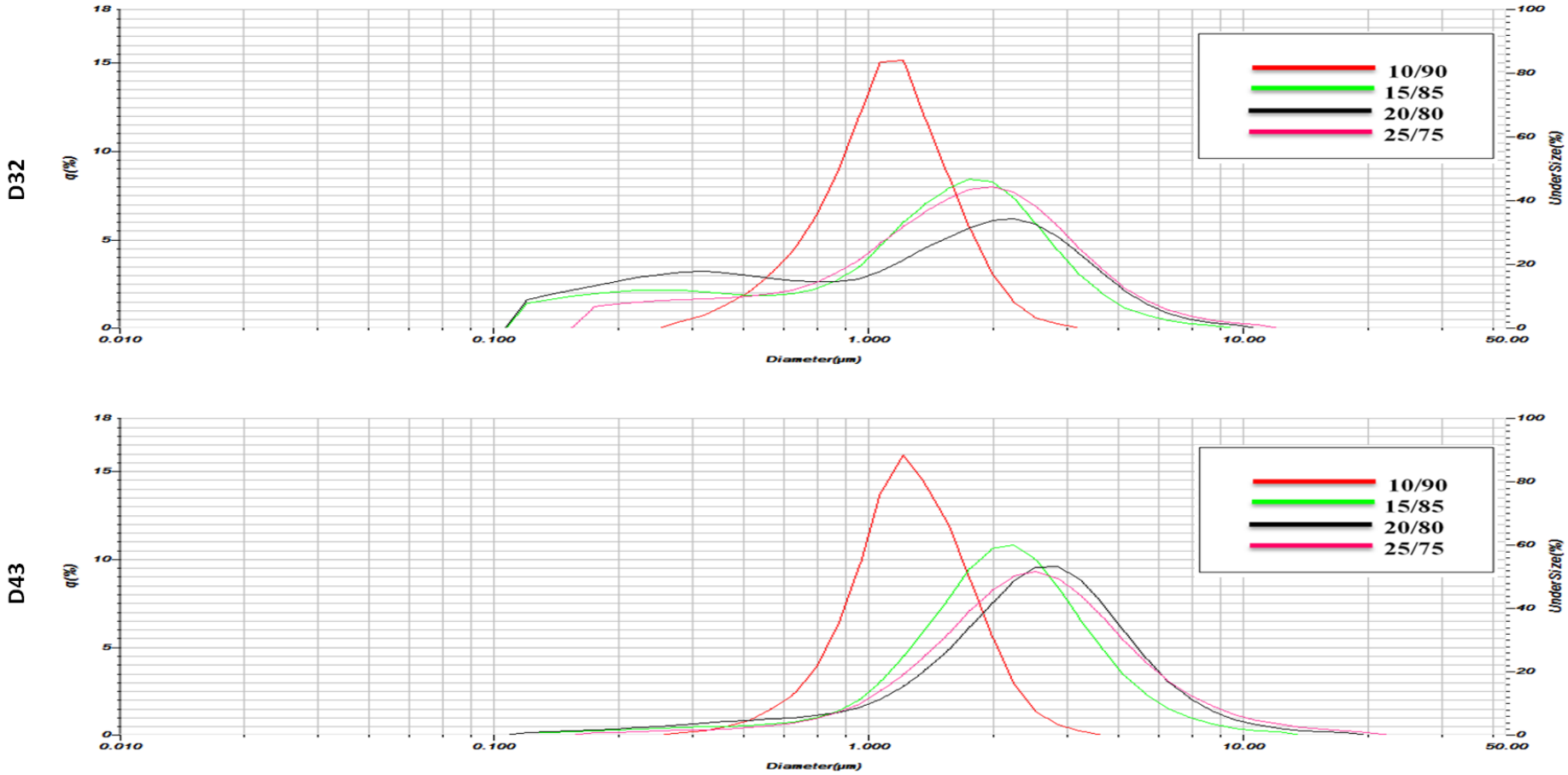

Figure 7. Particle size distribution graph of olive oil-in-bitter orange juice nano-emulsions prepared with SMP/SL with constant surfactant mixing ratio (SMP/SL= 90/10) as a function of O/S weight ratios of 10/90, 15/85, 20/80 and 25/75.

None of the emulsions prepared with SMP/SL were in nano-scale (Table 4), even though SL and SMP used in the current study have been shown to work synergistically (Compass Food Company, product specifications). Experimental results suggest that Tween80/SL and SMP/SL nano-emulsions cannot be as effective as Tween80/Span20 and Tween80/SMP nano-emulsions in preparation of olive oil-in-bitter orange juice (o/w) nano-emulsion at acidic conditions (pH 2.57).

4. CONCLUSIONS

The purpose of present study was to increase the use of bitter orange juice for flavoring and acidifying salads through raising consumer's awareness, and to promote healthy and fit life with daily diet routines. The results proved that it is possible to produce olive oil-in-bitter orange juice (o/w) nano-emulsion at acidic conditions (pH 2.57) by stabilizing binary combinations of Tween80/Span20, Tween80/SMP and Tween80/SL via phase inversion composition method. Nano-emulsions prepared with Tween80/Span20 and Tween80/SMP result with the highest transparency and relatively low particle diameters at O/S weight ratios of 15/85 and 10/90, while nano-emulsions prepared with Tween 80/SL was semi-transparent and were only in nano-scale at O/S weight ratios of 15/85 and 10/90. None of the emulsions prepared with SMP/SL were in nano-scale. Visual appearance, transparency, microstructure and particle size distribution of the nano-emulsions were influenced by surfactant blend composition and concentration. The information obtained from the current study is important for designing and commercialization of tailored olive oil-in-bitter orange juice (O/W) nano-emulsion based delivery systems with high transparency and fine droplets. For further studies, in vitro evaluation of the effectiveness of prepared nano-emulsions on weight control is suggested.

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Dedicated to the memory of Dr. Tharwat Tadros.

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PAPER

MANGO-SEED EXTRACT AND SULPHITES AS PROMOTERS OF COLOR AND BIOACTIVE COMPOUNDS RETENTION DURING TRAY DRYING OF MANGO SLICES

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ABSTRACT

The study objective was to evaluate effect of mango-seed extract alone or in combination with sodium metabisulphite on the content of vitamin C, free phenols, six phenols compounds, and total carotenes, and color in mango slices dried at 60ºC until 15% moisture. From drying curves were calculated effective diffusivities [1.17-1.35x10^{-9} $m^2 \bullet s^{-1}$] and drying rate constants [1.53±0.90x10^{-3}-2.27±0.80x10^{-3}] using Midilli's model. Results showed that combination of mango-seed extract with sodium metabisulphite has an important role in retention of vitamin C and carotenes, and an enrichment of phenolic compounds was found in dried mango slices.

Keywords: dried mango; mango-seed extract, sodium metabisulphite, free phenols; total carotenes; vitamin C

1. INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important fruits grown commercially in tropical and subtropical regions in the world. Since mango fruit is highly perishable, it is generally transformed to a dried product (LIN *et al*., 2016) for prolonged shelf life. Fresh mango is characterized by its high content of phenolic compounds, vitamin C and carotenes. Since these compounds are antioxidants, they are able to impart beneficial properties for the consumer health (SIDDIQ *et al*. 2013).

Mangoes have phenolic compounds derived from phenolic acids (SCHIEBER *et al*., 2000) such as gallic acid, caffeic acid, *p*-coumaric acid, etc. Gallic acid has antioxidant, anti-inflammatory, and anticarcinogenic activity (VELDERRAIN-RODRÍGUEZ *et a*l., 2018). SCHIEBER *et al*. (2000) reported that mango pulp has 5.9 mg gallic acid•(100 g dry mass)$^{-1}$. Other phenolic acids found in mangoes are caffeic acid [6.6 mg•(100 g dry mass)$^{-1}$] and ferulic acid [8.9 mg•(100 g dry mass)$^{-1}$] (ABDALLA *et al*., 2007). In addition, methyl gallate is a potent cell protector against oxidative stress, reduces lipid peroxidation (LPO) and reactive oxygen species (ROS) (WHANG *et al*., 2005). Lee *et al*. (2010) reported that methyl gallate suppresses T regulatory cells (Treg) in mice's malignant tumors. Mangiferin is a C-glucosyl-xanthone found in some mango varieties, such as Tommy Atkins, Haden and Ubi. This compound has a wide range of biological properties, because it is gastroprotective, analgesic, antibacterial and cytoprotective (MASIBO and HE, 2008). SCHIEBER *et al*. (2000) reported that mango pulp has 3.8 mg mangiferin•(100 g dry mass)$^{-1}$.

Vitamin C is one of the most abundant compounds in mango fruit and its concentration varies with the fruit maturity, as well as with the post-harvest handling and processing methods. ROCHA-RIBEIRO *et al*. (2007) reported a concentration of 94.0 mg AAE•(100 g dry mass)$^{-1}$ for vitamin C in fresh mango var Tommy Atkins grown in Brazil. Vitamin C decreases from thermal effects and exposure to air and light (LIU *et al*., 2014).

Mango is also a good source of carotenes. β-carotene is the most abundant in mango fruits (VARAKUMAR *et al*., 2011). β-carotene content is often used as an indicator of damage extent to mangoes during processing and storage (THARANATHAN *et al*., 2006). MANTHEY and PERKINS-VEAZIE (2009) reported a concentration of 32.9 to 59.1 mg of carotenes•(100 g dry mass)$^{-1}$ in mango Tommy Atkins Mexican mangoes.

During the mango convective drying process, considerable degradation of these bioactive compounds occurs (MÉNDEZ-CALDERÓN *et al*., 2018). Hence, to minimize bioactive compound degradation it may be beneficial to carry out mango pretreatments prior to the drying process (YAO *et al*., 2020). The pretreatment choice depends on the drying method and characteristics of desired product. Additionally, pretreatments may improve product quality by retaining color of fresh mango and reducing product darkening.

GUIAMBA *et al*. (2016) evaluated the retention of β-carotene and vitamin C (dehydroascorbic and ascorbic acids) in osmotically dried mango pretreated with calcium chloride or ascorbic acid. This study consisted in an initial osmotic dehydration using 45º Brix sucrose solutions added with 1% calcium chloride or 1% ascorbic acid. Then samples were dried in an air convection oven at 50ºC or 70ºC. The authors reported that both pretreatments significantly improved retention of vitamin C and *all-trans*-β-carotene in dried products. In other study, CHEN *et al*. (2007) performed mango drying experiments using hot drying air and freeze-drying in presence of 1% sodium hydrogen sulphite or 1% ascorbic acid. The authors reported that use of pretreatments during drying mangoes reduced carotenes degradation. This behavior also was observed by JIMÉNEZ-HERNÁNDEZ *et al*. (2017) when studied effect osmotic dehydration of mango slices in an emulsion based on inulin and piquin-pepper oleoresin. The results showed a retention of

68.8% of ascorbic acid and 95.5% of β-carotene at 30ºC. Also, this study found a strong decrease in retention of ascorbic acid (43.6%) when the process was carried out at 50ºC. While retention of β-carotene was 83.0%. Recently, DEREJE *et al*. (2020) dried mango slices using four pretreatments (lemon juice, salt solution dips, hot water blanching and control) and four drying methods (solar, tray, freeze and fluidized bed drying) to assess effect of pretreatments and drying methods on qualities of dried mango slices. The results showed that pretreatments and drying methods had significant effects on color antioxidants of the dried mango slices. The ascorbic acid and total phenol contents were affected by drying methods and had respective values of 33.18-41.24 mg AAE•(100 g dry mass)$^{-1}$ and 131.13-251.12 mg of gallic acid equivalents (GAE)•(100 g dry mass)$^{-1}$.

On the other hand, it has been reported that mango-seed extracts contain a significant amount of free phenols, 27.7±0.1 g GAE•(100 g dry mass)$^{-1}$ (Bernal-Mercado *et al*., 2018). For this reason, this same study used mango-seed extract as antioxidant agent of fresh-cut mango. The results showed that a solution mango-seed extract at 0.63% (*m*/*v*) contributed to preservation of fresh mangoes cubes due to increasing free phenols from 306.0 to 364.9 mg GAE•(100 g dry mass)$^{-1}$. Additionally, in this study found that mango-seed extract contains 60 mg of gallic acid•(100 g dry mass)$^{-1}$, 42.0 mg of mangiferin•(100 g dry mass)$^{-1}$, 77 mg of caffeic acid•(100 g dry mass)$^{-1}$ and 12.6 mg of *p*-coumaric acid•(100 g dry mass)$^{-1}$. Also, mango-seed extracts have been used to develop antioxidants films (ADILAH *et al*., 2018). To the best of our knowledge, there are no reports about use of mango-seed extracts as a fruit drying pretreatment.

Considering the above, aim of study was to evaluate effect of different pretreatments on retention of color and antioxidant compounds (free phenols, vitamin C and total carotenes) during tray drying of Tommy Atkins mango slices. Three pretreatments were used for tray drying of Tommy Atkins mango slices: 0.5% sodium metabisulphite (PT1), 1.44% mango-seed extract (PT2) and 0.5%/1.44% (*w*/*v*) sodium metabisulphite/mango-seed extract (PT3). HPLC was used to quantify the main phenolic compounds present in the dried products.

2. MATERIALS AND METHODS

2.1. Sampling procedure and sample preparation

Tommy Atkins mangoes (*Mangifera indica* L.) were obtained from Porfirio Díaz Market located in Huajuapan de León city, Oaxaca, México. Mango fruits were selected based on fruit size and pulp color as measured by CIELAB b* parameters, which defines yellow color. It was also verified that the fruits had no physical damage. Mangoes were peeled with a home peeler and slices of 6.0 ± 0.1 cm x 4.0 ± 0.1 cm (length x width) and 3.8 ± 0.4 mm thickness were obtained with a cutter.

2.2. Determination of mango physicochemical parameters

The physicochemical characteristics of fresh mangoes were evaluated from soluble solids content, moisture percentage, titratable acidity and pH, which are briefly described below.

Soluble solids content (AOAC 932.12). A drop of fresh mango juice was placed in Abbe refractometer and °Brix of sample was measured.

Moisture percentage (WROLSTAD *et al*., 2005). A known amount of mango pulp (3 g) was weighed into pre-weighed and dried crucibles. The samples were then placed in an oven

at 105°C for 24 h. After that, dried samples were placed in a desiccator for 30 min at room temperature and anhydrous conditions and were finally weighed.
Titratable acidity and pH (DEA *et al*., 2013). Mango pulp sample was blended for 2 min to homogenize the sample and the blend was filtered through a cotton plug. A potentiometer previously calibrated with standard solutions (pH 4 and 7) was used to measure sample´s titratable acidity. For titration 10 g of sample was used. A 0.1 N sodium hydroxide solution was continuously added, while the pH was measured until sample reached a pH of 8.3.

2.3. Preparation of the mango-seed extract

Tommy Atkins mango seeds were cut into small pieces, ground in a blender for 2 min and sieved through a #40 mesh to obtain a fine powder. Subsequently, 25 g of powder was weighed and mixed with 500 mL of 99.5% methanol and sonicated for 30 min at room temperature. Mixture was allowed to stand until a phase separation was observed. Supernatant was decanted and filtered through a Whatman filter paper #1. Pellet was treated with same extraction procedure three times. Following this, filtrates were collected, combined and evaporated on a rotary vacuum evaporator at 40°C until 11 g of solvent-free extract was obtained as a viscous reddish-brown liquid. Extract was dissolved in water to make a final volume of 100 mL in a volumetric flask. Finally, extract solution was stored at -20°C for preservation until further use.

2.4. Pretreatment of mango slices

700 g of mango slices were immersed for 3 min in 1.4 L of pretreatment solutions at 25ºC. Solutions were used only once, after they were discarded. Solutions used as pretreatment were 0.5% sodium metabisulphite solution (PT1), 1.44% mango-seed extract solution (PT2) and a combination of 0.5% sodium metabisulphite and 1.44% mango-seed extract solutions (PT3). Following this, slices were drained for 1 min. Mango slices without pretreatment was used as control.

2.5. Dryer and drying conditions

A tray dryer (SANTOS-SÁNCHEZ *et al*., 2012,) equipped with a tray rotating mechanism and a heating-air flow control, was used in this work to perform drying of 700 ± 8 g of mango slices. Mango slices were dried at 60°C, air velocity of 1.2 $m \bullet s^{-1}$ and 20 rpm tray rotation velocity. Moisture loss was quantified by weighing slices every 15 min until moisture content was about 15%. Drying time was around 110 min. For each determination, mass of three samples was measured.

2.6. Determination of effective diffusivity (D_{eff}) and drying rate constant (k)

Drying curves (moisture ratio *versus* drying time) were used to calculate effective diffusivity (D_{eff}) and drying rate constant (k). Drying curves were performed in triplicate. Moisture ratio (MR) of food slices can be predicted from Sherwood and Newman model, Equation 1. This equation relates MR with drying time (t), thickness of food slice (L) and effective diffusivity (D_{eff}). D_{eff} is related with Fourier number (F) through Equation 2. In this study, D_{eff} was calculated from curve slope expressed by simplified Sherwood and

Newman model, Equation 3 (ASHRAF *et al*., 2012). The k values were calculated from nonlinear regressions of Midilli equation (MIDILLI *et al*. 2002).

$$MR = \frac{MR_t - MR^*}{MR_o - MR^*} = \sum_{n=0}^{a} \frac{8}{\pi^2(2n+1)} \exp\left[-(2n+1)^2 \frac{\pi^2}{4} F\right] \quad (1)$$

$$F = \frac{D_{eff} * t}{L^2} \quad (2)$$

Where MR_t = moisture ratio at time t, MR_o = initial moisture ratio, MR* = equilibrium moisture ratio and F = Fourier number. Compared to values of MR_t and MR_o, the value of MR_e is relatively smaller, so the MR can be reduced to $MR = MR_t/MR_o$ (MEWA *et al*., 2018). Considering that for this study, n = 0 and diffusion occurs through two faces of mango slice (trays used for drying are perforated), MR can be expressed from Equation 3. D_{eff} values were obtained from slope of time *versus* ln *MR* curves.

$$\ln MR = \ln \frac{8}{\pi^2} - \left(\frac{\pi^2 D_{eff}}{4L^2}\right) t \quad (3)$$

Equation 4 was used to estimate k as well as n, a and b values, nonlinear regressions were applied to equation of MIDILLI *et al*. (2002), where MR is moisture ratio, t is the drying time, k is drying rate constant and a, b and n are the model constants. The regression was performed with InterReg 2014 (Kroll-Software).

$$MR = a \exp\left(-kt^n\right) + bt \quad (4)$$

2.7. Free phenols quantification of mango-seed extract and the mango slices

For free phenols determination, 0.2 g of mango seed was mixer with 3.1 mL of 60:40 % (*v*/*v*) ethanol:water or 2 g of mango pulp was mixed with 25 mL of 60:40 % (*v*/*v*) ethanol:water. The mixture was milled in a home blender for 1 min and subsequently filtered through cotton wool. Extracts were obtained in triplicate. Free phenols quantification was performed in a Biotek ELX-808 microplate reader using modified Folin-Ciocalteu method described by OCHOA-VELASCO *et al*. (2016). Extract or standard (40 μL) was pour in a microplate well with 40 μL of 0.1 M Folin-Ciocalteu reagent. The reaction mixture was allowed to stand for 3 min in microplate reader, and stirred for 15 s at low velocity. Subsequently, 40 μL of 0.5% sodium carbonate (*w*/*v*) was added and mixed by suction with a multichannel pipette. Mixture was allowed to stand for 30 min at 40°C, after which it was stirred at medium speed in microplate reader. Finally, sample absorbance was read at 765 nm. A calibration curve was prepared using gallic acid solutions with known concentrations. Free phenols content in mango-seed extract and mango samples was determined using calibration curve and was expressed in mg of GAE•(100 g dry mass)$^{-1}$. All measurements were made in triplicate.

2.8. Vitamin C quantification

The quantification of vitamin C was performed following procedure described by OCHOA-VELASCO *et al*. (2016). Briefly, 0.05 g of dried mango (0.3 g of fresh mango) was mixed with 1.00 mL of 1% metaphosphoric acid solution. Subsequently, mixture was sonicated in an 8510 sonicator (Branson Ultrasonics Co., USA) for 15 min at room temperature. Then mixture was centrifuged for 15 min at 900 *g*. Supernatant containing vitamin C was used for discoloration reaction of 2,6-dichloroindophenol sodium salt (DCIP) in a 96 well microplate plate. To carry out this reaction, 70 μL of extract was mixed with 70 μL of a solution of 30 ppm DCIP and allowed to stand for 1 min at room temperature in absence of light. Finally, mixture was stirred for 15 s and then absorbance at 515 nm was measured in a Biotek ELX-808 microplate reader. Vitamin C content in samples was determined using calibration curve and expressed in mg of ascorbic acid equivalents (AAE)•(100 g dry mass)$^{-1}$. All measurements were made in triplicate.

2.9. Phenolic compounds quantification by HPLC

A mixture of 0.2 g of dried mango and 2.5 mL of a 70:30% (*v*/*v*) methanol:water solution with 0.1% CH_3COOH was vortexed for 5 min. Subsequently, mixture was sonicated for 10 min at room temperature, followed by centrifugation for 10 min at 900 *g*. Supernatant was then removed and the pellet was re-extracted for a second time. Supernatants were pooled and dried on a rotary vacuum evaporator at 40°C. Extracts were obtained in triplicate. Dried extracts were redissolved in 500 mL of water and solution was cleaned up by eluting it through C18 SPE cartridges of 2.8 mL (Alltech, USA). The clean-up procedure consisted first in elution of 1 mL of deionized water through C18 SPE cartridge. Subsequently, extract solution was loaded into cartridge and eluted with 5 mL of water, followed by 1 mL of a 1:1 (v/v) methanol:water solution, and finally with 1 mL of methanol. Fractions were collected in HPLC vials. Phenolic compounds quantification was performed in an HPLC instrument equipped with a photodiode array detector (Water, alliance 2695, USA) and a C18 column of 4.6 mm x 250 and 5 μm inner diameter (Phenomex, USA). The following phenolic measuring standards were employed: mangiferin, methyl gallate, ferulic acid, gallic acid, caffeic acid and *p*-coumaric acid. A calibration curve was performed for each standard. A 10 μL injection volume and a 1 mL•min^{-1} flow was used. A binary mobile phase was used (A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile) with the following gradient program: 0 min 10% B, 5 min 10% B, 15 min 80% B and 30 min 100% B, 35 min 10% B. The mangiferin was monitored at 365 nm and phenolic acids and methyl gallate were monitored at 280 nm for (Rocha-Ribeiro *et al*., 2008). All measurements were made in triplicate.

2.10. Total carotenes quantification

A modification of method described by WROLSTAD *et al*. (2005) was used for total carotene quantification. Briefly, 0.3 g of dried mango was weighed and ground in a mortar with 3 mL of water. For fresh mango samples, 1.0 g pulp sample was homogenized and mixed with 2 mL of water. Mixture was placed in a 10 mL amber vial with subsequent addition of 4 mL of 95% ethanol was added, followed by vortexing for 4 min. This procedure was performed in triplicate. Then the mixture was vacuum filtered through filter paper. Supernatant was then poured through a 25 mL filtration funnel with 10 mL of hexane, and then manually stirred. Filtration funnel was allowed to stand for 2 min.

Subsequently, ethanolic phase was removed. Absorbance of hexane phase was measured at 450 nm in a Lambda 32 UV-vis spectrophotometer (Perkin Elmer, USA). Sample measurements were performed in triplicate. Equation 5 was used for the calculation of total carotenes of sample expressed as mg of β-carotene•(100 g dry mass)$^{-1}$. All measurements were made in triplicate.

$$[\text{Total carotenes}] = \left(\frac{A_{450} \bullet \text{Vol}_{\text{solution}}(\text{mL})}{258.84\ \text{mL} \bullet \text{mg}^{-1} \bullet \text{sample mass (g)}}\right) \bullet 100 \qquad (5)$$

2.11. Sulphites quantification

Sulphites quantification in dried mango slices obtained from PT1 and PT3 pretreatments was carried out analogously to reported by LI and ZHAO (2006). Briefly, solutions used to prepare samples and standards were 0.1 mM ethylenediaminetetraacetic disodium salt (EDTA) solution, 1000 ppm tris(hydroxymethyl)aminomethane (Tris) buffer solution at pH 8 and 0.3 mM 5,5-dithio-bis-(2-nitrobenzoic acid) [DTNB, also called Ellman's Reagent]. 0.1 mM EDTA and Tris buffer solutions were prepared with deionized and degassed water for 25 min at 25ºC. Subsequently, EDTA 0.1 mM solution was used to prepare 1000 ppm sodium metabisulphite solution. Tris buffer solution was used to prepare 0.3 mM DTNB solution. Before being employed, all solutions were degassed for 15 min at 25ºC. On the other hand, around 1 g of dried mango slices (PT2 and PT3), were dry-milled in a mortar to form a homogeneous paste. 20 mg of this paste was mixed with 1 mL of 0.1 mM EDTA. Later, samples were shaken in a vortex for 2 min and degassed for 15 min at 25ºC on ultrasound, followed by a centrifugation for 5 min at 900 *g*. Supernatants were separated and then used to carry out colorimetric reaction with 0.3 mM DTNB. This consisted of a mix 60 μL of sample or standard with 60 μL of 0.3 mM DTNB in a 96-well plate. Also, two reaction blanks were prepared, the first was prepared by mixing 60 μL of sample with 60 μL of 0.1 mM EDTA, while the second blank was prepared by mixing 60 μL of 0.3 mM DTNB with 60 μL of 0.1 mM EDTA. Reaction mixtures were allowed to stand for 5 min at 25ºC, then stirred for 30 s in a BioTek® model ELX808 microplate reader and absorbance at 405 nm was measured. Calibration curve was built with five sulphites standards, which were prepared at concentrations in interval of 6 to 20 ppm from 1000 ppm sodium metabisulphite solution. All measurements were made in triplicate.

2.12. Color determination

A HunterLab Ultra ScanVis (USA) spectrophotometric colorimeter was used to determine CIELAB color parameters of mango samples. D65 illuminant was used with an observation angle of 10° and a 0.9525 cm slit. For each drying data point, the CIELAB color parameters (L*, a* and b*) of mango slices were measured at ten different points to obtain an average. Also, angle Hue* was calculated with Equation 6. Three mango slices were measured for every drying data point.

$$Hue^* = arc\ tan\left(\frac{b^*}{a^*}\right) \qquad (6)$$

2.13 Statistical analysis

A randomized experimental design was applied in this study, one-factor ANOVA tests and Duncan´s means comparison method were used with a significance level $\alpha = 0.05$ between treatments and variables. Design-Expert® v.6.0 software was employed for these analyses.

3. RESULTS AND DISCUSSION

3.1. Physicochemical parameters of the mango pulp

Soluble sugars content and titratable acidity are related to fruit ripeness stage. The determinations of these parameters for fresh mango pulp are listed in Table 1 and are similar to values reported by SIDDIQ *et al*. (2013) for ripe mango samples. Visual color scale reported by BRECHT (2010) for Tommy Atkins mango was used to report state of mango ripeness in this study. According to this scale, fresh mango fruits ripeness was 5. The L* value was 63.56±4.73, accounting for a low degree of darkness, while b* parameter (57.81±4.22) indicates an intense yellow color, which was greater than that reported by ROCHA-RIBEIRO *et al*. (2008) (Table 1).

Table 1. Physicochemical properties of fresh Tommy Atkins mango.

Parameter		This study	Literature
Mango weight (g)		671.46±80.47	-
Soluble solids (°Brix)		16.22±1.62	14-16[b]
Moisture (%)		88.30±1.09	-
pH		3.78±0.15	3.4±0.1[a]
Acidity %		0.47±0.07	0.9±0.0[a]
Color	L*	63.56±4.73	55.0-61.1[b]
	a*	11.45±1.95	11.5-14.4[b]
	b*	57.81±4.22	40.0-50.0[b]

Mean ± standard deviation, n = 3.
[a]Siddiq *et al*. (2013).
[b]Rocha-Ribeiro *et al*. (2008).

3.2. Physicochemical, chemical and antioxidant properties of mango seed

Tommy Atkins mango seeds of 24.6±7.5 g weight and a 6.5±0.5 cm length used for study had a 37.7±0.3% moisture content. Extraction yield was 12.2±0.3 g of extract•(100 g of seed mango)$^{-1}$, which was comparable with yields reported by DORTA *et al*. (2012) for mango-seed extracts var. Keitt using 50% aqueous acetone solvent and 60 min ultrasound, 12.0±1.0 g of extract•(100 g of seed mango)$^{-1}$. Concentration of feee phenols in mango seed extracts was 23.9±0.0 g GAE•(100 g dry mass)$^{-1}$. This concentration was superior to that reported by SOGI *et al*. (2013), for mango var. Tommy Atkins from USA, 20.03-11.23 g GAE•(100 g dry mass)$^{-1}$, which was previously dried using different drying methods

(freeze drying, tray drying, vacuum drying and infrared drying) to extraction. In this study, freeze drying allowed highest retention of phenols compounds in mango seed extract. On the other hand, BERNAL-MERCADO *et al*. (2018) obtained a total phenol content of 27.7±0.1 g GAE•(100 g dry mass)$^{-1}$ from mango var. Haden. This last extract was obtained from a maceration at 25ºC for 10 days in darkness. This indicates that extraction assisted by ultrasound, in addition to being fast, allows a high retention of phenolic compounds in extracts from mango seeds. Additionally, it is important to avoid drying mango-seed samples with hot air.

3.3. Drying curves

During the drying time from 0 to 15 min, free water heating and evaporation occur slowly for PT1 pretreatment. All drying curves presented a significant moisture reduction in range from 15 to 75 min, Fig. 1. This behavior can be explained considering that during this drying period mango slice surface is wet, thus forming a continuous free water film. Consequently, there is no resistance to water transfer from solid surface to surrounding air. On the other hand, drying rate decreased after 75 min of drying for all pretreatments and control, indicating start of a decreasing drying rate period.

3.4. Effective diffusivities and constant drying rate

Effective diffusivity, D_{eff}, for different drying pretreatments and control (data no showed) lied in range of 1.17-1.35 x 10^{-9} $m^2 \bullet s^{-1}$. These values are similar to those reported by DISSA *et al*. (2008) for 5 mm-thick mango slices, dried at 60°C. A comparative analysis of D_{eff} means attested that there are not significant differences between mango pretreatments.
Midilli's n, k and a constants were calculated with Equation 4. The determination coefficients (R^2) for all pretreatments were higher than 0.99. The b constant for this study was zero, and n and a constants were found in ranges of 1.42±0.09-1.54±0.13 and 7.16±0.04-7.75±0.90, respectively. Constant k is considered a measure of water evaporation rate from the mango slice. The k values for pretreatments and control were not significantly different as compared by the Duncan's mean comparison test ($p<0.05$) and were found in range of 1.53±0.90x10^{-3}-2.27±0.80 x 10^{-3}. The k values obtained in this study are similar to that reported by MURTHY and MANOHAR (2014) for slices of dried mango at 60ºC and air velocity of 2.25 $m \bullet s^{-1}$: k = 0.054, n = 1.022 and R^2 = 0.969. While n value, which corresponds to drying kinetics order, is higher in present study than in that reported by MURTHY and MANOHAR (2014). It should be noted that an order of drying kinetics close to unity is indicative that drying process depends almost exclusively on temperature and as n value increases, it is indicative that other variables are also contributing significantly to food drying process. These variables are drying air velocity, mango slices thickness, concentrations and types of pretreatments, among others.

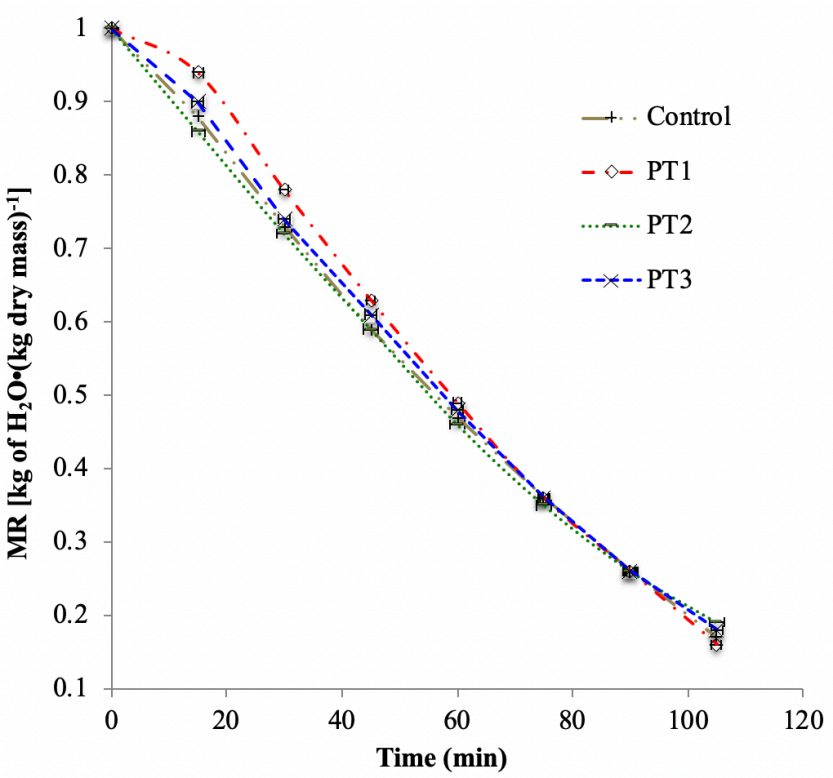

Figure 1. Drying curves for mango slices with different pretreatments. Control = Mango dried without pretreatment, PT1 = 0.5% (*w*/*v*) sodium metabisulphite, PT2 = 1.44% (*w*/*v*) seed extract mango, PT3 = seed extract mango/sodium metabisulphite [1.44%/0.5% (*w*/*v*)]. Each curve is mean of three drying replicates.

3.5. Free phenols

Fig. 2 shows free phenols content for dried and fresh mango slices. Free phenols content of fresh mango was 195.28±5.48 mg GAE•(100 g dry mass)$^{-1}$, which is within range reported by MANTHEY and PERKINS-VEAZIE (2009) of 171.8-257.3 mg GAE•(100 g dry mass)$^{-1}$, for a Mexican Tommy Atkins variety. From Duncan test comparisons, it can be implied that since control and PT1 pretreated mango slices (0.5% metabisulphite) are not statistically different ($p<0.05$), PT1 pretreatment did not have a significant effect on phenol retention (about 46%). This retention amount is similar to that reported by CHONG *et al.* (2013) (50.4%) who performed dried of mango slices using cold/hot air treatment. Treatment used by authors consisted of applying a flow of cold air (11.54±0.26°C) either at beginning or during dehydration process with hot air at 53.95±0.03°C.
On the other hand, dried samples which were pretreated with mango-seed extract (PT2 and PT3) had a much greater phenol content than corresponding values of both control and fresh mango samples (346.96±19.69, 368.00±11.84 mg GAE•(100 g dry mass)$^{-1}$, Fig. 2). This implies that, as opposed to bisulphites-only treatment (PT1), addition of mango-seed extract not only aided in retention of phenolic compounds but also in the increase of their concentration to 77.8 and 88.4% in PT2 and PT3 pretreatments, respectively. This effect appears to be due to the diffusion of phenolic compounds from pretreatment extract to the mango slices during the immersion period. It is also remarkable that in the case of PT3 pretreatment, addition of sodium metabisulphite caused a greater increase in total phenol content than that caused by use of the mango-seed extract alone (PT2) (Fig. 2). This seems

to indicate that sodium metabisulphite had a synergistic effect on retention and fortification of phenolic compounds during mango drying.

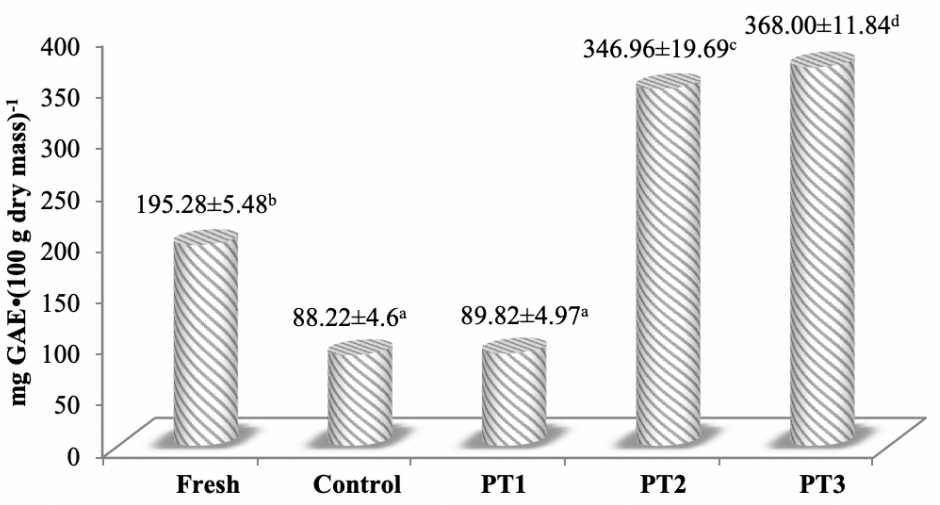

Figure 2. Free phenols content [mg gallic acid equivalents GAE•(100 g dry mass)$^{-1}$] in fresh mango and pretreated dried. Mean ± standard deviation, n = 3. Control = Mango dried without pretreatment, PT1 = 0.5% (*w*/*v*) sodium metabisulphite, PT2 = 1.44% (*w*/*v*) seed extract mango, PT3 = seed extract mango/sodium metabisulphite [1.44%/0.5% (*w*/*v*)]. Superscripts a-d showed a significant difference (α = 0.05) according to the Duncan test.

3.6. Profile of main phenolic compound in dried mango samples

The phenolic compound concentration profile of different pretreated samples was measured by HPLC for gallic acid, methyl gallate, mangiferin, caffeic acid, ferulic acid and *p*-coumaric acid (Table 2). Mangiferin concentrations were not affected by sample pretreatments with respect to control. Mango slices dried without pretreatment (control) had a similar behavior than mango sliced pretreated with metabisulphite (PT1), except for gallic acid. The PT1 pretreatment (0.5% sulphites only) caused a significant increase in the retention of gallic acid only (33.0%). On the other hand, PT2 pretreatment (1.44% mango-seed extract) significantly increased content of methyl gallate (27.4%), caffeic acid (70.9%), ferulic acid (244.4%), and *p*-coumaric acid (87%) with respect to control.

These results are also in agreement with total phenol assays in which PT2 samples had a higher phenolic content than both control and fresh samples, which confirms that PT2 dried products were enriched with phenolic compounds of mango-seed extract. This phenolic enrichment can be explained by considering that mango-seed extract solutions had a 10-fold higher concentration of free phenols than that present in fresh mango pulp, so molecular diffusion occurs from the extract to pulp by a concentration driving force.

In general, PT3 pretreatment samples (0.5% sulphites and 1.44% mango-seed extract) displayed greater increments in phenolic compounds concentration than those observed in PT2 pretreatment, with exception of methyl gallate, which is statistically equal in both treatments. In addition, PT3 samples presented a sharp increase in concentrations of gallic acid and caffeic acid with respect to PT2 pretreatment. This result agrees with free phenol

observations for PT2 and PT3 pretreatments, thus confirming synergic effect of bisulphites and mango extract on phenol enrichment of mango dried products.

Table 2. Phenolic compounds content in dried slices of Tommy Atkins mango quantified by HPLC.

Pretreatment	Compound [mg·(100 g dry mass)$^{-1}$]					
	Gallic acid	Methyl gallate	Mangiferin	Caffeic acid	Ferulic acid	*p*-Coumaric acid
Control	13.49±0.02^{a}	7.26±0.15^{a}	5.31±0.45^{a}	6.16±0.26^{a}	10.86±0.52^{a}	8.24±0.45^{a}
PT1	17.98±0.29^{b}	6.83±0.31^{a}	4.75±0.70^{a}	5.52±0.53^{a}	11.61±0.57^{a}	7.99±0.28^{a}
PT2	14.71±0.18^{c}	9.25±0.49^{b}	5.02±0.17^{a}	10.53±0.66^{b}	37.40±0.47^{b}	15.41±0.67^{b}
PT3	26.42±0.16^{d}	10.08±0.28^{b}	5.65±0.50^{a}	26.10±0.71^{c}	45.61±0.31^{c}	16.30±0.55^{c}

Control = Mango dried without pretreatment, PT1 = 0.5% (*w*/*v*) sodium metabisulfite, PT2 = 1.44% (*w*/*v*) seed extract mango, PT3 = seed extract mango/sodium metabisulfite [1.44%/0.5% (*w*/*v*)]. Mean ± standard deviation, n = 3. Superscripts a-d = mean difference significant in columns (α = 0.05) by Duncan test. In the calibration equation y = area and x = concentration of corresponding phenol compound.

3.7. Vitamin C content

Fresh mango samples had a vitamin C content of 135.59±3.40 AAE•(100 g dry mass)$^{-1}$ which is similar to that reported by ROCHA-RIBEIRO *et al*. (2007) (94.0 mg AAE•(100 g dry mass)$^{-1}$). Vitamin C contents in dried samples (Fig. 3) were greatly superior to those described by NDAWULA *et al*. (2004), who dried mango slices of 3-5 mm thickness in an open solar dryer. These authors reported a vitamin C content of 25.4 mg AAE•(100 g dry mass)$^{-1}$ and a 15.5% vitamin C retention in dried slices. Vitamin C content in PT2 (32.15±1.21 AAE•(100 g dry mass)$^{-1}$) pretreated samples (mango-seed extract only), was not significantly different than control (32.34±1.58 AAE•(100 g dry mass)$^{-1}$, $p<0.05$), thus indicating that PT2 pretreatment did not have a significant effect on retention of vitamin C in dried product. On the other hand, sulphites-added samples (PT1 and PT3) presented a higher vitamin C content than control samples, which indicates that bisulphites pretreatment efficiently promoted retention of vitamin C in dried mango (Fig. 3). Finally, PT3 pretreatment (combined sulphites and mango-seed extract) presented a 3-fold vitamin C content with respect to the control (96.52±5.09 AAE•(100 g dry mass)$^{-1}$, Fig. 3). Thus, similarly to phenol results, combined pretreatments of sulphites and mango-seed extract yielded an improved vitamin C retention in dried product (71.2%) as compared with individual pretreatments alone, which indicates a synergistic effect of such pretreatments on retention of antioxidant compounds during mango drying. However, in contrast to phenol results, sulphites pretreatment (PT1) was the most effective at maintaining ascorbic acid content of dried product.

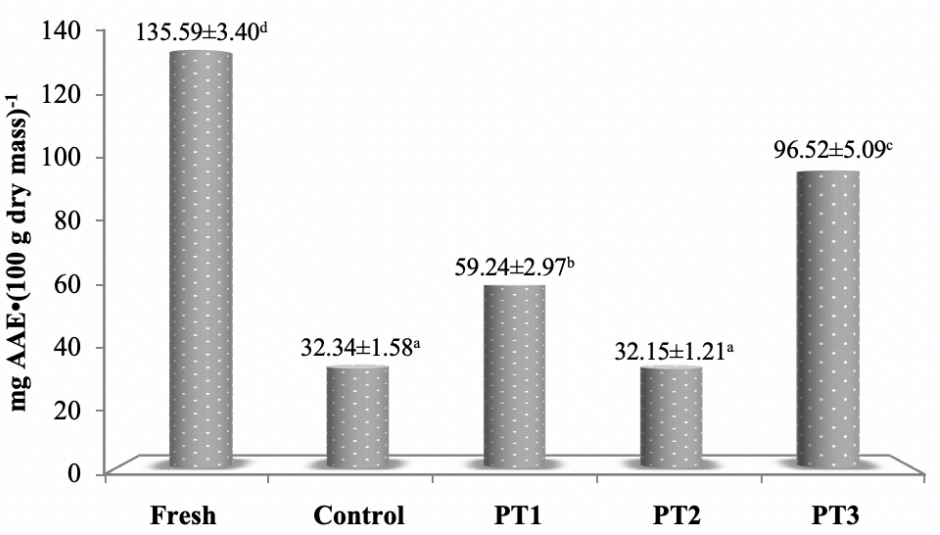

Figure 3. Content of vitamin C [mg ascorbic acid equivalents AAE•(100 g dry mass)$^{-1}$] in samples of fresh mango and pretreated dried. Mean ± standard deviation, n = 3. Control = without pretreatment, PT1 = 0.5 % (*w*/*v*) sodium metabisulphite, PT2 = 1.44% (*w*/*v*) mango seed extract, PT3 = [1.44%/0.5% (*w*/*v*)] mango seed extract/sodium metabisulphite. Superscripts a-d showed a significant difference (α = 0.05) according to the Duncan test.

3.8. Total carotenes content in dried products

The total carotenes content in fresh mango pulp was 25.54±0.81 mg of β-carotene•(100 g dry mass)$^{-1}$, Fig. 4, was within range reported by MANTHEY and PERKINS-Veazie (2009), who reported concentrations from 32.9 to 59.1 mg of carotenes•(100 g dry mass)$^{-1}$ in Mexican Tommy Atkins mangoes. Total carotenes content in sulphites-pretreated samples was 1.8 times higher than those reported by CHEN *et al*. (2007) for mango slices of 3 x 9 cm pretreated with 1% sodium bisulphite and dried with hot air at 60ºC. The variation in these results is probably due to different drying times and slice thickness. GUARTE *et al*. (2005) reported a carotene content of 6.80 mg•(100 g dry mass)$^{-1}$ for pulp mango, a very similar value to those measured for control and the PT2 samples in the present study.
From total carotene quantification of dried samples (Fig. 4), it was observed that mango-seed extract pretreatment (PT2) did not prevent carotene degradation since total carotene content with this pretreatment was similar than that of control [6.99±0.33 mg of β-carotene•(100 g dry mass)$^{-1}$, 7.06±0.321 mg of β-carotene•(100 g dry mass)$^{-1}$, respectively, Fig. 4]. Sulphites pretreatment (PT1) caused only a slight increase in total carotenes concentration with respect to control, which accounted for 29.3% of carotenes retention. On the other hand, PT3 combined pretreatment (sulphites and mango-seed extract) caused a remarkable increase in total carotenes content of dried sample with a 40.2% carotenes retention. Despite the fact that in this pretreatment carotenes retention was lower than 50%, total carotenes concentrations were 2.4 times higher than those obtained by CHEN *et al*. (2007) using a 1% sodium bisulphite treatment. In agreement with the previous results in this work, retention of carotenes was influenced synergistically by pretreatment with both the sulphites and mango-seed extract. Furthermore, the synergistic effect of combined pretreatments on carotene retention was more pronounced than with the other compounds, considering that in this case the individual pretreatments alone had little or no effect. A very high Pearson's correlation of total carotenes with vitamin C (r = 0.9580,

p = 0.0420) showed total carotenes are directly related with vitamin C content in mango slice during drying process.

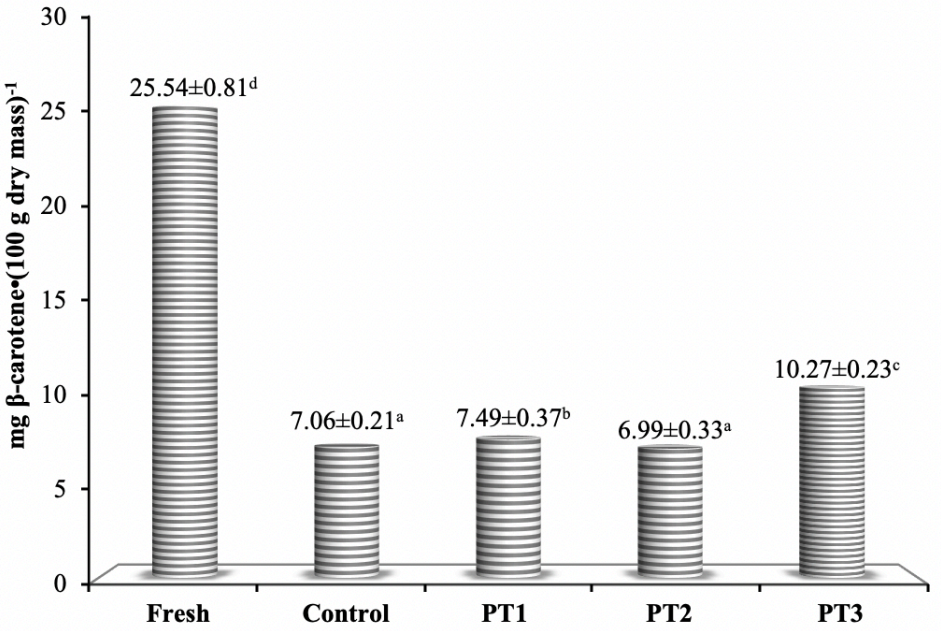

Figure 4. Content of total carotenes [mg β-carotene•(100 g dry mass)$^{-1}$] in samples of fresh mango and pretreated dried. Mean ± standard deviation, n = 3. Control = without pretreatment, PT1 = 0.5 % (*w*/*v*) sodium metabisulphite, PT2 = 1.44% (*w*/*v*) mango seed extract, PT3 = [1.44%/0.5% (*w*/*v*)] mango seed extract/sodium metabisulphite. Superscripts a-d showed a significant difference (α = 0.05) according to the Duncan test.

These results also could be ascribed to diffusion of antioxidant compounds from mango-seed extract/sodium metabisulphite solution to mango slice during pretreatment. This diffusion of antioxidant compounds could have contributed to maintain the solid structure of mango slices and thus reduce damage of mango pulp cells during drying process. The mango cells integrity during drying possibly reduced antioxidants compounds diffusion, including carotenes, from cell inside to mango slice surface. ADILETTA *et al*. (2016) provided evidence related to effect of pretreatments on solid structure preservation of foods during drying process. This study consisted in evaluating the pretreatment effect based NaCl 0.5% and trehalose 0.5% on eggplants drying. They observed by scanning electron microscopy (SEM) an increase in dried samples porosity, preserving its solid structure, while samples without pretreatment showed collapse and shrinkage phenomena. It is suggested that sodium metabisulphite behaves analogously to NaCl, promoting pores on the surface of mango slices during drying process, facilitating diffusion phenomena. Also, the combined effect of sodium metabisulphite with mango-seed extract, potency the decrease of antioxidant compounds degradation in mango slices.

3.9. Sulphites content in dried mango slices from PT1 and PT3

Sulphites are utilized as antioxidant additives for preventing oxidation, kept flavour and color, inhibit the growth of microorganisms that promote food spoilage, and also are anti-

browning agents for controlling enzymatic and non-enzymatic (Maillard) reactions (LOU *et al*., 2017). Otherwise, sulphites are allergenic components that can cause allergic reactions in asthma patients and people with diminished sulphite oxidase activity (SOUBRA *et al*., 2007). Additionally, these compounds can cause skin reactions (VALLY *et al*., 2009) and DNA damage (MENG *et al*., 2005). Hence, food safety organizations have considered an acceptable limit for sulphites in foods.

Sulphites concentrations in mango slices pretreated from PT1 and PT3 were 820.10±11.45 and 900.28±43.97 mg sulphites•(kg dry mass)$^{-1}$, respectively. Duncan's test showed a significant difference (α = 0.05) between samples PT1 and PT3. The latter showed approximately 9% more sulphites than PT1. Result indicates that mango-seed extract promoted an increase in sulphites diffusion towards the mango slice during immersion. Also, it is important to mention that sulphites concentrations in mango dried slices PT1 y PT3 are within the limit established by the Codex Alimentarius Commission, created by the World Health Organization and the United Nation's Food and Agriculture Organization (FAO), which supports a general maximum of 1250 mg•(kg SO_3^{-2})$^{-1}$ in dried fruits (LIAO *et al*., 2013).

3.10. Product color

The color parameters CIE L*a*b* and Hue° were determined for fresh and dried slices mango, Table 3.

Table 3. Color parameters CIE L*a*b* and Hue angle in dried slices and fresh mango.

Pretreatment	L*	a*	b*	Hue angle
Control	65.61±3.00^{b}	16.97±1.37^{c}	66.74±4.33^{b}	75.72±1.00^{a}
PT1	60.50±4.20^{a,b}	14.14±1.87^{b}	58.81±5.01^{a}	76.39±2.17^{a}
PT2	66.83±3.97^{b}	12.33±1.53^{a,b}	63.44±4.64^{a,b}	78.96±1.59^{b}
PT3	71.38±3.39^{b}	10.88±1.88^{a}	69.35±2.37^{b}	81.08±1.61^{b}
Fresh	63.56±4.73^{b}	11.45±1.95^{a}	57.81±4.22^{a}	78.84±1.43^{b}

Control = Mango dehydrated without pretreatment, PT1 = 0.5% (w/v) sodium metabisulfite, PT2 = 1.44% (w/v) seed extract mango, PT3 = seed extract mango/sodium metabisulfite [1.44%/0.5% (w/v)]. Mean ± standard deviation, n = 3. The letters a-c showed a significant difference in columns (α = 0.05) with Duncan test.

The values for L*, a*, b* and Hue° were found in ranges 60.50-71.38, 10.88-16.97, 57.81-69.35 and 75.72-81.08, respectively. Parameter L* indicates brightness degree of the sample on a scale from 0 (black) to 100 (white). The L* values allow affirm that pretreatment of mango slices with sulphites help to avoid the darkening of dried mango, as it expected. However, when sulphites are combined with extract of mango-seed, the effect is lost. This last behavior is attributed to the phenoloxidase enzymes present in seed mango extract that degrade the phenolic compounds to melanines. These compounds are responsible of darkening of mango slices during drying process. The temperature and sonication time used to obtain mango-seed extract, according to CHENG *et al*. (2013) do not inactivate phenoloxidase enzymes. These enzymes are inactivated only at temperatures above 62°C and ultrasound frequencies above 20 kHz.

Parameter a* indicates sample redness degree and as sample color is redder, a* has a bigger positive magnitude. Results obtained for a* show drying process induces an increase of redness in mango slices without mango-seed extract (control and PT1), Table 3. Parameter b* with positive values indicates yellowness degree of sample. Dried sample with 0.5% sodium metabisulphite and seed mango extract (PT3) show the higher valor of b*, 69.35±2.37. This result is concordant with carotenes content and indicates a combined effect protective of sodium metabisulphite and phenol compounds present in pretreatment (PT3).
Hue angle is one color property, defined as the degree to which a stimulus can be related with red, orange, green, yellow, green, blue and violet. A Hue° value of 90 represents a yellow tone. Therefore, from the data in Table 3 it can be affirm that samples pretreated with 0.5% sodium metabisulphite (PT2 and PT3) have a yellower tone than control and PT1 samples.

4. CONCLUSIONS

Midilli's model fitted very well to experimental data of mango slices dried without pretreatment and with the three pretreatments (PT1, PT2 and PT3) used in the present study. Comparative analysis of Midilli's constants, k, a and b using Duncan's means test showed pretreatments did not influence drying process of mango slices. Also, the results of antioxidant compounds quantification showed (1.44%/0.5%) mango-seed extract/sodium metabisulphite used as a pretreatment (PT3) has an important role on retention of vitamin C and carotenes in dried mango slices. The free phenols content was quadrupled compared to dried slices without pretreatment (control) and nearly were doubled compared to fresh mango. Mango slices pretreated with mango-seed extract were strongly enriched with gallic acid, caffeic acid, ferulic acid and *p*-coumaric acid, also with, methyl gallate to a lesser extent in relation to mango slices that did not receive a pretreatment (control). Furthermore, sulphites content of this dried product is within limit established by the Codex Alimentarius of FAO. Finally, this is the first study reporting combined use of mango-seed extract with sulphites as a pretreatment for drying mango pulp. Mango seed, which is generally an agro-industrial residue, used to obtain extracts rich in phenolic compounds and using these as a pretreatment for drying mango pulp and other foods could become commercially feasible in the proximate future.

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PAPER

INFLUENCE OF DIFFERENT PRETREATMENTS AND CHAPTALIZATION TYPES ON THE PHYSIOLOGICAL CHARACTERISTICS AND ANTIOXIDANT ACTIVITY OF APRICOT (*PRUNUS ARMENIACA* L.) WINE

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ABSTRACT

The effects of pretreatment (pectinase and $CaCO_3$) and chaptalization (sugar and puree concentrate) on the quality of apricot wine were investigated. Pectinase-treated apricot wines had increased amounts of total phenolics, flavonoid compounds, as well as antioxidant activities. The apricot wine chaptalized with puree concentrate and treated with pectinase (PCP) showed the highest total acidity and some organic acid contents, which resulted in the strongest sourness. In contrast, the apricot wine treated with pectinase and $CaCO_3$ (SCPC and PCPC) showed the lowest total acidity and least sourness. Antioxidant activities of PCP and PCPC wines were higher than other wines, and other pectinase-treated wines were also higher than the control wine. Volatile higher alcohols and terpenes increased in all the pectinase-treated wines, whereas volatile ester compounds were decreased. Sensory evaluation showed that SCPC, PCP, and PCPC wines obtained significantly high flavor scores, and SCPC and PCPC wines obtained the highest overall preference scores.

Keywords antioxidant, apricot wine, aroma profile, fruit wine, pretreatment

1. INTRODUCTION

Apricot (*Prunus armeniaca* L.) is a stone fruit mainly grown in China, the Mediterranean European countries, Turkey, and the USA (SOLIMAN, 2013). Consumption of apricot has shown human health benefits because of its antioxidant, anti-inflammatory, and immune-stimulating properties, which might be attributed to the presence of various phytochemicals, such as carotenoids, polyphenols, vitamins, and fiber (DRAGOVIC-UZELAC *et al*., 2007; HEGEDŰS *et al*., 2010; MADRAU *et al*., 2009). Due to the various advantages of apricot, the development of apricot wine has good potential for commercialization.

Despite the excellent functionality, the strong sourness of apricot, associated with its notably high acidity, has still not been acceptable, which prevents the development of apricot wine. Pretreatment of high-acid wines by deacidification offers a suitable resolution to this issue, and it is commonly carried out by physicochemical methods, such as carbonic amelioration, blending, chemical neutralization, and precipitation, and by biological methods, such as malolactic fermentation (LOIRA *et al*., 2018; VOLSCHENK *et al*., 2006). Among these methods, chemical neutralization by the addition of salts ($CaCO_3$) to deacidify fruit wines is usually preferred because it reduces the risk of increasing the pH levels and, additionally, prevents microbial problems (COSME *et al*., 2018; MATTICK *et al*., 1980).

Pectinases are enzymes that are generally added to maximize juice yield and act by degrading the pectins that interfere with extraction and clarification of most fruit juices (SHARMA *et al*., 2017). In addition, treatment of fruit juice with pectinase has been reported to increase the amounts of phenolics and anthocyanins, facilitate filtration, and contribute to the release of the molecules responsible for aroma and color, two of the major components that characterize a wine (PARDO *et al*., 1999; PINELO *et al*., 2006; WATSON *et al*., 1999).

Some fruits with low sugar content must be chaptalized to obtain sufficient sugar content for making wine (JARVIS, 1996; MIYAWAKI *et al*., 2016). Several researchers have used various technologies, such as freeze-concentration and nanofiltration, to decrease the levels of available water in fruits deficient in sugar content, thereby concentrating the sugar content (BANVOLGYI *et al*., 2006; CLARY *et al*., 2006; MIYAWAKI *et al*., 2016). Puree concentrate can also be a suitable alternative instead of chaptalization because of its concentrated sugar content and using the apricot puree concentrate could reduce labors and enhance productivity by skipping the process of washing the fruit and removing the seed for the industrial mass production of apricot wine.

This study aimed to improve the quality of apricot wine. Apricot wines were prepared following different types of pretreatments, including pectinase and $CaCO_3$, and chaptalization, by the addition of sugar and puree concentrate, and their physicochemical parameters, volatile aromatic profiles, antioxidant activities, and sensory characteristics were investigated.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Trolox, Folin–Ciocalteu reagent, methanol (HPLC grade), potassium

metabisulfite ($K_2S_2O_5$), organic acids, and all other standards were obtained from Sigma–Aldrich (St Louis, MO, USA). White table sugar (CJ Co., Seoul, South Korea), used to adjust the sugar content of the must, was bought from the local market. Apricot puree concentrate (30.7°Bx, pH 4.1, and acidity 1.31%) was procured from Aftun Gida Ltd. (Yenisehir, Mersin, Turkey). Rapidase® X-Press L (pectinase+hemicellulase, 180,000 AVJP/g) was purchased from DSM Food Specialties (Delft, Netherlands). The fermentation agent *Saccharomyces cerevisiae* var. *bayanus* EC-1118 yeast was purchased from Lallemand Inc. (Montreal, Canada). $CaCO_3$ was acquired from Daejung Co. (Siheung, South Korea).

2.2. Apricot fruit samples

Fully-ripened apricot fruit (*P. armeniaca* L.) were bought from local farms in Yeongcheon (Gyeongsangbuk-do, South Korea) during the 2017 harvest season. "Harcot" apricot fruit was selected for uniformity of size, color, and absence of decay or rot. Fruit was stored at –18°C until further use.

2.3. Apricot fruit must preparation and pretreatment conditions

Apricot fruit was washed with tap water, the seeds removed manually. The deseeded fruit was blended using a household juicer (NJ-9300A, NUC Juicer, Daegu, South Korea) and then combined immediately with 0.02% (w/v) $K_2S_2O_5$ to prevent bacterial contamination and oxidation. To determine the most suitable amount of enzyme and $CaCO_3$ (for deacidification), a part of the apricot fruit pulp was divided into four portions of 300 mL each. The first portion was used as the control while the three remaining portions were treated with pectinase (Rapidase® X-Press L) at 0.05%, 0.1% and 0.2% (v/w), respectively. For the deacidification process, $CaCO_3$ was added at 0.1%, 0.2%, and 0.3% (w/w), respectively. Pectinase treatment or deacidification occurred for 2 h under constant agitation using a shaking incubator (30°C, 200 rpm). The pulp samples were centrifuged at 3,578 × g for 10 min, and the obtained juices were analyzed and compared for pH level, total acidity, total soluble solids, and reducing sugars.

2.4. Apricot wine-making

Apricot fruit pulp was divided into five wine-making trial batches (5 kg), from which wines were prepared in triplicate and, subsequently, treated before fermentation. The chaptalization and pretreatment conditions are listed in Table 1. In the first batch, namely, the control batch (SC), the apricot pulps were chaptalized with white sugar to obtain 22°Bx. In the second batch (SCP), the apricot pulps were chaptalized to 22°Bx with white sugar and then treated with 0.1% (v/w) pectinase. In the third batch (SCPC), the apricot pulps were chaptalized to 22°Bx with white sugar, treated with 0.1% (v/w) pectinase, and then deacidified with 0.3% $CaCO_3$. In the fourth batch (PCP), the apricot pulps were chaptalized to 22°Bx with apricot puree concentrate and then treated with 0.1% (v/w) pectinase. In the fifth batch (PCPC), the apricot pulps were chaptalized to 22°Bx with apricot puree concentrate, treated with 0.1% (v/w) pectinase, and then deacidified with 0.3% (w/w) $CaCO_3$. Each treatment process lasted for 2 h under constant agitation (30°C, 200 rpm), 200 mg/L of $K_2S_2O_5$ was added to prevent bacterial contamination, and then the batches were centrifuged at 3,578 × g for 10 min. The apricot wine was fermented with 1–2 × 10^6 CFU mL^{-1} *S. cerevisiae* var. *bayanus* EC-1118 that was rehydrated by sterile distilled

water at 40°C for 30 minutes, according to the manufacturer's instruction. Each sample was fermented without shaking at 20°C for 7 days until complete fermentation. The final wine samples were filter-sterilized, poured into wine bottles with 50 mg/L of $K_2S_2O_5$, and stored at 4°C for further analysis and sensory assessment.

Table 1. List of ingredients used in apricot wine-making.

Ingredients (g)	Chaptalization and pretreatment conditions				
	SC	SCP	SCPC	PCP	PCPC
Apricot pulp	4,472.5	4,472.5	4,472.5	2,430	2,430
Sugar	527.5	527.5	527.5		
Apricot puree concentrate				2,570	2,570
Pectinase		1	1	1	1
$CaCO_3$			15		15

SC sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$

2.5. Physicochemical parameters

The physicochemical analysis was undertaken on the supernatant obtained from centrifugation of the wine samples at 3,578 × g for 10 min. The pH was measured using a pH meter (MP225K, Mettler-Toledo CH, Seoul, South Korea). Soluble solids (°Bx) were determined using a refractometer (RA250, Atago, Tokyo, Japan). A vinometer was used to evaluate the alcohol content at 15°C. Titratable acidity was assayed using NaOH solution (0.1 N) until neutralization of the organic acids to pH 8.2-8.3, and the results were expressed as a percentage of citric acid/100 g.

2.6. Total phenolic compounds

The total phenolic compounds in the apricot wine samples were estimated, as detailed by OUGH and AMERINE (1988), with some modifications. Wine samples (2 mL) were mixed with 2 mL of 1:1 (v/v) Folin–Ciocalteu reagent and incubated at room temperature for 3 min. Afterward, each tube was added with 2 mL of 10% Na_2CO_3, vortexed, and allowed to stand at room temperature for 1 h. The absorbance was measured at 700 nm. The results were expressed as gallic acid equivalents in mg/mL of apricot wine.

2.7. Total flavonoid content

The total flavonoid contents of the apricot wines were determined, as described by ZHISHEN *et al*. (1999) with minor modifications. The wine samples were examined spectrophotometrically at 510 nm against a blank solution containing all reagents and 200 µL of distilled water instead of wine samples using a spectrophotometer (UV-1601, Shimadzu Co.). First, 430 µL of 50% ethanol, 70 µL of wine sample, and 50 µL of 5% $NaNO_2$ were combined in a test tube. After 30 min of incubation, samples were combined with 50 µL of 10% $Al(NO_3)_3 \cdot 9H_2O$. Six minutes later, 500 µL of NaOH (1 N) was added,

and the solutions vortexed. The results were expressed as rutin equivalents in mg/mL of apricot wine.

2.8. DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method previously described by OSZMIAŃSKI *et al*. (2011). Here, 100 μM of DPPH was dissolved in pure ethanol (96%). The radical stock solution was prepared just before experimentation. Then, 1 mL of DPPH was added to 1 mL of apricot wine sample and 3 mL of 96% ethanol. The mixture was thoroughly shaken and placed at room temperature in the dark for 10 min. The decrease in absorbance of the resulting solution was observed at 517 nm at 10 min. The results were corrected for dilution and expressed in μM of Trolox/mL of apricot wine. Absorbance was measured using a spectrophotometer (UV-1601, Shimadzu Co.).

2.9. ABTS radical scavenging activity

ABTS radical scavenging activity was measured based on the method previously reported by OSZMIAŃSKI *et al*. (2011). ABTS was dissolved in water to make a 7 μM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting the ABTS stock solution with 2.45 of μM potassium persulfate (final concentration) and kept in the dark at room temperature for 12–16 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. The samples containing $ABTS^{\cdot+}$ solution were diluted with redistilled water to an absorbance of 0.700±0.02 at 734 nm and equilibrated at 30°C. After adding 3.0 mL of diluted $ABTS^{\cdot+}$ solution ($A_{734\ nm}$ = 0.700±0.02) to 30 μL of apricot wine sample, the absorbance was read at exactly 6 min after initial mixing. The results were corrected for dilution and expressed in μM Trolox/1 mL of apricot wine. Absorbance was measured using a spectrophotometer (UV-1601, Shimadzu Co.).

2.10. FRAP assay

Ferric ion reducing antioxidant power was measured according to the method previously described by OSZMIAŃSKI *et al*. (2011). The assay was based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}), with the latter forming a blue complex (Fe^{2+}/TPTZ) that increases absorbance at 593 nm. Moreover, FRAP reagent was prepared by mixing with an acetate buffer (300 μM, pH 3.6), a solution of 10 μM of TPTZ in 40 μM of HCl and 20 μM of $FeCl_3$ at a ratio of 10:1:1 (v/v/v). The reagent (300 μL) and apricot wine sample solutions (10 μL) were added to each well and thoroughly mixed. The absorbance was measured at 593 nm after 10 min. A standard curve was plotted using different Trolox concentrations. All solutions were prepared on the same day of experimentation. The results were corrected for dilution and expressed in μM of Trolox/1 mL of apricot wine. Absorbance was measured using a spectrophotometer (UV-1601, Shimadzu Co.).

2.11. Free sugar and organic acid analyses

The free sugar and organic acid contents in the wine samples were identified and quantified using a Prominence HPLC instrument (Shimadzu Co.) with a refractive index detector (RID-10A, Shimadzu Co.), as described by KIM *et al*. (2018). The wine samples were centrifuged at 3,578 × g for 10 min, and the resultant supernatants were filtered

through a Millex-HV 0.45-μm membrane filter (Millipore Co., Bedford, MA, USA) to obtain analytical samples. Free sugar content was determined using a Sugar-Pak I column (6.5 mm × 300 mm, 10 μm; Waters, Milford, MA, USA). The mobile phase was Ca–EDTA buffer (50 mg/L) at a flow rate of 0.5 mL/min at 90°C. Organic acids were quantified using a Shodex RSpak KC-811 column (8.0 mm × 300 mm, 6 μm; Showa Denko KK, Kawasaki, Japan), and a mobile phase of 0.1% H_3PO_4 at a flow rate of 1 mL/min at 65°C. Standard curves were plotted using different concentrations of each compound. The results were expressed as each compound's equivalents in g/L of apricot wine.

2.12. Analysis of volatile compounds

Volatile compounds were analyzed as described by LEE *et al*. (2016) with minor modifications, using a 7890A GC–MS system (Agilent, Santa Clara, CA, USA). Volatile compounds were separated using a DB-WAX column (60 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent, Santa Clara, CA, USA) and detected using an Agilent 5975C TAD inert XL MSD. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The temperature of the GC oven was initially held at 40°C for 2 min, increased at a rate of 2°C/min until 220°C, and then increased at 20°C/min to 240°C, and maintained at 240°C for 5 min. Volatile compounds were collected using a headspace (HS) solid-phase microextraction (SPME) fiber (10 mm length, 50/30 μm DVB/CAR/PDMS; Supelco, Bellefonte, PA, USA) with magnetic stirring. Five milliliters of each sample was placed in a HS vial (20 mL, 23 × 75 mm, PTFE/silicone septum, magnetic cap, Agilent, Santa Clara, CA, USA) and then 1.25 g NaCl was added to increase the efficiency of salting-out of volatile aromatic compounds in the HS. Prior to extraction, the sample was shaken in a water bath at 35°C for 20 min to achieve equilibrium. Afterward, the SPME fiber was inserted into the vial and incubated at 35°C for 40 min. The chemical standards for volatile ester compounds were customized by Chem Service Inc. (West Chester, PA, USA). Other volatile compound standards were purchased from Sigma-Aldrich (St Louis, MO, USA). Volatile compounds were identified by comparing their retention times and mass spectra against the Wiley 9 spectral library (John Wiley and Sons, Hoboken, NJ, USA) using NIST 0.8 (version 5.0; NIST, Gaithersburg, MD, USA). For the quantitative analysis of each compound in the wine, a calibration curve was established by plotting the peak area against the concentration of the chemical standards. Some chemicals that were commercially unavailable were quantified using standard curves of volatile compounds that had similar molecular properties. The results were expressed as each volatile alcohol compound's equivalents in mg/L of apricot wine and each volatile ester and terpene compound's equivalents in μg/L of apricot wine, respectively.

2.13. Sensory evaluation

A seven-point hedonic scale was used for sensory evaluation. Each apricot wine was placed in a sample bottle and left undisturbed at room temperature for 1 h, with the bottle lid still closed before being subjected to sensory evaluation. After opening the lid, each wine was poured into wine glasses to evaluate color, sweetness, sourness, and overall preference. Clarity and turbidity levels were considered as part of the parameters for color evaluation. The well-trained panel was composed of 20 students (13 males and 7 females aged 20–29 years old) from the School of Food Science and Biotechnology, Kyungpook National University, Korea. Each panelist evaluated the apricot wines with at least a 3-min

interval between samples, and water was provided to cleanse their palate. Sensory scores ranged from 1 (very poor) to 7 (excellent).

2.14. Statistical analysis

All experiments were conducted at least three times or more. Statistical significance was determined by the Student's *t*-test for independent means using Microsoft Excel (Microsoft, Redmond, WA, USA). One-way analysis of variance and Duncan's multiple range test were used to determine significant differences between means. Statistical significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of different pretreatment conditions on the physicochemical parameters of apricot juice

The effects of different pretreatments on the physicochemical parameters of apricot juice are listed in Table 2. The yield of apricot juices subjected to pectinase treatment were higher by 5.66%, 10.02%, and 10.38% in apricot pulp containing 0.05%, 0.1%, and 0.2% pectinase, respectively, compared with the control juice, but the yields of juices treated with 0.1% and 0.2% pectinase enzyme were not considerably different. In addition, juices treated with pectinase enzyme had a statistically lower pH and higher total acid contents relative to the control juice. The reducing sugar contents of apricot juices also increased with increasing pectinase enzyme concentrations, but no significant differences were found between pectinase-treated juices. Apricot juices treated with 0.05%, 0.1%, and 0.2% pectinase had reducing sugar contents of 15.65%, 15.83%, and 15.90%, respectively. The pH and total acid contents of apricot juices by deacidification significantly increased and decreased, respectively, with increasing $CaCO_3$ concentration compared with those of non-treated apricot juice.

Table 2. Effects of pectinase enzyme and $CaCO_3$ concentrations on the physicochemical properties of apricot juices.

Treatment	Pectinase enzyme			
	Non-treated	0.05%	0.1%	0.2%
Juice yield (%)	67.20±0.12^{d}	72.86±0.03^{c}	77.22±0.05^{b}	77.58±0.05^{a}
pH	3.16±0.02^{a}	3.11±0.01^{b}	3.10±0.04^{b}	3.10±0.07^{b}
Total acidity (%)	2.56±0.02^{b}	2.62±0.03^{a}	2.63±0.04^{a}	2.64±0.3^{a}
Soluble solids (°Bx)	16.2±0.05^{b}	16.4±0.08^{a}	16.4±0.05^{a}	16.4±0.09^{a}
Reducing sugars (%)	14.5±0.04^{b}	15.65±0.10^{a}	15.83±0.08^{a}	15.90±0.12^{a}
Treatment	**Deacidification ($CaCO_3$)**			
	Non-treated	**0.1%**	**0.2%**	**0.3%**
pH	3.14±0.06^{d}	3.22±0.01^{c}	3.33±0.02^{b}	3.42±0.01^{a}
Total acidity (%)	2.56±0.10^{a}	2.48±0.03^{a}	2.30±0.05^{b}	2.17±0.04^{c}

All data are expressed as mean±standard deviation (n = 3).
Different letters in the same row indicate significant differences at $p<0.05$.

Fruits other than grape, such as apricot, have high acidity, which needs to be controlled before, during, or after fermentation, for producing a suitable final wine (VELIĆ *et al.*, 2018). In this study, each 0.1% pectinase treatment and 0.3% $CaCO_3$ treatment improved the juice yield and appropriate physicochemical changes in apricot juice, so we further investigated the appropriate combination of these pretreatment conditions for apricot wine.

3.2. Effects of different chaptalization types and the combination of pretreatments on the fermentation and physicochemical properties of apricot wine

The influences of the various chaptalization techniques and the combination of pretreatments on the changes in fermentation characteristics during alcohol fermentation and physicochemical properties of fully fermented apricot wine are provided in Fig. 1 and Table 3. The soluble solid and alcohol contents of all the apricot wines similarly decreased and increased, respectively, for the first 3 days of fermentation. After then, all the pectinase-treated apricot wines showed higher soluble solid and alcohol contents, compared with the control wine because of increased juice yield and reducing sugar caused by 0.1% pectinase treatment. The pH and total acidity of all the apricot wines decreased and increased, respectively, for first or second days of fermentation, then steadily increased and slightly decreased, respectively, until complete fermentation. The pH and total acidity of apricot wines treated with $CaCO_3$ (SCP and PCP wines) were significantly lower and higher, respectively, than those of other apricot wines from beginning to end of the fermentation process. The total phenolic and total flavonoid contents of all the apricot wines were significantly superior to those of the control wine because pectinase released phenols and polyphenols from the plant cell wall (CHANG *et al.*, 1995). In addition, PCP and PCPC wines that were chaptalized with puree concentrate presented higher total acidity, as well as total phenolic and flavonoid contents, compared with those of SCP and SCPC wines that were chaptalized with sugar, because all of these compounds were concentrated in the added apricot puree concentrate. Although the total phenolic and total flavonoid contents of pectinase-treated apricot wines were relatively higher than those of other groups, the lower pH and higher total acid content of PCP wine may be negatively associated with the sensory properties. On the contrary, PCPC wine contained similar contents of functional compounds but better palatability compared to PCP wine because of deacidification.

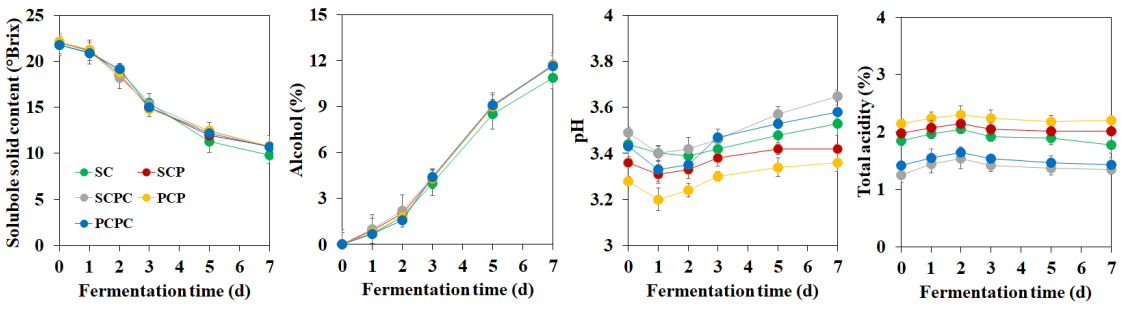

Figure 1. Changes in the soluble solid, alcohol, pH, and total acidity of apricot wines during fermentation. *SC* sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$

Table 3. Effects of different chaptalization types and pretreatment conditions on the physicochemical parameters of apricot wines.

Parameter	Wine				
	SC	SCP	SCPC	PCP	PCPC
Soluble solids (°Bx)	9.80±0.20^{b}	10.75±0.10^{a}	10.75±0.10^{a}	10.80±0.20^{a}	10.70±0.10^{a}
Alcohol (%)	10.9±0.10^{b}	11.74±0.20^{a}	11.72±0.10^{a}	11.70±0.10^{a}	11.64±0.10^{a}
pH	3.53±0.10^{ab}	3.42±0.09^{b}	3.65±0.05^{a}	3.36±0.03^{c}	3.58±0.05^{a}
Total acidity (%)	1.78±0.04^{c}	2.02±0.01^{b}	1.35±0.01^{e}	2.20±0.03^{a}	1.43±0.04^{d}
Total phenolic compounds (mg/mL)	11.41±0.37^{c}	16.95±3.11^{b}	17.15±2.03^{b}	21.87±0.96^{a}	21.43±1.21^{a}
Total flavonoids (mg/mL)	0.39±0.00^{b}	0.41±0.01^{a}	0.42±0.01^{a}	0.43±0.01^{a}	0.43±0.01^{a}

All data are expressed as mean±standard deviation (n = 3).
Different letters in the same row indicate significant differences at $p<0.05$.
SC sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$

3.3. Free sugar and organic acid contents of apricot wines

The impacts of different chaptalization types and the combination of pretreatments on the free sugar and organic acid contents in apricot wines are evident in Table 4. After alcoholic fermentation, sucrose, glucose, galactose, and fructose were identified in the apricot wines. Fructose was the most abundant reducing sugar (0.599±0.014–4.662±0.019 g/L) in all the apricot wines. Marked differences in the organic acids were observed between each apricot wine. Citric acid and quinic acid of SCP and SCPC wines were significantly decreased and increased compared with SC wine, respectively, whereas tartaric acid and malic acid of SCPC wine were the lowest among all the apricot wines. Citric acid and quinic acid contents of PCP and PCPC wines were significantly higher than other wines because various components of apricot were concentrated during puree concentrate preparation, whereas tartaric acid of PCPC wine was significantly lower than PCP wine due to deacidification. Succinic acid levels were comparable among all the apricot wines, and acetic acid of pectinase-treated apricot wines was slightly increased compared with control apricot wine. According to AMERINE *et al*. (1965), the decreasing order of sourness intensity of organic acids is malic acid, tartaric acid, citric acid, and lactic acid. $CaCO_3$ treatment was reported to reduce wine acidity by inducing the precipitation of tartrate and malate (MATTICK *et al*., 1980). Thus, the combination of pectinase and $CaCO_3$ treatments increased the yield of apricot juice and reduced the acidity in apricot wine.

3.4. Antioxidant activity of apricot wines

The various antioxidant activities, such as DPPH radical scavenging activity, ABTS radical scavenging activity, and FRAP of apricot wines are shown in Fig. 2. All of the antioxidant activities were highest in PCP and PCPC wines, followed by SCP and SCPC wines, and then SC wine, which might be attributed to the release of pigment compounds, such as flavonoids, by pectinase (all the pectinase-treated apricot wines) and the concentration of those compounds in the added puree concentrate (PCP and PCPC wines).

Table 4. Composition of free sugar and organic acid contents (g/L) of apricot wines depending on different chaptalization types and pretreatment conditions.

Parameter	Wine				
	SC	SCP	SCPC	PCP	PCPC
Free sugars					
Sucrose	0.08±0.01^{a}	ND	ND	ND	ND
Glucose	0.16±0.06^{c}	0.28±0.03^{b}	0.25±0.01^{b}	0.70±0.02^{a}	0.67±0.03^{a}
Galactose	0.23±0.04^{c}	0.86±0.01^{b}	0.82±0.04^{b}	1.58±0.02^{a}	1.50±0.06^{a}
Fructose	0.60±0.01^{c}	2.66±0.01^{b}	2.64±0.01^{b}	4.56±0.02^{a}	4.66±0.02^{a}
Organic acid					
Citric acid	11.58±0.35^{b}	9.89±0.25^{c}	9.61±0.34^{c}	14.40±0.51^{a}	14.25±0.48^{a}
Tartaric acid	2.83±0.08^{b}	2.84±0.11^{b}	0.42±0.04^{d}	3.11±0.12^{a}	0.73±0.06^{c}
Malic acid	5.61±0.12^{a}	4.39±0.12^{b}	3.20±0.09^{c}	4.29±0.09^{b}	2.96±0.10^{d}
Quinic acid	7.37±0.16^{c}	11.48±0.34^{b}	11.34±0.33^{b}	34.17±1.03^{a}	32.87±1.17^{a}
Succinic acid	0.50±0.02^{a}	0.54±0.04^{a}	0.52±0.04^{a}	0.45±0.02^{b}	0.44±0.02^{b}
Acetic acid	0.18±0.01^{c}	0.31±0.04^{b}	0.29±0.01^{b}	0.42±0.02^{a}	0.40±0.02^{a}

All data are expressed as mean±standard deviation (n = 3).
Different letters in the same row indicate significant differences at $p<0.05$.
SC sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *ND* not detected

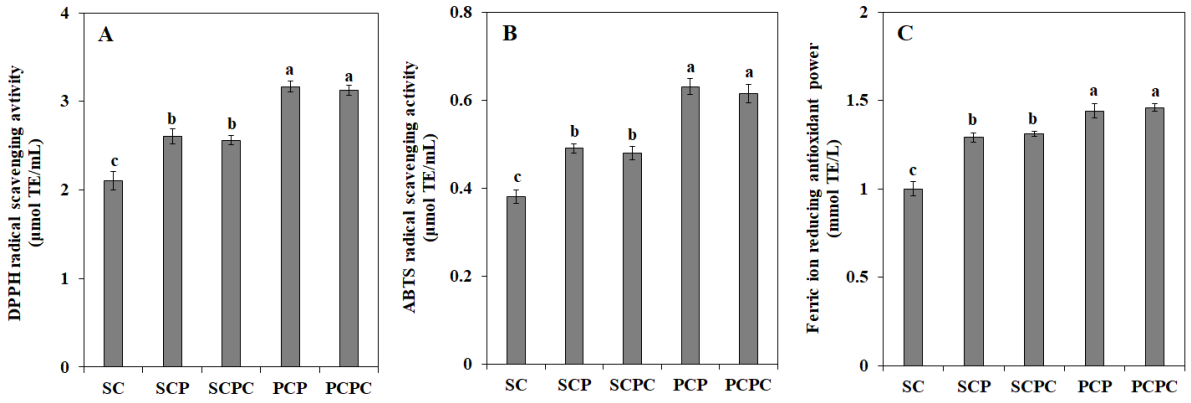

Figure 2. Effects of different chaptalization types and pretreatment conditions on the DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), and ferric ion reducing power (C) antioxidant activities of apricot wines.
Different letters indicate significant differences at $p<0.05$.
L-AA L-ascorbic acid, *TE* Trolox equivalents, *SC* sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$

Apricot contains numerous phenolic compounds, including catechin, epicatechin, *p*-coumaric acid, caffeic acid, and ferulic acid, that contribute to the antioxidant activity and nutritional benefits (CAMPBELL and PADILLA-ZAKOUR, 2013; SOCHOR *et al*., 2010). ARNOUS *et al*., (2002) mentioned that total polyphenol and total flavonol compounds could significantly contribute to the overall antioxidant activity of wine. As such, in the present study, the high antioxidant activities displayed by the apricot wines depended on the increased total phenolic and flavonoid compounds released by pectinase pretreatment and concentrated by puree concentrate chaptalization.

3.5. Volatile aromatic compounds of apricot wines

The volatile aromatic compounds of apricot wines are given in Table 5. The volatile higher alcohol compounds were more abundant in pectinase-treated apricot wines than control apricot wine. In PCP and PCPC wines, most of the volatile higher alcohols, except for 1-propanol, were detected at levels lower than in SCP and SCPC wines, respectively. Moreover, SCPC wine showed the highest amount of 1-propanol, isobutanol, isoamyl alcohol, 1-hexanol, 3-ethoxypropanol, 1-decanol, and benzyl alcohol, among all the apricot wines. A higher amount of 2,3-butanediol, which is an unattractive compound in wine because of its buttery aroma (BARTOWSKY and HENSCHKE, 2004), was detected in greater quantities in SC and SCP wines than in the other apricot wines examined. Total volatile ester compounds were the highest in SC wine, as those of pectinase-treated apricot wines were evaporated during pectinase treatment at 30°C for 2 h. Furthermore, PCP and PCPC wines presented significantly lower total volatile ester compounds than those of the other wines, which is considered to be due to the loss of their corresponding precursors during heat treatment of the puree concentrate production process. SC wine contained the highest amounts of isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl-9-decanoate, as well as ethyl decanoate. These compounds primarily influenced the changes in the amount of total volatile ester compounds. Volatile terpenes were higher in all the pectinase-treated apricot wines than control apricot wine. In particular, linalool and α-terpineol of PCP and PCPC wines were significantly higher than those of the other wines. The group of higher alcohols is well known as one of the dominant chemical constituents in wine, in which they play a major role as ester precursors (LAMBRECHTS and PRETORIUS, 2000). Esters are well recognized as the most abundant aromatic compounds in wine (ROJAS *et al*., 2001) and are produced by yeasts during alcoholic fermentation, whereas terpenes are only present in small amounts in some fruits, such as grape (especially in aromatic cultivars), apricot, and peach. However, terpenes can mostly affect the floral properties of wines with low odor thresholds (100-400 ppb) (MAICAS and MATEO, 2005). In the present study, significantly decreased contents of volatile ester compounds were detected in the pretreated apricot wines compared with non-treated apricot wine, but the levels of volatile higher alcohols and terpenes were greater, which might have assisted in improving the sensory properties of apricot wine.

Table 5. The concentration of volatile aromatic compounds in apricot wines depending on different chaptalization types and pretreatment conditions.

Compound	Odor description	Threshold (mg/L)	Amount of volatile aromatic compound				
			SC	SCP	SCPC	PCP	PCPC
1-Propanol	Alcohol, ripe fruity[1]	306[1]	85.48±6.55^{b}	97.17±9.45^{b}	169.76±14.11^{a}	103.27±10.11^{b}	178.63±13.28^{a}
Isobutanol	Alcohol, solvent, green, bitter[1]	75[1]	159.27±12.09^{b}	176.03±16.23^{ab}	199.71±20.56^{a}	141.00±12.24^{b}	162.13±15.06^{b}
Isoamyl alcohol	Solvent, sweet, nail polish[2]	60[2]	2605.68±233.17^{a}	2863.76±256.18^{a}	3024.25±306.50^{a}	2729.15±250.06^{a}	2896.57±269.77^{a}
1-Hexanol	Herbaceous, grass, woody[1]	1.1[1]	20.10±1.94^{c}	30.68±3.31^{b}	37.41±3.42^{a}	23.85±2.65^{c}	32.64±3.04^{ab}
3-Ethoxypropanol	Fruity[1]	0.1[1]	10.04±1.11^{a}	10.27±0.98^{a}	10.73±0.94^{a}	6.82±0.61^{b}	6.77±0.64^{b}
1-Octanol	Jasmine, lemon[1]	0.8[1]	13.62±1.26^{b}	89.27±7.24^{a}	10.10±0.88^{b}	8.83±0.77^{b}	5.04±0.62^{c}
2,3-Butanediol	Floral, fruity, herbal, buttery[2,3]	150[2]	14.70±1.32^{a}	14.24±1.52^{a}	11.21±1.05^{b}	9.52±0.89^{b}	8.16±0.72^{b}
1-Decanol	Floral, fruity, bitter, winey[2]	0.4[2]	5.13±0.44^{b}	6.21±0.56^{a}	6.51±0.52^{a}	4.44±0.41^{b}	4.50±0.39^{b}
Benzyl alcohol	Roasted, sweet, fruity[1]	200[1]	20.07±2.12^{c}	52.63±5.10^{a}	60.63±6.12^{a}	35.17±3.41^{b}	41.17±4.41^{b}
Phenylethyl alcohol	Rose, honey[1]	14[1]	201.16±19.43^{a}	242.63±22.73^{a}	245.14±26.18^{a}	249.67±24.07^{a}	243.81±23.58^{a}
∑Alcohols			3135.25±279.43^{a}	3582.89±323.30^{a}	3775.46±380.28^{a}	3311.73±305.22^{a}	3579.42±331.51^{a}
Methyl acetate			ND	13.93±1.30^{b}	15.67±1.51^{b}	23.25±2.16^{a}	25.17±2.32^{a}
Ethyl acetate	Pineapple, fruity, balsamic[2]	12[2]	729.35±74.28^{a}	668.56±64.86^{a}	760.26±71.34^{a}	726.78±70.86^{a}	810.59±78.50^{a}
Ethyl propionate	Fruity[4]	1.8[4]	18.65±1.56^{a}	17.24±1.55^{a}	19.18±1.68^{a}	15.43±1.62^{a}	16.22±1.55^{a}
Ethyl isobutyrate	Sweet, rubber[4]	0.015[4]	11.51±1.05^{a}	9.51±0.92^{ab}	11.11±1.06^{a}	7.77±0.74^{b}	8.95±0.78^{b}
Propyl acetate	Sweet, fruity[4]	4.7[4]	24.83±2.62^{a}	18.04±1.77^{b}	21.16±2.04^{ab}	19.21±1.78^{b}	22.04±2.04^{ab}
Isobutyl acetate	Fruity, apple, banana[4]	1.6[4]	42.19±3.84^{a}	30.40±3.13^{b}	34.23±2.99^{b}	23.79±2.24^{c}	25.64±2.82^{bc}
Ethyl butanoate	Banana, pineapple, strawberry[1]	0.4[1]	43.24±4.13^{a}	28.37±2.47^{b}	29.59±3.41^{b}	22.59±2.01^{b}	23.10±1.98^{b}
Butyl acetate	Fruity[5]		4.88±0.43^{a}	3.96±0.35^{ab}	4.37±0.56^{a}	3.35±0.36^{b}	3.84±0.33^{ab}
Isoamyl acetate	Banana[1]	0.16[1]	2472.35±242.56^{a}	1403.56±142.53^{b}	1541.19±136.04^{b}	871.05±82.60^{c}	972.42±88.09^{c}
Ethyl pentanoate	Yeast, fruity[4]	0.094[4]	4.24±0.36^{a}	2.89±0.27^{b}	3.62±0.35^{a}	4.19±0.40^{a}	4.63±0.51^{a}
Ethyl hexanoate	Banana, green apple[1]	0.08[1]	749.92±72.65^{a}	515.85±49.06^{b}	535.92±55.50^{b}	388.25±36.12^{c}	442.16±43.69^{bc}
Hexyl acetate	Apple, cherry, pear, floral[1]	1.5[1]	60.09±7.32^{a}	47.19±4.53^{b}	58.32±5.36^{a}	18.63±1.92^{c}	22.85±2.12^{c}
Ethyl heptanoate	Fruit[4]	0.22[4]	9.26±0.87^{a}	6.41±0.67^{b}	5.27±0.61^{bc}	4.04±0.38^{c}	4.36±0.41^{c}

Methyl octanoate	Orange[4]		34.89±3.57^{a}	40.63±3.88^{a}	43.31±4.10^{a}	35.28±3.11^{a}	41.49±3.89^{a}
Ethyl octanoate	Fruity, sweet, banana, pear[1,2]	0.24-0.58[1,2]	2552.69±226.39^{a}	1521.94±126.93^{b}	1668.65±171.03^{b}	890.86±82.62^{d}	1114.51±103.43^{c}
Geranyl acetate	Floral, rose[6]		96.95±9.32^{b}	120.62±11.05^{a}	102.99±10.23^{ab}	67.65±6.59^{c}	62.78±6.32^{c}
Ethyl nonanoate			44.32±4.34^{a}	32.19±3.36^{b}	33.67±3.42^{b}	36.19±3.54^{b}	35.20±3.17^{b}
Methyl decanoate	Wine[4]	1.2[4]	14.46±1.28^{a}	12.63±1.01^{a}	13.90±1.21^{a}	9.91±0.79^{b}	10.33±0.92^{b}
Ethyl decanoate	Fatty acids, fruity, soap[1,2]	0.2[1,2]	1840.95±156.98^{a}	878.86±90.09^{b}	957.66±92.06^{b}	464.04±42.22^{c}	504.38±48.56^{c}
Ethyl benzoate	Heavy, floral, fruity[4]	5.75[4]	315.49±33.65^{b}	577.75±46.60^{a}	589.05±54.98^{a}	606.73±61.17^{a}	631.40±56.77^{a}
Ethyl 9-decenoate	Fruity[4]	0.1[4]	231.95±24.25^{a}	32.81±3.14^{b}	27.38±2.67^{b}	7.50±0.73^{c}	5.53±0.50^{c}
Methyl salicylate	Pepper, mint[4]		11.38±1.14^{b}	15.12±1.87^{a}	16.64±1.52^{a}	12.13±1.10^{b}	12.44±1.39^{b}
Ethyl phenylacetate	Fruity, sweet[4]		2.03±0.15^{b}	1.88±0.23^{b}	2.08±0.19^{b}	3.26±0.33^{a}	3.41±0.31^{a}
2-Phenylethyl acetate	Fruity, rose[1]	1.8[1]	41.38±3.36^{a}	34.34±3.18^{a}	35.77±3.48^{a}	24.39±2.31^{b}	26.72±2.43^{b}
Ethyl dodecanoate	Oily, fatty, fruity[1]	1.5[1]	175.20±18.21^{b}	178.56±15.56^{b}	223.61±21.13^{a}	120.02±10.65^{d}	154.12±12.98^{c}
∑Esters			9532.22±894.31^{a}	6213.25±580.31^{b}	6754.61±648.47^{b}	4406.30±418.35^{c}	4984.28±465.81^{c}
Linalool	Flowery, muscat[1]	0.025[1]	731.91±71.03^{c}	1007.80±96.32^{b}	897.27±90.43^{bc}	1424.51±153.07^{a}	1369.53±128.25^{a}
α-Terpineol	Lilac, floral, sweet[1]	0.25[1]	135.46±12.63^{c}	184.23±16.70^{b}	159.49±14.17^{bc}	302.61±28.65^{a}	288.09±26.72^{a}
Citronellol	Rose[1]	0.1[1]	18.48±1.72^{c}	28.88±2.64^{b}	27.68±2.60^{b}	58.05±5.57^{a}	60.98±5.78^{a}
Geraniol	Citric, geranium[1]	0.02[1]	40.89±4.65^{b}	53.12±5.35^{a}	49.27±4.55^{ab}	56.91±5.43^{a}	51.64±5.33^{a}
∑Terpenes			926.75±90.03^{c}	1274.03±121.01^{b}	1133.72±111.75^{bc}	1842.09±192.72^{a}	1770.24±166.08^{a}

All data are expressed as mean±SD (n = 3).
Different letters in the same row indicate statistically significant differences at $p<0.05$.
SC sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *ND* not detected
[1] CAI *et al.*, 2014; [2] BUTKHUP *et al.*, 2011; [3] BARTOWSKY and HENSCHKE, 2004; [4] ZHANG *et al.*, 2015; [5] NATTAPORN and PRANEE, 2011; [6] NISHIMURA, 1995

3.6. Sensory evaluation of apricot wines

The sensory evaluation results of apricot wines are shown in Fig. 3. All the pectinase-treated apricot wines obtained higher color scores compared with control apricot wine, due to clarification by pectinase enzyme. The flavor scores of SCPC wine, containing the highest amount of total volatile higher alcohols, and PCP and PCPC wines, which recorded the greatest abundance of total volatile terpenes, were significantly higher relative to the other apricot wines. The sweetness scores of pectinase-treated apricot wines were slightly higher than control apricot wine because of some remaining free sugars. The sourness of PCP wine was the strongest, whereas that of SCPC wine was the weakest because these wines contained, respectively, the highest and lowest presence of tartaric acid and malic acid, which are the two strongest organic acids. PCPC wine also obtained low sourness score because of its low tartaric acid and malic acid levels. In the overall preference, SCPC and PCPC wines, having the most reduced sourness, obtained the highest scores among all the apricot wines. SCP and PCP wines also obtained higher scores when compared with SC wine.

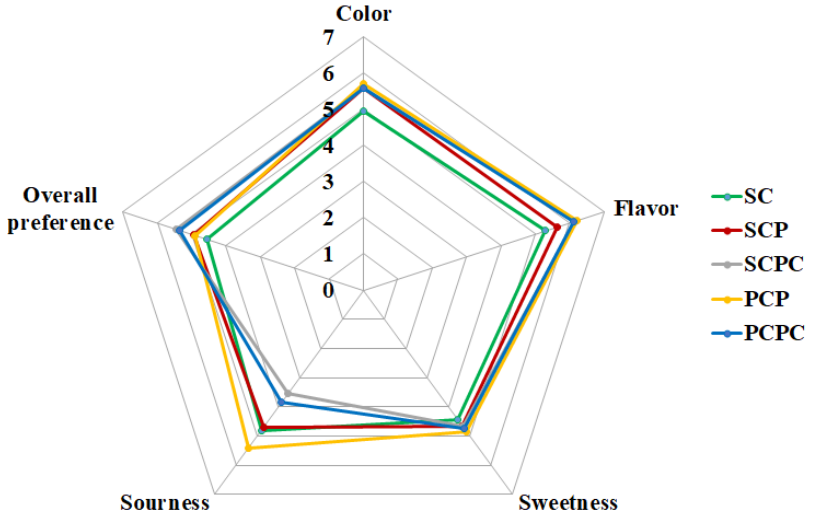

Figure 3. Sensory evaluation of apricot wines depending on different chaptalization types and pretreatment conditions.
SC sugar chaptalization, *SCP* sugar chaptalization treated with 0.1% pectinase, *SCPC* sugar chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$, *PCP* puree concentrate chaptalization treated with 0.1% pectinase, *PCPC* puree concentrate chaptalization treated with 0.1% pectinase and 0.3% $CaCO_3$

In this study, we investigated the effects of puree concentrate chaptalization and various pretreatments on the quality of apricot wine. The results demonstrated that apricot wines chaptalized with puree concentrate have shown not only higher antioxidant activity and total volatile terpene compounds than sugar-chaptalized apricot wines but also higher acidity that negatively affects the sensory properties of wine. Pectinase and $CaCO_3$ pretreatments can clarify the appearance apricot wines and reduce the acidity of apricot wines, indicating that combining puree concentrate chaptalization and various pretreatments may result to improved apricot wine quality.

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PAPER

METABOLOMIC PROFILING, ANTIOXIDANT, ANTIPROLIFERATIVE AND ANTIMICROBIAL ACTIVITY OF MEDEMIA ARGUN PALM

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ABSTRACT

Metabolomic profiling, antioxidant, anticancer and antimicrobial activity of three parts (leaves, male parts and fruits) of the *Medemia argun* palm were evaluated. Secondary metabolites content showed a significant difference among the evaluated parts. Multivariate data analysis (MVDA) classified the parts into three groups based on their metabolomic profiling and the total antioxidant capacity (TAC) of their extracts. The highest content of secondary metabolites, particularly in the leaves and fruits, was reflected in the DPPH radical scavenging activity and consequently in the IC_{50} of their extracts. The leaves and fruits extracts showed the lowest IC_{50}, followed by the male parts extract (62.8, 78.9 and 134.4 μg/ ml, respectively). Individual polyphenols were also determined by HPLC, which revealed the dominance of rutin, spigenin-7-glucoside, vanillic and rosmarinic acids, and kaempferol in the leaves. *p*-hydroxybenzoic, caffeic, syringic, sinapic, and cinnamic acids and chrysin were the dominant polyphenols in the male parts extract. Ferulic acid, luteolin and apigenin were the dominant polyphenols in the fruits extract. *Medemia argun* extracts showed very strong antiproliferative activity against hepatocellular carcinoma (HepG-2) and lung cancer cell lines (A549). Of the three parts that showed very strong antiproliferative activity, the male parts extract showed prominent antiproliferative activity against HepG-2, while the leaves extract showed more

prominent activity against A549 (IC_{50} was 0.587 and 1.038 μg/ml, respectively). The leaves and fruits extracts showed antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* (Gram-positive bacteria) and *Pseudomonas aeruginosa* (Gram-negative bacteria). The male parts showed moderate antibacterial activity only against *B. subtilis*. No extracts affected the growth of *Escherichia coli*, *Candida albicans* and *Aspergillus flavus*. The biological activity of the *M. argun* palm will be discussed in the light of secondary metabolites content in the plant. To the best of our knowledge, this is the first study concerning the biological activity of *M. argun*.

Keywords: antiproliferative activity, antimicrobial activity, DPPH, HPLC, *Medemia argun*, polyphenols

1. INTRODUCTION

Recently, the search for natural compounds as antioxidants from plant materials has become a topic of interest for scientists due to the safe nature and low cost of these sources (LINDENSCHMIDT *et al.*, 1986; GURIB-FAKIM, 2006); to avoid the utilisation of synthetic compounds which have negative side effects; and because of the very high costs of the available synthetic compounds (ODUJE *et al.*, 2016). Polyphenols is a group of natural plant compounds that showed very strong antioxidant activity against free radicals, which cause many oxidative processes (LANGLEY-EVANS, 2000; PANDEY and RIZVI, 2009). More than 2000 years ago, the consumption of food rich in antioxidant compounds such as polyphenols, anthocyanins, saponins and carotenoids was recommended in traditional Chinese medicine due to their health benefits (LANGLEY-EVANS, 2000; MOHAMED, 2009).

The *Medemia* palm tree is a rare and mysterious genus found in the south of Egypt and the north of Sudan. It has only one species (*M. argun*), which resembles *Hyphaene,* particularly in its leaf, flower and inflorescence morphology (IBRAHIM and BAKER, 2009).

There is a lack of information about the metabolomics and biological activity of *M. argun*. Very few studies have been conducted on *Medemia argun,* and most of these focused on the profiling of essential oils and the proanthocyanidin fraction of the fruits by targeted metabolomics analysis, which was unable to reveal the whole picture of the *Medemia* metabolome. The essential oils from different parts of the fruits (mesocarp and headspace of seeds) were profiled and the results indicated that there was a significant variation in essential oils among the evaluated parts of the fruits (HAMED *et al.*, 2012). Higher performance liquid chromatography and electrospray ionisation mass spectroscopy revealed that the *M. argun* nut is a rich source of proanthocyanidin (HAMED *et al.*, 2014). In another study, incubation of the proanthocyanidin fraction with blood platelets and plasma reduced the formation of 3-nitrotyrosine and diminished the oxidation of thiol groups, in addition to the reduction of the level of carbonyl groups in proteins caused by treatment with peroxynitrite (MOREL *et al.*, 2014). Masullo *et al.* (2016) investigated the butanol extract of *Medemia* fruits, revealing the presence of eight compounds.

Neither a complete picture of *Medemia* metabolome of different parts such as leaves, fruits and male parts, nor the biological activity of these parts is evaluated. The objective of this study is to evaluate different parts such as leaves, male parts and fruits for their metabolomic content using targeted and non-targeted analysis in combination with MVDA, and to assess their biological activity against different microorganisms and against human carcinogenic cell lines.

2. MATERIALS AND METHODS

2.1. Plant materials

Parts of the *M. argun* palm (leaves, male parts and fruits) were collected from Aswan University desert garden. They were dried, separately ground into powder and stored in closed containers until used.

2.1.1 Plant extraction

100 mg of dried plant materials was dissolved in 4 ml of methanol-water (80%). The mixture was vortexed for one min and then placed in a 60ºC water bath for 1 h. The mixtures were centrifuged at 800 rpm for 10 min and the supernatants were used for determination of secondary metabolites such as saponins, phenolics, flavonoids, flavonols and tannins.

2.2. Plant analysis

2.2.1 Determination of carbohydrates

Carbohydrates were determined where the absorbance was read at 620 nm with a spectrophotometer (Thermo Spectronic Genesys 5) (MORRIS, 1948). The concentrations of carbohydrates in different parts of *M. argun* were calculated and expressed as mg/ g DW.

2.2.2 Determination of anthocyanins

Plant samples were dissolved in acidified methanol in brown tubes or in well-closed tubes covered with aluminum foil and incubated at +4ºC for 24 h (PADMAVATI *et al.,* 1997). The absorbances of the supernatants after centrifugation were recorded at 530 nm and 657 nm. The anthocyanins content was calculated using the following equation:

$$\text{Anthocyanin concentration } (\mu\text{mol/ g}) = ([A530 - 0.33 \times A657]/31.6) \times (\text{volume [ml]}/\text{weight [g]}).$$

2.2.3 Determination of saponins

Saponins were determined using vanillin reagent and the absorbance of samples and standard was read at 473 nm. Total saponins content was expressed as mg saponins equivalent (mg SE/ g extract) (EBRAHIMZADEH and NIKNAM, 1998).

2.2.4 Determination of total phenolics content

Folin-Ciocalteu reagent was used to determine total phenolics content in different parts of *M. argun*. The absorbance of samples and standard (gallic acid) was read at 700 nm (SINGLETON *et al.,* 1999). Total phenolics content was expressed as mg gallic acid equivalent (mg GAE/ g extract).

2.2.5 Determination of flavonoids

Aluminium chloride was used to determine flavonoids content in different parts of *M. argun*. The absorbance of samples and standard (quercetin) was measured at 510 nm (ZHISHEN *et al.,* 1999). The content of flavonoids was expressed as mg quercetin equivalent (mg QE/ g extract).

2.2.6 Determination of flavonols

Flavonols content was determined spectrophotometrically using aluminium chloride and sodium acetate. The absorbance of samples and standard (quercetin) was read at 440 nm (KUMARAN and KARUNAKARAN, 2007). The content of flavonols was expressed as mg of quercetin equivalent (mg QE/ 100 g extract).

2.2.7 Determination of total tannins

Tannins content was determined using vanillin reagent. The absorbance of samples and standard (catechol) was read at 550 nm (JULKUNEN-TITTO, 1985). The amount of total tannins was expressed as mg of catechol equivalent (mg CE/ g extract).

2.2.8 Determination of total antioxidant capacity (TAC) and 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging activity assay

Total antioxidant capacity and DPPH radical scavenging activity were estimated based on PRIETO *et al.* (1999) and BLOIS (1958), respectively. The experimental details of TAC and DPPH radical scavenging activity were reported (ABDEL-FARID *et al.,* 2014). DPPH radical scavenging activity (%) of different concentrations of the plant crude extracts was calculated from the equation: %DPPH = (A_0-A_s) / A_0 X 100, where A_0 is the absorbance of the control and A_s is the absorbance of the evaluated sample. Then the % inhibitions were plotted against concentrations and IC_{50} was calculated from the graph. The experiment was performed in triplicate and average absorption was recorded for each concentration.

2.3. Profiling of polyphenols in different parts of *M. argun* using HPLC

HPLC analysis was performed using Agilent Technologies 1100 series liquid chromatography equipped with an auto sampler and a diode-array detector. The mobile phase was formed from acetonitrile (solvent A) and acetic acid in water (2% v/v) (solvent B). 0.45 μm Acrodisc syringe filters (Gelman Laboratory, MI) were used to filtrate samples before injection. 20 μl injection volume, 0.8 ml/ min flow rate and 60 min run time was used with the following gradient programme: 100% B to 85% B for 30 min, 85% B to 50% B for 20 min, 50% B to 0% B for 5 min and 0% B to 100% B for 5 min. Simultaneously, peaks were monitored at 280, 320 and 360 nm and identified by congruent retention times and UV spectra with comparison with the standards (KIM *et al.*, 2006; MANSOUR *et al.*, 2018).

2.4. Biological activity

2.4.1 Antiproliferative activity

2.4.1.1 Cancer cell lines, medium and in vitro antiproliferative activity

Human hepatocellular carcinoma HepG-2 and lung carcinoma cell lines (A549) were obtained from an American culture collection. PRMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2 ml glutamine containing 100 U/ml streptomycin and 100 U/ml penicillin at 37ºC/5% CO_2 were used for cell culturing. From each *M. argun* extract, several concentrations were prepared in 1% DMSO (0.049, 0.098, 0.195, 0.391, 0.781, 1.56, 3.13, 6.25, 12.5 and 25 μg/ ml).

2.4.1.2 Determination of inhibition concentration 50% (IC_{50}) for Medemia palm extracts using sulforhodamine B (SRB) colorimetric assay

The antiproliferative activity of *M. argun* extracts was assessed using SRB assay based on Vichai and Kirtikara (2006). The antiproliferative test procedures, from cell harvesting to measurement of colour intensity using a microplate reader and calculation of IC_{50} of each extract, were described in VICHAI and KIRTIKARA, 2006 and EL-NAGGAR *et al.*, 2015.

2.4.2 Antimicrobial activity

Antimicrobial activity of the evaluated extracts was determined using disc diffusion assay (BAUER, *et al.*, 1966). Discs were impregnated with the stock solutions of the evaluated parts of *M. argun*, where each disc received 500 µg from the *Medemia* extracts. Positive control was also prepared where each disc received 200 µg of ampicillin and amphotericin (antibacterial and antifungal agents), respectively. Mueller-Hinton agar plates were inoculated with Gram (+) bacteria (*Staphylococcus aureus* and *Bacillus subtilis*); Gram (-) bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were incubated at 35-37ºC for 24-48 h. *Candida albicans* was incubated at 30ºC for 24-48 h and filamentous fungi as *Aspergillus flavus* was incubated at 25ºC for 48 h (BAUER *et al.*, 1966). The inhibition zone was measured in millimetres, each experiment was repeated three times, and the average of three readings of the diameters of the inhibition zones was calculated.

2.5. Statistical analysis

Spectrophotometer data was subjected to multivariate data analysis (MVDA) such as principal component analysis (PCA) and hierarchical clustering analysis (HCA) using SIMCA-P software (version 12.0). The statistical differences among the content of secondary metabolites in different parts of *M. argun* were evaluated using analysis of variance (ANOVA) from Minitab (version 12.21). The correlation between the determined metabolites in different parts and TAC was assessed using Pearson's correlation test. Data was presented as the average of three readings ± the standard deviation.

3. RESULTS

3.1. Phytochemical analysis and metabolomic profiling of different parts of *M. argun* (argun palm) using spectrophotometer and HPLC combined with multivariate data analysis

The metabolites content of different parts (leaves, male parts and fruits) of *M. argun* is shown in Table 1. The flavonols content differed significantly in the leaves and fruits ($P<0.05$). Carbohydrates showed no significant difference among the evaluated parts ($P>0.05$). Fruits were characterised by a higher content of total saponins, total phenolics, total flavonoids and total condensed tannins. Phenolics and flavonoids content showed significant differences among the evaluated parts, while for total saponins and tannins content the only significant difference was observed between the leaves and fruits and the male parts and fruits (Table 1). Male parts were characterised by higher anthocyanins content, which showed a significant difference among the evaluated parts ($P<0.05$).

Table 1. Phytochemical analysis, total antioxidant capacity, DPPH radical scavenging activity and IC_{50} of different parts of *Medemia argun* palm.

Metabolites	Leaves	Male parts	Fruits
Carbohydrates (mg/g DW)	124.5±0.2[a]	122.9±0.05[a]	122.2±0.41[a]
Saponins (mg saponins equivalent/g)	15.3±0.7[a]	16.2±0.7[a]	18.7±0.04[b]
Phenolics (mg/ gallic acid equivalent/ g)	13.3±0.9[a]	36.04±0.06[b]	72.3±3.9[c]
Flavonoids (mg quercetin equivalent/ g)	13.3±0.9[a]	27.6±3.6[b]	43.1±4.4[c]
Flavonols (mg quercetin equivalent/ 100 g)	76.12±1.2[a]	67.9±9.35[ab]	45.2±7.0[b]
Anthocyanin (μmole/ g)	0.34±0.04[a]	0.41±0.01[b]	0.26±0.02[c]
Tannins (mg catechols equivalent/ g)	2.28±0.45[a]	2.47±0.09[a]	5.53±0.5[b]
TAC (ascorbic acid equivalent μg/ g)	0.305±0.007[a]	0.285±0.032[a]	0.153±0.006[c]
DPPH (100 μg/ ml)	85.7±1.34[a]	37.5±4.9[b]	72.5±0.42[c]
IC_{50} (μg/ ml)	62.8±9.9[a]	134.4±3.5[b]	78.9±2.9[a]

Different letters in the same row means significant difference at $P<0.05$.

To reduce the dimensionality of the metabolomic data and to evaluate the similarity and dissimilarity between different parts of *M. argun* from the perspective of their phytochemical composition and metabolomic profiling, the data of the three parts of *M. argun* was subjected to PCA, followed by HCA. PCA separated the parts into three groups: leaves, fruits and male parts, as shown in the score scatter plot of PC1 vs. PC2 (Fig. 1A). The score scatter plot also showed that there is a similarity between the leaves and male parts resulting from the approximation of the two parts in the negative part of PC1 (left hand side of the ellipse) (Fig. 1A). The score loading plot (Fig. 1B) revealed the metabolites contributed to the separation obtained in the score scatter plot.

Leaves had higher content of carbohydrates, flavonols, TAC and DPPH radical scavenging activity. Fruits had higher content of saponins, tannins, flavonoids and phenolics. The male parts had higher content of anthocyanins. The score biplot confirmed the results of the PCA score scatter and score loading plots (Fig. 2A). HCA showed the classification of the three parts in three groups with a similarity between the leaves and male parts (Fig. 2B).

Metabolomic profiling of individual polyphenols in different parts of the *M. argun* palm was evaluated using HPLC. 24 standards were injected and their peaks are shown in Fig. 3. In the evaluated parts, 18 individual polyphenols were detected and their concentrations were determined (Table 2). Rutin, apigenin-7-glucoside, kaempferol, and rosmarinic and vanillic acids were the dominant polyphenols in the leaves extract of *M. argun*. Caffeic, *p*-hydroxybenzoic, sinapic, syringic, and cinnamic acids and chrysin were the dominant polyphenols detected in the male parts extract, where ferulic acid, apigenin and luteolin were the dominant polyphenols in the fruits (Table 2).

Metabolomic profiling of individual polyphenols in different parts of *M. argun* palm was evaluated using HPLC. 24 standards were injected and their peaks are shown in Fig. 3. In the evaluated parts, 18 individual polyphenols were detected and their concentrations were determined (Table 2). Rutin, apigenin-7-glucoside, kaempferol, rosmarinic and vanillic acids were the dominant polyphenols in leaves extract of *M. argun*. Caffeic, *p*-hydroxybenzoic, sinapic, syringic, cinnamic acids and chrysin were the dominant polyphenols detected in male parts extract, where ferulic acid, apigenin and luteolin were the dominant polyphenols in fruits (Table 2).

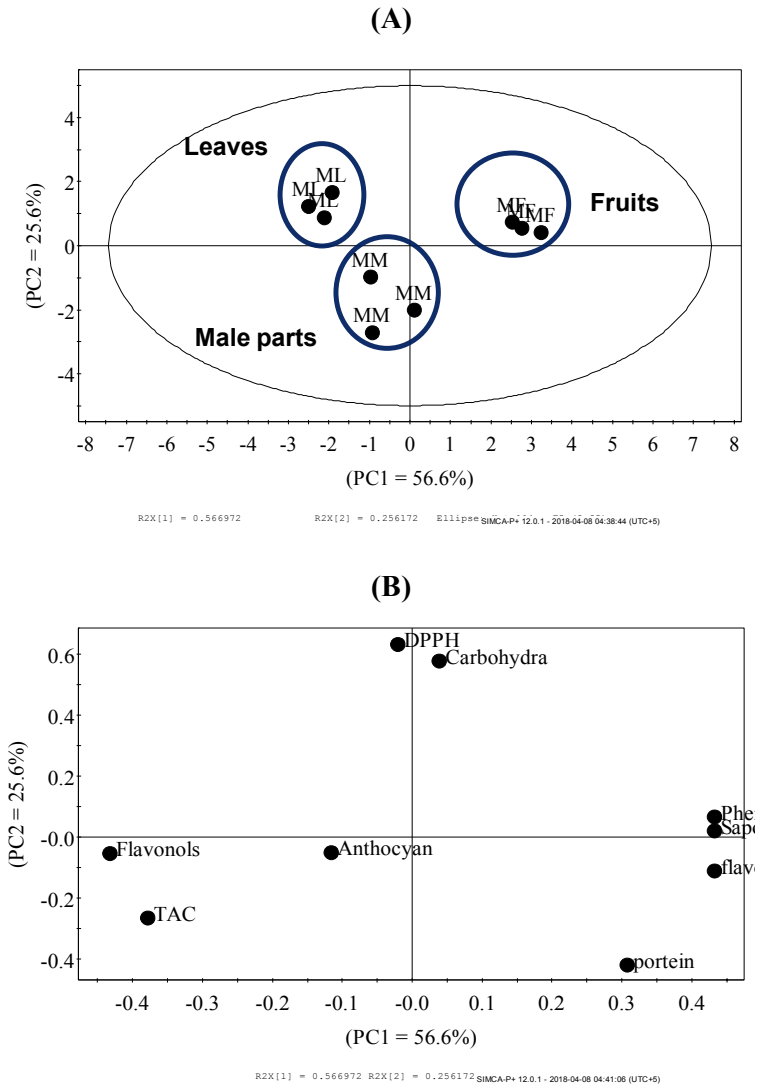

Figure 1. Score scatter plot of PC1 vs. PC2 (A), and the score loading plot of PC1 vs. PC2 of the metabolomic profiling of different parts of *Medemia argun* palm (B). ML = *Medemia argun* leaves, MM = *Medemia argun* male parts and MF = *Medemia argun* fruits.

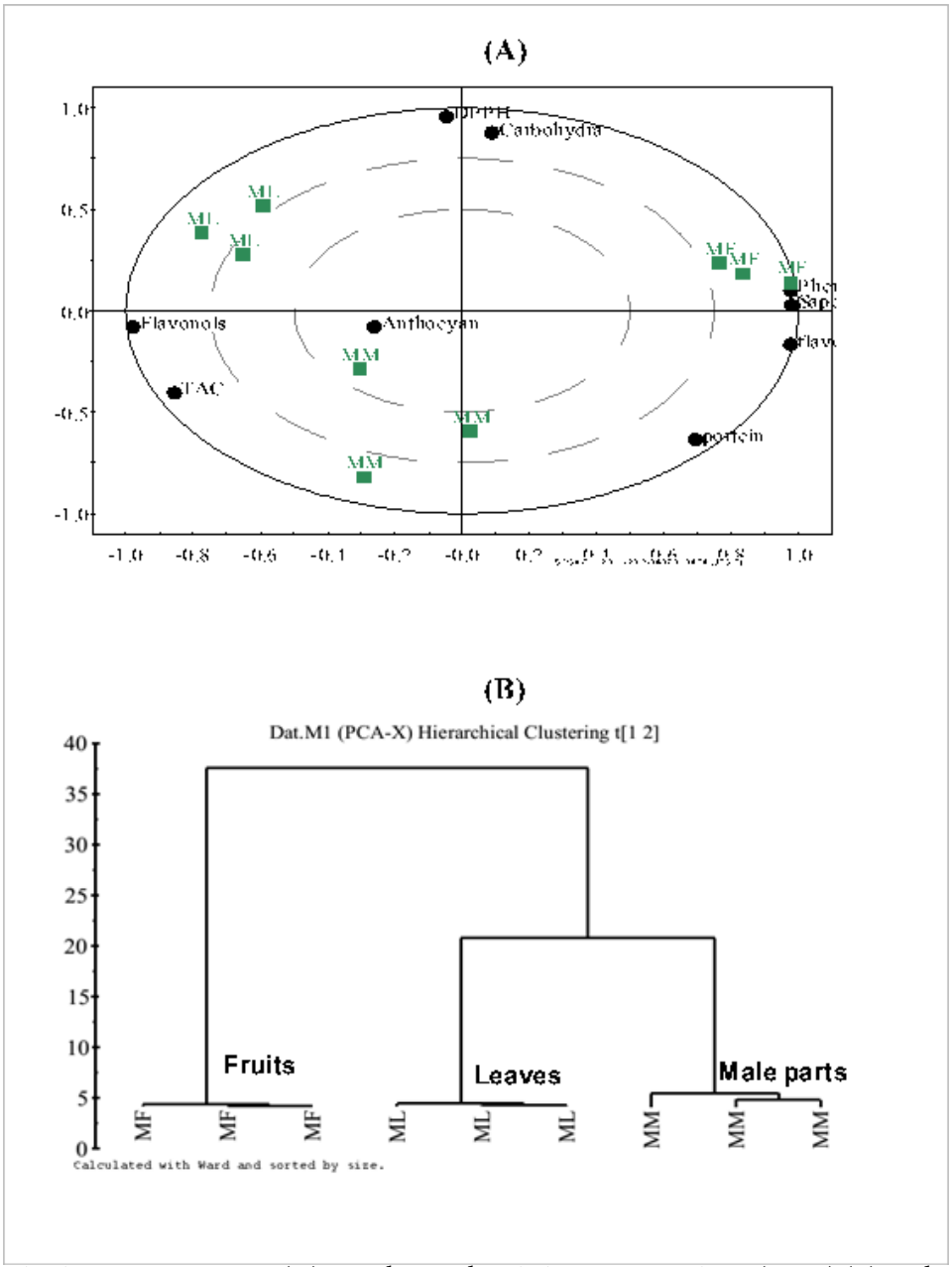

Figure 2. Score biplot of PC1 vs. PC2 (A), and hierarchical clustering analysis (HCA) (B) of the metabolomic profiling of different parts of *Medemia argun* palm. Labeling of each group is the same as in Fig. 1.

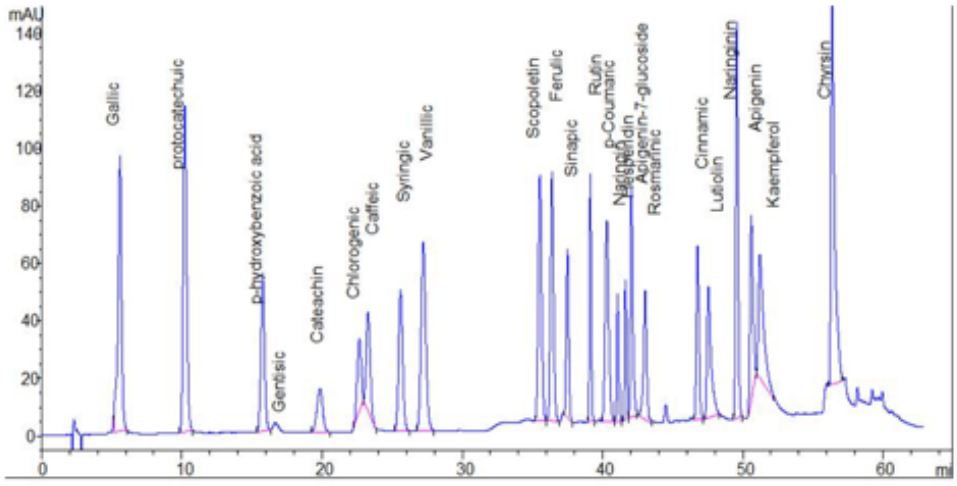

Figure 3. HPLC chromatogram of 24 polyphenols standards at 280 nm (TAHA *et al.,* 2020).

Table 2. Individual polyphenols (mg/ g) in different parts of *M. argun* palm.

	Leaves	Male parts	Fruits
Protocatechuic acid	0.167	0.152	ND
p-hydroxybenzoic acid	0.158	0.371	0.110
Catechins	0.316	0.295	ND
Chlorogenic acid	0.071	ND	ND
Caffeic acid	ND	0.206	ND
Syringic acid	0.112	0.267	0.081
Vanillic acid	0.066	ND	0.05
Ferulic acid	0.05	0.010	0.033
Sinapic acid	ND	0.179	0.169
Rutin	12.55	2.352	0.538
p-coumaric acid	0.821	0.722	0.70
Apigenin-7-glucoside	2.85	0.288	0.255
Rosmarinic acid	0.631	0.127	0.111
Cinnamic acid	0.012	0.088	0.017
Luteolin	ND	ND	0.033
Apigenin	ND	0.079	0.229
Kaempferol	0.211	0.131	0.073
Chrysin	ND	0.044	ND

ND = non-detectable.

3.2. Antioxidant, DPPH free radical scavenging activity and IC_{50} of *M. argun* palm

DPPH values differed significantly among the evaluated parts ($P<0.05$). TAC showed a significant difference between the leaves and fruits and the male parts and fruits (Table 1). The leaves and fruits had the highest values of DPPH radical scavenging activity (85.7 and 72.5%, respectively), whereas the male parts showed the lowest percentage (37.5%). The richness of leaves and fruits in bioactive metabolites was reflected in the DPPH radical scavenging activity and also in the IC_{50} of the extracts under evaluation. The leaves and fruits showed the lowest IC_{50} compared to the male parts (62.8, 79.9 and 134.4 μg/ ml, respectively) (Table 1).
The higher the free radical scavenging activity, the lower the IC_{50} and vice versa. This means that leaves and fruits are more active than male parts as antioxidant agents.
Pearson's correlation was performed to assess the relation between the estimated metabolites and TAC in the three evaluated parts of the *M. argun* palm. TAC positively correlated with the total flavonols and anthocyanins content ($P<0.05$), and r values: 0.799 and 0.913, respectively.

3.3. Antiproliferative activity of different parts of *M. argun*

The results of the IC_{50} of different parts of the *M. argun* palm are shown in Table 3. The three evaluated parts of *M. argun* showed very strong antiproliferative activity against hepatocellular carcinoma (HepG-2) and lung cancer cell lines (A549). The male parts of the *M. argun* palm showed the lowest IC_{50} against HepG-2 cell lines (0.587 μg/ ml), followed by the fruits and leaves extracts with IC_{50}: 1.247 and 1.476 μg/ ml, respectively. The leaves extract of *M. argun* was the strongest antiproliferative extract against lung carcinoma cell lines (A549) with the lowest IC_{50} (1.038 μg/ml), followed by the male parts and fruits extracts (IC_{50}: 2.369 and 3.551 μg/ ml, respectively) (Table 3). The dead cells in both cell lines incubated with *M. argun* were dose dependent as they increased with the concentrations used (Fig. 4). Although all extracts showed very strong antiproliferative activity against hepatocellular carcinoma and lung cancer cell lines, the effect of the male parts and fruits extracts was more pronounced than that of the leaves extract on HepG-2 cell lines, and the leaves and male parts extracts exerted stronger antiproliferative activity against lung cancer cell lines than the fruits extract (Table 3 and Fig. 4). Hepatocellular carcinoma (HepG-2) revealed more susceptibility to *M. argun* extracts than lung cancer cell lines (A549) (Fig. 4).

Table 3. IC_{50} (μg/ ml) of *M. argun* extracts against hepatocellular carcinoma (HepG-2) and lung cancer cell line (A549).

	HepG-2	A549
Leaves	1.476	1.038
Male parts	0.587	2.369
Fruits	1.247	3.551

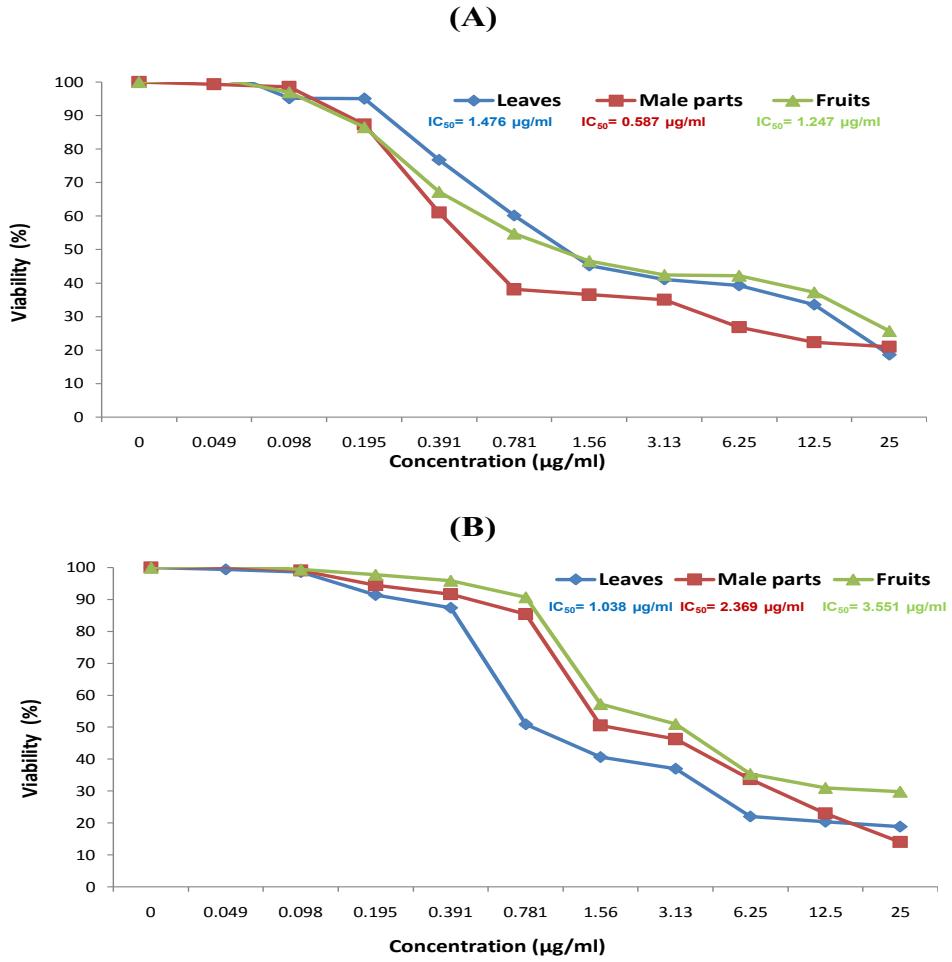

Figure 4. The viability percentage (%) of different parts of *Medemia* palm extracts against hepatocellular carcinoma HepG-2 (A) and lung cell line carcinoma A549 (B). Each experiment was repeated three times.

3.4. Antimicrobial activity of different parts of *M. argun*

The antimicrobial activity of different parts of an *M. argun* palm was evaluated using paper diffusion disc assay. Only the leaves and fruits extracts of *M. argun* exerted antimicrobial activity against *B. subtilis, S. aureus* and *P. aeruginosa* (Table 4). The effect of the fruits extract was more prominent than the leaves extract on Gram-positive bacteria. The leaves extract showed more effect than the fruits extract against *P. aeruginosa* (Table 4). Gram-positive bacteria showed more susceptibility than Gram-negative bacteria to the methanol extracts of the *M. argun* palm (Table 4). Only the male parts extract showed antibacterial activity against *B. subtilis* and no extract from the evaluated parts showed antimicrobial activity against *C. albicans* and *A. flavus*, meaning that fungal strains had more resistance than bacterial strains against *M. argun* palm extracts.

Table 4. Antimicrobial activity (in term of clear zones) of different parts of *M. argun*.

		Zone of inhibition (mm)			
		Control	**Leaves**	**Male parts**	**Fruits**
Gram +ve bacteria	*Bacillus subtilis*	28.0±2.0	11.3±2.5*	9.0±0.0*	12.7±0.06*
	Staphylococcus aureus	31.0±6.5	12.3±2.3*	0.0±0.0*	12.3±2.3*
Gram -ve bacteria	*Pseudomonas aeruginosa*	32.4±1.1	12.7±1.5*	0.0±0.0*	11.0±2.0*
	Escherichia coli	29.0±1.7	0.0±0.0*	0.0±0.0*	0.0±0.0*
Fungi	*Aspergillus flavus*	14.6±1.5	0.0±0.0*	0.0±0.0*	0.0±0.0*
	Candida albicans	16.3±1.1	0.0±0.0*	0.0±0.0*	0.0±0.0*

+ Control is ampicillin as antibacterial agent and amphotericin B as antifungal agent. Each disc was impregnated with 500 μg from each extract; whereas the disc of positive control was impregnated with 200 μg from each control. The data presented is a mean of 3 replicates with the standard deviation. *mean there a significant difference between the diameter of clear zones with the plant extracts and that of the positive control at $P<0.05$.

4. DISCUSSION

Under normal conditions, plants produce many bioactive water soluble secondary metabolites such as polyphenols, terpenoids, steroids, alkaloids, saponins, glucosinolates, isothiocyanates and tannins (KRUSE et al., 2000; DIXON, 2001). These metabolites are very important not only for a plant itself for its defense against pathogens and herbivores and tolerance for different biotic and abiotic stresses (PANDEY and RIZVI, 2009), but also for humans, as the metabolites are the main source of medicines as antioxidant and free radical scavenging activities for different oxidative stress linked diseases (OLAJUYIGBE and AFOLAYAN, 2011). Polyphenols are groups of secondary metabolites such as phenolic and cinnamic acids, flavonoids, anthocyanins, stilbene and lignans; they are considered as potentially health beneficial antioxidants. Consumption of a polyphenol-rich diet provides protection against a series of dangerous diseases such as cancers, diabetes, cardiovascular diseases, neurodegenerative disease and osteoporosis (PANDEY and RIZVI, 2009).

To assess the biological activities of plant extracts, the phytochemical and metabolomic profiling of plants is very important in attributing any biological activity to the plants' metabolites content. To reveal the whole picture of metabolomics of a given plant, many analytical techniques should be used in combination with MVDA. *M. argun* has received very little attention from researchers, and search engine queries for the plant found only five articles that focus only on the targeted phytochemical studies of *M. argun* (HAMED *et al.,* 2012, 2014, MOREL *et al.,* 2014; MASULLO *et al.,* 2016; SAID *et al.,* 2017). The significant variations in the bioactive secondary metabolites content in different parts of *M. argun* were in line with many previous reports, which showed clearly that each part of a given plant has its own phytoanticipins and metabolomic content (HAMED *et al.,* 2012; ABDEL-FARID *et al.,* 2014; TAHA *et al.,* 2020).

The highest percentages of DPPH free radical scavenging activity of the leaves and fruits of *M. argun* can probably be attributed to the richness of these parts in bioactive secondary metabolites such as polyphenols, saponins and tannins, as explored by spectrophotometer

and HPLC analysis. The positive correlation between TAC and DPPH free radical scavenging activity and secondary metabolites content, particularly polyphenols, was well documented in many previous studies (BASAR *et al.*, 2013; ABDEL-FARID *et al.*, 2014; DIACONEASA *et al.*, 2015; MANSOUR *et al.*, 2016).

The highest content of these previously mentioned secondary metabolites not only affected the DPPH radical scavenging activity and TAC, they also positively affected the antiproliferative activity of *M. argun* extracts. The cytotoxic activity of some Saudi and Egyptian desert plants against hepatocellular, breast and lung cancer cell lines was attributed to the highest content of these secondary metabolites in their extracts (ALENAD *et al.*, 2013; EL-NAGGAR *et al.*, 2015; TAHA *et al.*, 2020). The individual polyphenols in *M. argun* extracts such as rutin, catechin, kaempferol, rosmarinic, vanillic, rosmarinic, caffeic, *p*-hydroxybenzoic, protocatechuic, sinapic, syringic, ferulic, cinnamic acids, apigenin-7-glucoside, luteolin, apigenin and chrysin were well known as anticancer agents that worked individually or in combination (synergistic effect) (FUCHS and MILBRADT, 1993; VELIKA and KRON, 2012; FONSECA *et al.*, 2015; WILKINS *et al.*, 2017; CHANDRA and VISWANATHSWAMY, 2018; YAMAGATA *et al.*, 2018). The interaction of some of these active secondary metabolites with cancer-associated receptors seems to trigger specific mechanisms and consequently causes the death of cancer cells (WANG *et al.*, 2008).

The antimicrobial activity of *M. argun* was also attributed to its active metabolites content, particularly polyphenols. The suppression of proteases and/or inactivation of the microbial adhesions may be the mechanism of polyphenols toxicity against microorganisms (COWAN, 1999). The correlation between the content of secondary metabolites such as polyphenols and saponins and the antimicrobial potentiality was reported (CISOWSKA *et al.*, 2011; ALVES *et al.*, 2013; NAYAKA *et al.*, 2014; GULL *et al.*, 2015). The different responses of Gram-negative and -positive bacteria to *M. argun* extracts may be due to the nature of the cell wall, which varies between Gram-positive and -negative bacteria. Not only the type of bacteria but also the metabolites content of the plant part used and also the extraction method and the extraction solvent used may control the response of bacteria to the extract (GULL *et al.*, 2015). *M. argun* extracts showed no antifungal activity. Similar to these results, the proanthocyanidin fraction isolated from the *M. argun* nut has not affected the growth of *Cephalosporium gramineum* (MARTYNIUK *et al.*, 2017).

5. CONCLUSION

M. argun has high bioactive secondary metabolites content, such as polyphenols (with its different classes) and saponins, which were affected positively on potentiality against hepatocellular carcinoma and lung cancer cell lines. The highest content of these metabolites was reflected in the antiproliferative activity of *M. argun* against carcinogenic cell lines, and it also extended to its TAC and free radical scavenging activity. Moreover, the high secondary metabolites content, particularly polyphenols in the *M. argun* palm, was affected positively on potentiality as antibacterial activity. *M. argun* will be a promising plant in future for industrial and pharmaceutical medicines. Pharmacological and pharmaceutical studies are required in which separated individual polyphenols as well as plant extracts should be tested against different carcinogenic cell lines in vitro and in vivo in animals in amelioration experiments. The effect of *M. argun* extracts on diabetes in mice is also desirable.

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PAPER

PROPERTIES OF STRAWBERRIES PUREE STORED IN THE FREEZER

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ABSTRACT

Four different strawberry varieties were used for puree production and stored at -18 ºC for 12 months. Every three months samples were tested for rheological parameters, polyphenols content and antioxidant activity. Mathematical models for rheological behaviour of the samples were determined together with consistency coefficient (k) and flow behaviour index (n). Fluidity of the samples increased over time, but pseudoplastic behaviour remained through tested period. The biggest decrease of polyphenol content was observed between 9 and 12 months of storage, while antioxidant activity decreased the most during first three months by DPPH and ABTS method.

Keywords: antioxidant activity, polyphenols, rheology, strawberries

1. INTRODUCTION

Current guidelines for fruit and vegetable consumption recommend five portions per day (HARTMANN *et al*., 2008). Due to seasonal character of many fruits, as well as hectic lifestyle, these recommendations are not easily achievable especially when targeting fresh fruit and vegetables. Thus, replacement of one or several of portions by fruit juices, concentrates or purees is suggested (HARTMANN *et al*., 2008). Consumers have increased interest to high-value food products, especially fruit, vegetables and other functional foods (BISHARAT *et al*., 2013). Therefore, food manufacturers are facing the challenge to create new products which, beyond basic nutrients, also provide certain health-promoting properties (OBRADOVIĆ *et al*., 2015). Strawberries are one of the most consumed berries worldwide. In European market average strawberries intake is 2.16 kg/year per person including raw and processed fruit (GASPEROTTI *et al*. 2015). These popular fruits are favoured for their attractive taste, and are considered as rich source of micronutrients and phytochemical compounds such as water soluble vitamin C and polyphenols (phenolic acids, anthocyanins, flavonols, tannins and other) (KLOPOTEK *et al*., 2005; OSZMIAŃSKI *et al*., 2009; BODELÓN *et al*., 2013; ŻEBROWSKA *et al*., 2019). Diet rich in fruit and vegetables is beneficial for human body. It lowers the risk of many diseases: diabetes, atherosclerosis, cardiovascular disease, inflammatory-related illnesses, and cancer. This is attributed to vitamins, dietary fiber and polyphenols (NOWICKA *et al*., 2019). Strawberries have very strong antioxidant activity. They have 1.3 times activity of oranges, 2 times that of red grapes, 5 times that of apples and bananas and 13 times that of honeydew melon (OSZMIAŃSKI *et al*., 2009). For all mentioned reasons, strawberries are considered as a functional food although exact mechanism involved is still generally unclear (GASPEROTTI *et al*., 2015). Unfortunately, strawberries are very perishable due to high water content and soft structure, and consequently have extremely short postharvest shelf-life (HOLZWARTH *et al*., 2012; PEINADO *et al*., 2012). Therefore, there is a huge demand for strawberries puree for use as a base product for preparation of juices and soft drinks, for addition to ice-creams and yoghurts (BODELÓN *et al*., 2013), or it can be sold directly to consumers in canned or frozen forms (DIAMANTE *et al*., 2016). Purees are usually preserved by freezing or by heat. Freezing results in cell destruction allowing reactions between genuine enzyme activities and their corresponding substrates. Thawing is especially critical since polyphenoloxidases (PPO) are responsible for polyphenols destruction (HOLZWARTH *et al*., 2012). In order to create puree of satisfying quality and nutritional value it is necessary to determine optimal storage time. Therefore, the objective of this study was to explore influence of freezing on strawberry purees. Rheological properties, polyphenol content and antioxidant activity were evaluated. Changes in the rheological properties of fruit purees that have undergone freezing or freeze–thaw treatments are of practical significance for their acceptance and consumption (DIAMANTE *et al*., 2016). Reduction in antioxidant activity during processing and storage may reduce the health beneficial effects of food products (OSZMIAŃSKI *et al*., 2009) and that was the reason for test puree samples for above mentioned parameters. Some works have reported the rheological characterization of different fruits like mango and papaya (EL-MANSY *et al*., 2005), blueberry (ANTONIO *et al*., 2007, NINDO *et al*., 2007), raspberry, strawberry, prune, peach (MACEIRAS *et al*., 2007, ERGOVIĆ RAVANČIĆ *et al*., 2012), nectarine and blackberry (ERGOVIĆ *et al*., 2009; ERGOVIĆ *et al*., 2010), but to the best of our knowledge they haven't followed rheological parameters together with nutritional characteristics over a period of time in order to determine how storage time in the freezer affects mentioned characteristics.

2. MATERIALS AND METHODS

2.1. Sample preparation

Sample S-1 was prepared from wild strawberries harvested in woods near Požega town, Slavonia region, Croatia. Strawberries for sample S-2 (Albion variety) and S-3 (Clery variety) were purchased from the local farmers and for sample S-4 (Joly variety) in the local supermarket. Fruits were cleaned and blended in kitchen blender at room temperature for 3 minutes, divided in small portions (100 mL), sealed in polyvinyl chloride freezer bags (at atmospheric pressure and temperature, vacuum or modified atmosphere haven't been used) and kept in chamber freezer at -18 ºC, until the analysis. Every three months three bags were thawed at room temperature as parallels for the analysis.

2.2. Sugar and acidity determination

Total and reducing sugars were determined according to the Luff-Schoorl method (GAFTA, 2018). Total acidity was determined by potentiometric titration.

2.3. Extract preparation

1 g of strawberry puree was extracted with 20 mL of acidified methanol (methanol/2% HCl, 95:5) at room temperature for 60 min with constant shaking in temperature-controlled shaker (Kottermann labortechnik) at 200 rpm and centrifuged (Tehtnica, Centric 322A). Glasses were covered with aluminium folium to prevent evaporation of **solvent.**

2.4. Total phenol content

Polyphenols were determined according the Folin-Ciocalteu method (OBRADOVIĆ *et al*. 2015, with modifications). An aliquot of the extract (200 μL) was mixed with 2 mL water and 100 μL Folin-Ciocalteu reagent (Kemika, Croatia). The mixture was allowed to equilibrate for 5 min, and then 300 μL of sodium carbonate solution (20%) was added. After incubation at room temperature in dark for 30 min, the absorbance of the mixture was read at 725 nm (Camspec M501, UK). Acidified methanol was used as a blank. Total polyphenols were determined with 3 replications. Gallic acid (Carlo Erba reagents, Italy) was used as a standard (calibration curve $y = 1.1979x - 0.0188$, $R^2 = 0.9984$), and results were expressed in mg of gallic acid equivalents per 100 g of sample.

2.5. Antioxidant activity determination (ABTS)

$ABTS^{\cdot+}$ radical was obtained by mixing 7.4 mM ABTS (Fluka, Switzerland) solution and 2.6 mM solution of ammonium persulfate in 1:1 ratio. Solution was left in dark through the night in order to develop stable radical, and then radical solution was diluted with ethanol in 2:70 ratio to obtain absorbance approximately 1.100 (A_{ABTS}). An aliquot of extract (0.2 mL), was mixed with 3.2 mL of diluted $ABTS^{\cdot+}$ radical. After incubation at room temperature in dark for 95 min, the absorbance of the mixture was read at 734 nm (A_{EXTR}), and ΔA was calculated as $A_{ABTS} - A_{EXTR}$. Trolox (Sigma Aldrich, USA) was used as a standard. Decrease in absorbance caused by trolox was done in the same way as for the samples, and standard curve ΔA/trolox concentration was created ($y = 489.13x - 17.903$, $R^2 = 0.9952$).

Determination of antioxidant activity was done in 3 replications. Results were expressed in μmol of the trolox equivalents per gram (OBRADOVIĆ *et al*., 2015).

2.6. Antioxidant activity determination (DPPH)

An aliquot of extract (50 μL) was mixed with 2 mL DPPH radical solution (0,1mM in ethanol). The absorbance of the mixture was read at 517 nm during period of 30 min, results were expressed as the mean of 3 replications. Pure ethanol was used as a blank.

$$\% \text{ inhibition} = [(A_0 - A_t)/A_0] \times 100 \quad (1)$$

A_0 - absorbance of DPPH radical solution,
A_t – absorbance after 30 minutes.

2.7. Rheological properties determination

The rheological properties were measured before storage in the freezer and after 3, 6, 9 and 12 months of storage by rotation rheometer, model VT 550 362-0001 HAAKE with concentric cylinders (RheoWin Pro 2.91 software). Diameter of inner rotating cylinder was 36 mm, inner diameter of outer stationary cap was 40 mm, gap between cylinders was 4 mm. The measurements were carried out in triplicate at 40°C, at shear rates 0 – 60 1/s. Temperature of 40°C has been selected as the closest to the temperature of "ready to eat" food containing puree. Puree is not expected to be eaten alone, it is usually used as a filling for some cakes like strudel, or as a dressing for pancakes and this is temperature of "ready to eat" product, close to the body temperature. For each shear rate computer recorded shear stress which was provided by the strawberry puree during rotation of the measuring cylinder of the rheometer. Flow curves are presented as the mean value of recorded results. Rheological parameters determined with experimental flow curves were fitted to Ostwald-de Waele (power law) model using a software (Excel 2016, USA).

$$\tau = k \cdot D^n \quad (2)$$

τ - shear stress (Pa),
k - consistency coefficient (Pas^n),
D - shear rate (1/s),
n - flow behavior indeks.
Samples were taken from the freezer and after thawing and reaching room temperature, rheological parameters were determined. Relation between shear rate and shear stress were presented graphically and determination coefficient (R^2) was calculated for each curve.

2.8. Data analysis

Chemical composition data were analysed by Statistica 12 software, using *post hoc LSD* at 95% level.

3. RESULTS AND DISCUSSION

Initial composition of strawberries puree samples is presented in Table 1. Parameters were tested as an indicator of ripeness to initially assess the starting material. S-1 sample, wild strawberries, had the highest sugar content and the lowest acidity. Sample S-2 is following with slightly lower content of sugars, but with much higher acidity, and the samples S-3 and S-4 were similar in sugar content, but sample S-3 had the highest acidity among all samples. These parameters were not expected to be significantly changed during storage, so they were not measured every three months.

Table 1. Composition of purees before storage.

	S-1	S-2	S-3	S-4
Total sugars (%)	15.40±0.22	13.42±0.16	8.22±0.26	8.64±0.04
Reducing sugars (%)	10.56±0,06	8.96±0.12	7.64±0.08	8.16±0.06
Total acidity (mmol/100g)	7.96±0.04	17.52±0.02	24.40±0.06	13.60±0.10

There are diverse phenolic compounds in strawberries, not only coloured anthocyanins, but also colourless phenols like ellagic acid, ellagitannins, *p*-coumaric acid and quercetins (HARTMANN *et al*., 2008). GASPEROTTI *et al*. (2015) identified and quantified 56 individual compounds in strawberries, with concentrations ranging from 1 μg/100 g to 40 mg/100 g. They also highlighted that this is not a complete list of polyphenols present in strawberries. Total phenol content in puree samples before and after 3, 6, 9 and 12 months of storage is presented in Table 2.

Table 2. Total phenol content in samples during 12 months of storage [A, B].

Sample	Total phenols (mg_{GAE}/100 g)				
	Before storage	After 3 months	After 6 months	After 9 months	After 12 months
S-1	422.59^{e}±4.72	415.10^{d}±2.82	404.60^{c}±5.06	400.89^{b}±1.55	368.98^{a}±0.44
S-2	196.92^{d}±6.96	192.66^{c}±0.16	189.96^{b}±0.98	188.12^{b}±0.46	170.49^{a}±1.47
S-3	164.68^{d}±0.99	152.15^{c}±0.83	151.62^{c}±3.39	147.49^{b}±1.41	135.21^{a}±0.47
S-4	185.70^{d}±3.12	165.69^{c}±0.89	163.35^{b}±3.94	163.91^{b}±4.53	151.47^{a}±1.19

[A]Results are expressed as mean of three repetitions ± standard deviation.
[B]Means followed by the same letter in the lines are not statistically different at 5% probability.

As presented by YILDIZ *et al* (2014) and DIAMANTI *et al* (2014), initial polyphenols content in wild strawberries puree (sample S-1) was more than double compared to cultivated strawberries purees (samples S-2 till S-4). Polyphenols content is in direct relation to the ripeness stage (sugar content and acidity presented in Table 1). Obtained results are similar to the values presented by GALOBURDA *et al*. (2014) and KLOPOTEK *et al*. (2005). It is already documented that strawberry phenolics such as pelargonidin, ellagic acid, *p*-coumaric acid, quercetin and kampferol derivatives are very unstable and undergo destruction during fruits transformation in frozen products especially in the thawing process by native and microbiological enzymes and by nonenzymatic oxidation

(AABY *et al*., 2007; OSZMIAŃSKI *et al*., 2009), but we couldn't find recommendations for storage time in freezer in order to preserve reasonable high level of polyphenols, and if initial value influences degree of degradation.
As it can be seen in Table 3, samples S-1 and S-2 had relatively low level of polyphenols destruction during first three months in the freezer (1.77 and 2.17%, respectively). Level of degradation continued over next months of storage, so both samples had percentage of degradation approximately 5% after 9 months of storage, which can be considered as good result. Between 9 and 12 months of storage a large decrease in polyphenol content can be seen and percentage of degradation during that period was higher than in previous 9 months. On the other hand, samples S-3 and S-4 had relatively high degradation during period of first three months (7.61 and 10.78%, respectively), which haven't changed significantly until the 9 months, so at the end of that period degradation was 10.44 and 11.74%. Still, the highest degradation was between 9 and 12 months. Therefore, it can be concluded that storage time of strawberry puree shouldn't be longer than 9 months in order to preserve phenolic compounds. In the end, percentage of degradation after 12 months of storage was higher in samples with lower initial values of polyphenols. It can be explained by the protective role of polyphenols (higher level of polyphenols-higher level of protection). Similar effect can be found in wines where red wines are less susceptible to degradation and require less chemical protection than white wines. HARTMANN *et al*. (2008) concluded that every processing step during production of juices and purees reduces the content of polyphenols, but they are better retained in purees than in juices. They also recommended a short enzymatic treatment of the mash with maceration enzymes in order to achieve maximal yield of polyphenols and antioxidant capacity. It is very important to obtain short enzymatic treatment because longer mash standing actually increases the loss. At the same time enzymatically treated puree was less viscous and smoother. While the nonenzymatically treated puree registered a water phase separation in less than 3 weeks of storage, which wasn't the case in this research. HOLZWARTH *et al*. (2012) reported that freezing technique did not have significant influence on polyphenols as well as on colour and ascorbic acid. Thawing method was, on the other hand, very important factor affecting mentioned parameters. Thawing at 20ºC and microwave thawing were favourable methods (compared to 4 ºC and 37ºC thawing). As previously explained, in this research samples were thawed at room temperature.

Table 3. Percentage of polyphenols degradation during storage compared to the initial value.

Sample	Degradation (%)			
	After 3 months	After 6 months	After 9 months	After 12 months
S-1	1.77	4.26	5.13	12.68
S-2	2.17	3.54	4.47	13.42
S-3	7.61	7.93	10.44	17.90
S-4	10.78	12.04	11.74	18.43

ABTS and DPPH methods have gained popularity for the study of antioxidant activity due to their speed and simplicity, and both of them are based on free-radical scavenging activity. Antioxidant activity by ABTS method is presented in Table 4 and by DPPH method in Table 5. Values are in accordance with the results presented by KLOPOTEK *et*

al. (2005) and NOWICKA *et al*. (2019). As expected, wild strawberries puree (S-1) had much higher antioxidant activity than other samples. Samples with higher content of polyphenols also had higher level of antioxidant activity by both methods. Contrary to polyphenols degradation, antioxidant activity by ABTS method in sample S-1 had the biggest decrease in first six months of storage. Sample S-3 (which had the lowest antioxidant activity at the beginning) lost almost 50% of the initial value by the end of a storage. Results obtained by DPPH method also show the biggest decrease in antioxidant activity during first three months. During the rest of the storage period further decrease was slower compared to first three months. Although polyphenols are the most important and the most popular antioxidants, there are also other molecules with antioxidant properties like products of Maillard reactions (OBRADOVIĆ *et al*., 2015), so direct correlation between polyphenols content and antioxidant activity is not always the case and it depends on method used. BAIANO *et al.* (2009) showed low correlation between amount of polyphenols in wines and antioxidant activity. They also concluded that beside previously mentioned antioxidants, antioxidant activity depends not only on the phenolic concentration, but also on the specific chemical structure of each phenolic compound.

Table 4. Antioxidant activity of samples during 12 months of storage (ABTS method) A, B.

Sample	Antioxidant activity (ABTS) (μmol TE/g)				
	Before storage	After 3 months	After 6 months	After 9 months	After 12 months
S-1	30.20^{d}±0.59	27.65^{c}±0.53	24.31^{a}±0.21	25.70^{b}±0.45	25.71^{b}±0.32
S-2	10.79^{e}±0.37	9.96^{d}±0.56	9.16^{c}±0.33	7.98^{b}±0.20	7.16^{a}±0.18
S-3	7.62^{e}±0.08	6.22^{d}±0.04	5.46^{c}±0.15	4.52^{b}±0.39	3.94^{a}±0.29
S-4	8.63^{d}±0.04	5.52^{c}±0.14	5.59^{c}±0.13	5.10^{b}±0.48	4.70^{a}±0.41

[A]Results are expressed as mean of three repetitions ± standard deviation

[B]Means followed by the same letter in the lines are not statistically different at 5% probability.

Table 5. Inhibition of DPPH radical after 30 minutes[A, B].

Sample	Inhibition (%)				
	Before storage	After 3 months	After 6 months	After 9 months	After 12 months
S-1	80.02^{d}±1.82	71.80^{c}±2.04	70.54^{b}±1.94	68.38^{a}±2.20	68.13^{a}±1.56
S-2	49.39^{c}±0.96	39.63^{b}±1.14	39.93^{b}±1.55	39.49^{b}±1.24	38.95^{a}±0.93
S-3	43.94^{d}±1.06	36.14^{c}±0.58	36.60^{c}±1.86	32.75^{b}±0,64	31.44^{a}±0.88
S-4	49.74^{d}±1.58	40.35^{c}±0.98	38.20^{b}±0.60	38.27^{b}±1,46	37.21^{a}±1.38

[A]Results are expressed as mean of three repetitions ± standard deviation.

[B]Means followed by the same letter in the lines are not statistically different at 5% probability.

Beside chemical and nutritional properties, knowledge of the rheological properties of food products is important for process design, control of the process, and consumer acceptability of a product. Rheological properties provide information on how to control flow properties of the product so that the desired product can be prepared. Rheological

properties are explained by rheological parameters: flow behavior index (n) and consistency coefficient (k) (LOVRIĆ, 2003; OSORIO *et al*., 2008). Fruit purees are suspensions of solid matter in fluid media and have been categorized as time-independent non-Newtonian fluids showing a pseudoplastic behavior (RUDRA *et al*., 2007; SOROUR *et al*., 2016).

Rheological properties of strawberry puree samples are presented in Fig. 1. Shape of the curve in Fig. 1. shows that strawberry puree is pseudoplastic system. Pseudoplastic non-Newtonian flow behavior occurs when shear stress is increasing at a diminishing rate, while increasing shear stress decrease when fluid is subjected to higher shear rates. It leads to a convex profile curve in which tangential slope is decreasing with increasing shear rate (KREITH, 1999; FIGURA and TEIXEIRA 2007). This behavior is caused by decreasing molecular interactions within the molecular structure of the fluid during flow. Pseudoplastic behavior of all samples remains regardless of storage time. It can be seen that freezing caused decrease of shear stress values in all samples compared to the starting sample. Deviations presented in stress-strain graphs are result of a samples' inhomogeneity, but still, measured values fit well to the Ostwald de Waele model for pseudoplastic systems (Table 6, R^2 ranging from 0,911 till 0,994). The same trends were obtained by several authors. ALVAREZ *et al*. (2006) studied the rheological behavior of strawberry jam, MACEIRAS *et al*. (2007), BUKUROV *et al*. (2012) and YALÇINÖZ and ERÇELEBI (2016) researched the rheological properties of strawberry puree. All mentioned authors fitted results of rheological measurements to the same model.

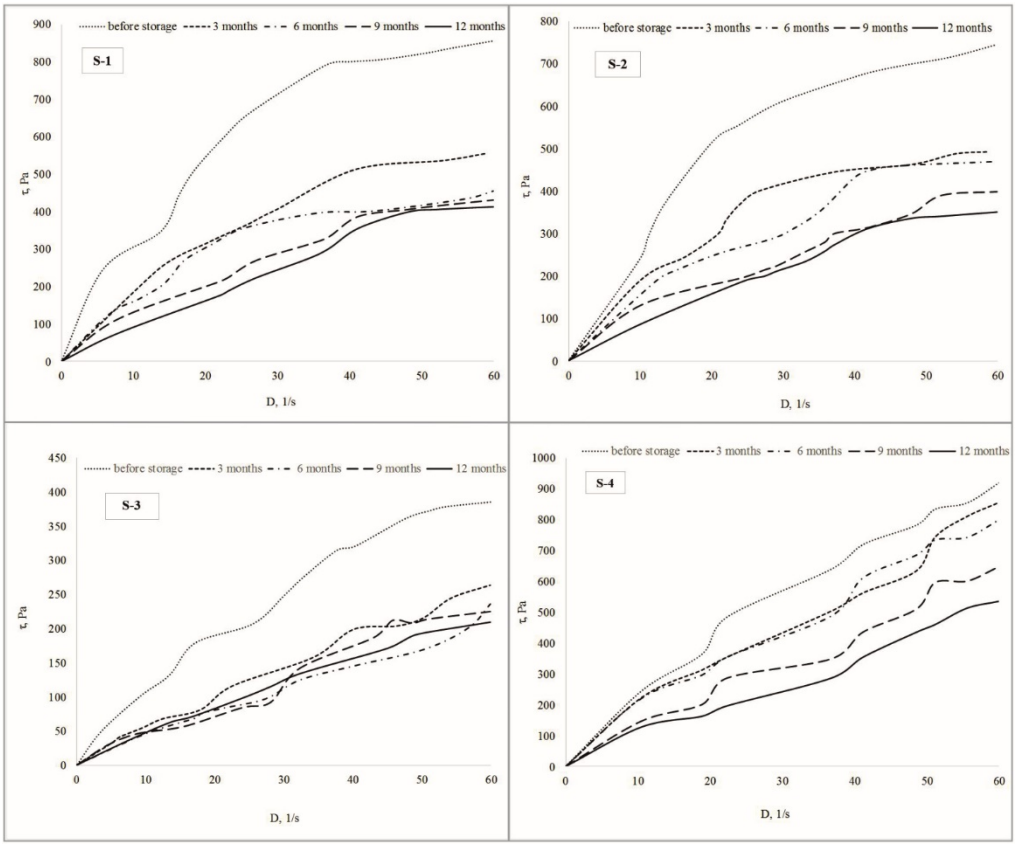

Figure 1. Rheological properties of strawberry purees during storage in the freezer.

Shear stress values were the highest in sample S-4 before and during storage with maximum shear stress 921 Pa and the lowest in sample S-3 with maximum shear stress 379 Pa at the shear rate 60 s^{-1}.

Table 6. Rheological parameters of strawberries puree samples during storage.

		Sample			
		S-1	**S-2**	**S-3**	**S-4**
Before storage	n	0.573	0.572	0.753	0.758
	k, Pa·s^n	91.285	79.818	19.289	41.910
	R^2	0.953	0.911	0.994	0.991
After 3 months of storage	n	0.575	0.545	0.837	0.769
	k, Pa·s^n	56.559	58.425	8.322	32.848
	R^2	0.985	0.930	0.994	0.979
After 6 months of storage	n	0.588	0.646	0.878	0.778
	k, Pa·s^n	45.301	36.274	5.785	32.674
	R^2	0.941	0.953	0.988	0.983
After 9 months of storage	n	0.701	0.675	0.873	0.873
	k, Pa·s^n	26.044	24.786	5.957	17.495
	R^2	0.987	0.962	0.961	0.971
After 12 months of storage	n	0.895	0.831	0.879	0.867
	k, Pa·s^n	11.622	12.956	6.067	14.375
	R^2	0.990	0.9863	0.998	0.961

As shown in Table 6, flow behavior index values are within 0 and 1 (0,545-0,878), characteristic for pseudoplastic systems. Pseudoplastic fluid behavior is explained by cracking of the molecule structure when exposed to hydrodynamic forces and increasing the alignment of the constituent molecules. It can be seen that from the start samples S-1 and S-2 had lower values for flow behavior index than samples S-3 and S-4. This difference is obvious till 9 months of a storage, while after that flow behavior index is practically the same for all samples. Strawberry purees before storage in the freezer had the lowest values of flow behavior index and the highest values of consistency coefficient. Consistency coefficient is constantly decreasing during time in all samples. Before storage, samples S-1 and S-2 had higher k values than samples S-3 and S-4 (lower fluidity). Decrease in k value overtime indicates that fluidity of the sample increased during storage time. Moreover, weak physical bonds like electrostatic and hydrophobic forces might have been destroyed easily during shearing (ISANGA AND ZHANG, 2009). Destruction of cellular structure during freezing and thawing caused increase of flow behavior index value and decrease of consistency coefficient value. Although it is obvious that rheological behavior is changed during storage with increased fluidity, pseudoplastic behavior remained. Depending on the final purpose of puree, improvement of fluidity can be achieved by addition of hydrocolloids (ERGOVIĆ *et al*., 2010).

4. CONCLUSIONS

Based on the results of this paper it can be concluded that strawberry puree shouldn't be stored in the freezer longer than 9 months to avoid excessive polyphenol degradation because after 9 months of storage degradation of polyphenols accelerated in all samples. Regardless of the method used, antioxidant activity of all samples decreased significantly within the first three months of storage and continued to decrease further during the rest of the time. Fluidity of all samples increased during time, consistency coefficient decreased, flow behaviour index increased, but rheological parameters stayed within the limits for pseudoplastic systems. After all, depending on the final purpose of the puree optimal values for flow behaviour index and coefficient of consistency should be determined. Shelf life cannot be evaluated only based on polyphenols and additional tests are required.

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PAPER

COLLOIDAL, TRIBOLOGICAL AND SENSORY PROPERTIES OF ORAL NUTRITIONAL SUPPLEMENTS

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ABSTRACT

This study aims to evaluate the physicochemical and sensory properties of oral nutritional supplements (ONSs). High physical stability was measured in ONSs with mean particle sizes <0.33 μm and viscosity >19.3 mPa·s. ONSs formulated with dairy-soy protein mixtures displayed low friction coefficients, whereas ONSs containing dairy proteins alone had high friction coefficient values in the boundary regime. Sensory analysis revealed low to medium liking across the products and the highest preference was found in samples with the highest perceived 'sweetness', 'vanilla aroma' and 'thickness'. The results will underpin the formulation of novel ONSs with good physical stability and sensory acceptability.

Keywords: colloidal stability, oral nutritional supplement, tribology, sensory properties

1. INTRODUCTION

The global market in foods for medical purposes was valued at USD 12.3 billion in 2015 and is expected to grow because of the projected increase in nutritional deficiencies and chronic diseases, as well as the increasing global geriatric population (VMR, 2019). Foods for medical purposes are receiving increasing attention due to their ability to support the nutrient requirements of patients, to prevent and control nutrition-related diseases and to decrease medical complications and hospital re-admissions in hospitalized patients (MUELLER, 2003; SIRÓ *et al.*, 2008; CAMILO, 2003; SMITH *et al.*, 2020).
Oral nutritional supplements (ONSs) represent a specific product category, which includes beverages highly dense in macro- and micro-nutrients that are thermally treated to provide a 12 month shelf-life (MCCLEMENTS, 2015a). From a colloidal perspective, ONSs are oil-in-water emulsions in which the lipid phase, containing a mixture of vegetable oils (e.g., sunflower and soy) and lipophilic bioactive compounds, is dispersed into a water phase enriched in water-soluble vitamins, salts, polysaccharides, in addition to proteins. The proteins are derived from dairy or plant sources, and it is common that mixtures of both are used. Surface-active molecules such as proteins, polysaccharides and phospholipids are usually added to create a protective layer at the oil-water interface and prevent the oil droplets from coalescing. It is well known that oil-in-water emulsions display an inherent tendency to destabilise through a variety of physicochemical mechanisms, including gravitational separation and droplet aggregation (MCCLEMENTS, 2015a, MCCLEMENTS, 2020).
The ingredients selected in the formulation of ONSs ultimately determine their sensory properties, such as appearance, aroma and texture, which contribute to consumer liking. Previous studies have reported that ONSs possess poor sensory properties and low consumer acceptability which, in turn, can negatively affect consumption rates (GOSNEY, 2003; THOMAS *et al.*, 2018; REGAN *et al.*, 2019). Given that these beverages may function as a sole source of nutrition, this is highly problematic. Considering the importance of sensory properties and consumer acceptability for ONSs, tribology can serve as a useful instrumental means of understanding how these products are perceived during consumption in the oral cavity. Tribological parameters (i.e., coefficient of friction at the boundary and mixed regimes) have been linked to important sensory attributes, such as astringency and creaminess (MALONE *et al.*, 2003; DE HOOG *et al.*, 2006; DRESSELHUIS *et al.*, 2008; VARDHANABHUTI *et al.*, 2011; CAMPBELL *et al.*, 2017; PRIYANKA *et al.*, 2020). VARDHANABHUTI *et al.* (2011) found an increase in both astringency perception and coefficient of friction in dairy protein suspensions with increasing protein content from 0.5 to 10% w/w at acidic pH. Moreover, a strong correlation between perceived creaminess and tribological parameters as a function of fat content was identified in several studies of emulsion systems (MALONE *et al.*, 2003; DE WIJK and PRINZ, 2005).
ONSs, due to their high nutrient density, and diverse ingredient combinations, represent a highly complex food matrix and to the best of our knowledge, information on ingredient interactions, colloidal properties, physical stability and consumer acceptability of ONSs are not widely available. Therefore, the aim of this work was to undertake a comprehensive assessment of the physicochemical and sensory properties of ONSs with protein, fat and carbohydrate contents ranging from 4.0-10.0%, 2.6-6.7%, 8.7-20.4%, respectively. The information gathered will help underpin the formulation of ONSs with high physical stability and sensory acceptability.

2. MATERIALS AND METHODS

2.1. Oral nutritional supplements

Forty commercial ONSs in different flavours (e.g., vanilla, strawberry, chocolate) were procured from six different suppliers and reviewed. In order to have a broad overview of the physicochemical properties of ONS, six samples at low (~4%), medium (~6%) and high (~10%) protein content were selected for further analysis. The ONS samples were obtained from three different suppliers and all samples had a common flavour (i.e., vanilla). The ONSs were packaged in 250 mL plastic bottles, with the exception of sample A, which was packaged in a 250 mL metallic can. The samples were stored at 22±1°C prior to testing and were analysed within 4 months of their manufacture, according to the supplier information. Table 1 shows the macro-nutrient composition of the samples along with their ingredient lists.

Table 1. Macronutrient content (% w/v of product) and ingredient declaration for protein, carbohydrate and fat for the oral nutritional supplements studied A - F.

Macronutrient	A	B	C	D	E	F
Protein (%)	4.0	4.3	6.4	6.8	10.0	10.0
Ingredients	*Milk proteins, soy protein isolate*	*Milk proteins, soy protein isolate*	*Milk proteins, soy protein isolate*	*Milk proteins*	*Milk proteins*	*Milk proteins, soy protein isolate*
Carbohydrate (%)	13.5	8.7	20.4	18.8	12.4	15.0
Ingredients	*Sucrose, maltodextrin*	*Sucromalt, maltodextrin, fructo oligosaccharide*	*Sucrose, hydrolysed corn starch, fructo oligosaccharide, oat fibre, gum Arabic, carboxymethyl cellulose, soy polysaccharide*	*Sucrose, hydrolysed corn starch, gum Arabic, soy poly saccharide*	*Sucrose, maltodextrin*	*Glucose syrup, maltodextrin*
Fat (%)	3.3	3.5	5.1	2.6	6.7	5.6
Ingredients	*Canola oil, high oleic sunflower oil*	*Canola oil, high oleic sunflower oil*	*Canola oil, high oleic sunflower oil, corn oil*	*Medium chain triacyl-glycerol from palm kernel, canola and soy*	*Rapeseed oil, sunflower oil*	*Vegetable oils*

2.2. Analysis

2.2.1 Colour

Colour analysis was carried out using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The

instrument was standardised against a white tile before measurements. Colour was expressed in L* (luminosity), a* (green to red components for negative and positive values, respectively) and b* (blue to yellow components for negative and positive values, respectively) parameters.

2.2.2 Particle size distribution

Particle size distribution of the ONSs was determined using a laser light diffraction unit (Mastersizer 3000, Malvern Instruments Ltd, Worcestershire, UK) equipped with a 300 RF (reverse fourier) lens and He-Ne laser (λ of 633 nm). Sample was introduced to the mixing chamber and dispersed in ultrapure water until a laser obscuration of 12% (± 0.5%) was reached. The water refractive index was set at 1.33, while that of the ONSs was measured using a refractometer (Atago™ R–5000 Hand-Held Refractometer, ATAGO CO., LTD) and ranged between 1.34 and 1.38 at 20°C. Data were presented as volume-based particle size distribution together with $D_{4,3}$ values, which represent the volume mean diameter.

2.2.3 Rheological properties

The rheological properties of the selected ONSs were determined using a controlled-stress rheometer (TA Discovery Hybrid Rheometer, TA Instruments, Crawley, West Sussex, UK) equipped with a concentric cylinder geometry. Viscosity was measured as a function of shear rate in the range 1-300 s^{-1} at 22°C.
The power law (eq.1) was applied to the data obtained:

$$\tau = K\gamma^n \qquad \text{(eq. 1)}$$

where τ is shear stress (Pa), K is consistency coefficient (Pa·s^n), $\dot{\gamma}$ is shear rate (s^{-1}) and n is flow behaviour index (ANEMA *et al.*, 2014). All the curves showed an $R^2 \geq 0.99$. The results are reported as apparent viscosity at 50 s^{-1}, with this shear rate previously demonstrated to relate best to thickness perception during sensory assessments (Ross *et al*., 2019).

2.2.4 Accelerated physical stability

An analytical centrifuge (LUMiSizer®, L.U.M. GmbH, Berlin, Germany), which measures the intensity of transmitted near infra-red (NIR) light as a function of centrifugation time and position over the length of a cell held horizontally over the light path, was used to measure the rate and the extent of separation in ONSs. Polycarbonate cells (2 mm light path) were filled with 400 µL of ONSs with a wide-bore needle. Measurements were performed at 25°C and 2200g for 60 min. For calculating the change in transmission over time, integration limits were set at 109 and 130 mm. Data were reported as integral transmission (%) as a function of the running time (CROWLEY *et al.*, 2016) and the creaming rate was calculated by linear regression analysis.

2.2.5 Tribology

Tribological assessment was conducted on the selected ONSs to determine variations in friction and lubrication properties using a method as described by Batchelor *et al*. (2015). Tribological measurements were conducted using a mini traction machine (MTM2 PCS Instruments, London, UK) consisting of a 19.05 mm stainless steel ball loaded onto a 46

mm diameter silicone elastomer disc, both of which are independently driven, allowing for different motion between the two, and the temperature of measurement was maintained at 20°C. This temperature was chosen since the products are retained in the mouth for less than 20 s and therefore, it is a more representative temperature than body temperature (i.e., 37°C) (BATCHELOR *et al.*, 2015).
Stribeck curves were constructed for all of the investigated systems by measuring traction in the range 1-200 mm/s with a normal force of 2 N, which is the normal force typically applied to dairy-based products during oral processing (HORI *et al.*, 2009; MILLS *et al.*, 2013; BATCHELOR *et al.*, 2015; NGUYEN *et al.*, 2016).

2.2.6 Sensory analysis

The sensory analysis was conducted using untrained assessors (n=25) recruited from University College Cork (Ireland) in the range 21–50 years. Selection criteria for assessors were availability for testing and motivation to participate on all days of the experiment and the assessors also needed to be regular dairy beverage consumers. ONSs were coded with a randomly selected 3-digit code and presented in duplicate (STONE *et al.*, 2012). Consumers evaluated both intensity attributes and hedonic in the same session, but separated by an interval to allow training and descriptor explanation with reference to a table of descriptions provided (FELLENDORF *et al.*, 2017). The assessors were asked to rate the samples on a continuous line scale from 1 to 10 cm, in which 1 corresponded to 'extremely low descriptor intensity', and 10 to 'extremely high descriptor intensity' (Ranking Descriptive Analysis, RDA) (RICHTER *et al.*, 2010). Additionally, each assessor was asked to indicate their degree of liking on a 10-cm line scale ranging from 0 (dislike extremely) at the left to 10 (like extremely). Sessions were carried out at 22°C under white light and sensory evaluators were instructed to use still water provided to cleanse their palates between tastings.

2.3. Statistical data analysis

The results presented are the average of at least three measurements and are reported as mean value±standard deviation. Statistical analysis was performed using R v.2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and the Tukey test was used to determine statistically significant differences among means ($P<0.05$). Linear regression analysis by least squares minimisation was performed using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). The goodness of fit was evaluated based on correlation coefficients (R^2) and P values. Correlation analysis between instrumental measurements and sensory attributes was carried out using Statistica (Statistica for Windows v. 10, StatSoft, Inc.).

3. RESULTS AND DISCUSSION

3.1. Colloidal properties of oral nutritional supplements

3.1.1 Colour

Selected ONSs were firstly characterized for their colour coordinates (Table 2). High luminosity and yellowness values, indicated by L* and b* co-ordinates, with values ranging between 62.6 to 74.3 and 14.7 to 26.2, respectively, were recorded for ONS samples. Low red point values, indicated by a*, with values ranging between -0.1 and 2.8 were displayed in the samples. Similar results have been reported for nutritional beverages enriched in dairy protein and carbohydrates and have been attributed to brown melanoidin-based pigments produced in the latter stages of the Maillard reaction upon sterilisation (VAN BOEKEL, 1998; LIU and ZHONG, 2015; CHEN and O'MAHONY, 2016; DRAPALA *et al.*, 2017). LIU and ZHONG (2015) reported L*, a* and b* values ranging between 73.0 and 66.3, -2.9 and 1.2, 2.8 and 37.0, respectively, in mixtures of whey protein isolate, maltodextrin and lactose at pH 3.0-7.0 thermally treated at 130°C for 30 min. Similar results have been observed in nutritional beverages containing 8.5% milk protein isolate and 5% carbohydrate (i.e., maltodextrin, corn syrup or glucose) at near-neutral pH (pH 6.48-6.78) subjected to thermal treatment at 121°C for 15 min (CHEN and O'MAHONY, 2016).

Table 2. Chromaticity co-ordinates (L*, a*, b*), particle size mean diameter ($D_{4,3}$), rheological properties and traction coefficient for the oral nutritional supplements studied A - F.

Sample	Chromaticity co-ordinates			Particle size distribution parameter	Rheological properties		Traction Coefficient (-)	
	L*	a* (-)	b*	$D_{4,3}$ (μm)	Flow behaviour index (-)	Viscosity at 50 s^{-1} (mPa·s)	Boundary Regime (2.5 mm s^{-1})	Mixed Regime (25 mm s^{-1})
A	72.1±0.0^{d}	0.6±0.0^{d}	14.7±0.0^{f}	0.30±0.00^{c}	0.954±0.021^{ab}	7.2±0.0^{e}	0.239±0.021^{a}	0.128±0.005^{c}
B	71.4±0.0^{c}	0.6±0.0^{d}	18.5±0.0^{d}	0.31±0.00^{e}	0.801±0.001^{d}	17.3±0.0^{d}	0.221±0.025^{b}	0.143±0.014^{a}
C	68.1±0.0^{f}	0.8±0.0^{b}	22.8±0.0^{b}	8.16±0.05^{a}	0.948±0.005^{bc}	43.4±1.0^{b}	0.217±0.018^{b}	0.093±0.019^{d}
D	62.6±0.0^{e}	-0.1±0.0^{e}	26.2±0.0^{a}	2.05±0.02^{b}	0.891±0.003^{c}	19.3±0.1^{dc}	0.243±0.033^{a}	0.131±0.013^{a}
E	74.3±0.0^{a}	2.8±0.0^{a}	15.8±0.0^{e}	0.64±0.00^{c}	0.968±0.021^{a}	20.6±0.1^{c}	0.231±0.021^{a}	0.115±0.013^{ab}
F	72.7±0.0^{b}	0.7±0.0^{c}	19.7±0.0^{c}	0.37±0.00^{d}	0.927±0.003^{cd}	56.0±0.5^{a}	0.204±0.024^{c}	0.092±0.008^{d}

$^{a, b, c, d}$: means with different letters in the same column are significantly different ($P<0.05$).
n.a.: not applicable.

3.1.2 Particle size distribution

Samples A, B, E and F displayed particle size distributions with a dominant peak in the nano-sized region (0.01-1 μm) and $D_{4,3}$ ranged between 0.30 to 0.64 μm (Fig. 1A, B, E, F and Table 2). A bimodal distribution, with two distinct peaks in the nano-sized region and in the micron-sized region (1-100 μm) was observed for both samples C and D; $D_{4,3}$ values for

both samples C and D were 8.16 and 2.05 μm respectively (Fig. 1c, d; Table 2). The presence of a micron-sized peak may be attributed to hydrocolloids (i.e., including gum arabic, oat fibre and carboxy-methyl cellulose) present in the formulations, in agreement with the work of HUANG *et al.* (2001), who reported a particle size distribution similar to those in this study for emulsions containing hydrocolloids (0.5%) including methylcellulose and gum arabic. The results indicated that dairy and soy protein (i.e., A, B, E and F) aided the formation of nano-sized particle distributions, perhaps due to synergistic emulsifying properties of dairy and plant proteins when blended (HO *et al.*, 2018).

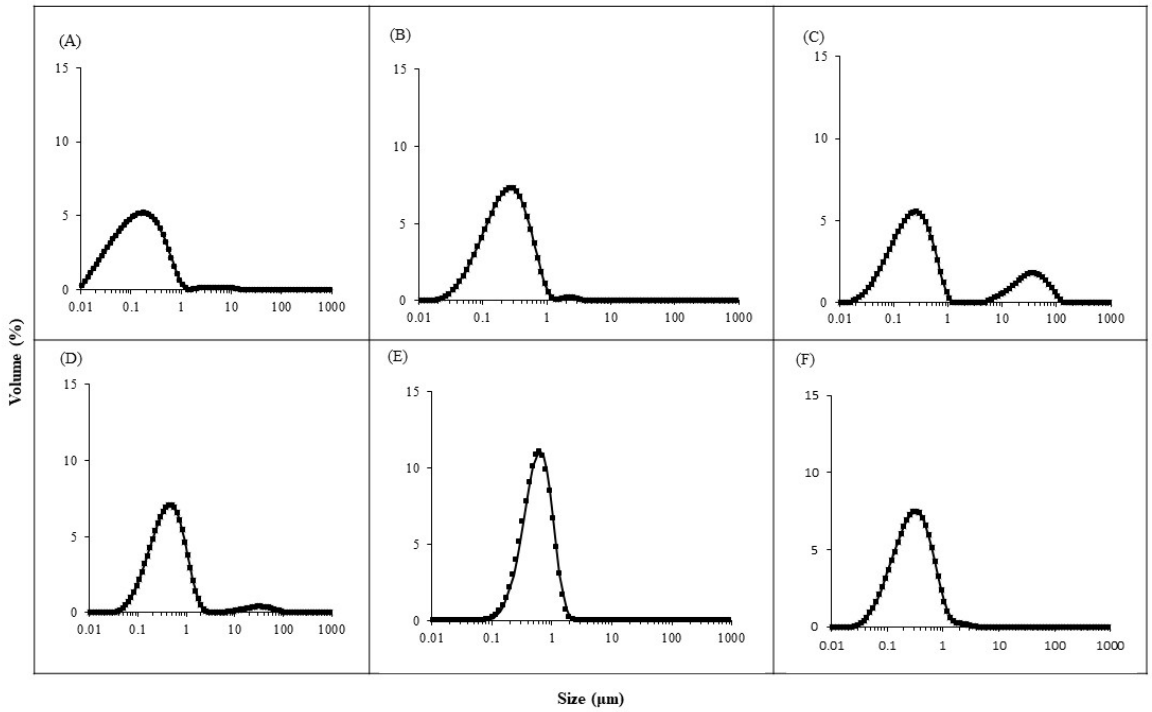


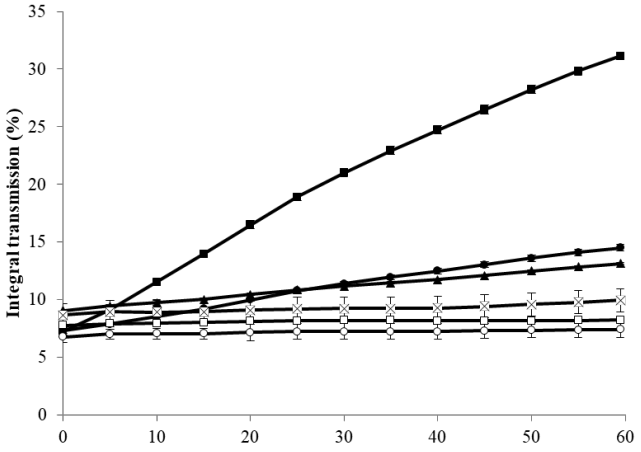
Figure 1. Particle size distribution data for the oral nutritional supplements studied A - F.

3.1.3 Rheological properties

The rheological properties, including flow behaviour index and apparent viscosity at 50 s^{-1} of the ONSs, are reported in Table 2. All the samples displayed shear thinning behaviour with flow behaviour index (n) ranging between 0.801 and 0.968. These values are typical of protein suspensions, which form weak structures that are disrupted upon application of shear stress (WALSTRA *et al.*, 2006). The viscosity ranged between 7.2 and 56.0 mPa·s across all the ONSs, with the samples ranked from lowest to highest as follows: A < B < D < E < C < F (Table 2). Samples A and B, having the lowest protein content (4%), displayed the lowest viscosity (7.2 and 17.3 mPa·s), whereas in samples D and E higher viscosity was observed with values ranging between 19.3 and 20.6 mPa·s. The highest viscosity values were recorded in ONS C and F, with values of 43.0 and 56.0 mPa·s, respectively, and may be attributed to the high proportions of carbohydrates (greater than 15%), proteins (greater than 6%) and fat (greater than 5%) in these samples (DICKINSON, 2003; PITKOWSKI *et al.*, 2009; HUPPERTZ *et al.*, 2017; QUINZIO *et al.*, 2018).

3.1.4 Physical stability

The physical stability of ONSs was investigated by measuring the changes in light transmission during centrifugation of the samples. The integral transmission as a function of centrifugation time, together with the clarification rate are shown in Fig. 2.
An increase in integral transmission with progressive centrifugation time was observed, indicative of phase separation. Samples A and B displayed clarification rates of 0.41 and 0.124 integral transmission·min^{-1} (IT%·min^{-1}), respectively, while in sample C, a slight change was observed (0.068 IT%·min^{-1}). On the other hand, no changes in the integral transmission were observed in samples D, E and F, thus indicating high stability of the ONSs (Fig. 2). The highest clarification rate (i.e., 0.41 IT%·min^{-1}), and therefore the lowest physical stability observed in sample A may be ascribed to its low viscosity (i.e., 7 mPa·s), whereas, the relatively high stability of samples C, D, E and F may be attributed to viscosity values higher than 18 mPa·s, associated with the presence of hydrocolloids (e.g., hydrolysed corn starch, oat fibre, gum arabic) in samples C and D. It is well known that hydrocolloids are able to improve the physical stability of emulsion-based systems by increasing the viscosity (MCCLEMENTS, 2015b). It has been demonstrated that the addition of guar gum from 0 to 0.5% (w/w) in an oil-in-water emulsion containing 10% oil resulted in reduction in the creaming rate from 10 to 100 min (VÉLEZ *et al.*, 2003; YOUSEFI and JAFARI, 2019).



Sample	Physical stability (Integral transmission·min^{-1})	R^2
A	0.410±0.003	0.993
B	0.124±0.003	0.999
C	0.068±0.003	0.995
D	n.a.	n.a.
E	n.a.	n.a.
F	n.a.	n.a.

Figure 2. Changes in transmission profile during centrifugation at 2200*g* for 60 min at 20°C for the oral nutritional supplements studied A (■), B (●), C (▲), D (×), E (□), F (o). Shown inset is the separation rate expressed as integral transmission·min^{-1} and R^2 computed from the slopes of the linear regression at 2200*g* for the oral nutritional supplements studied A - F.

3.2. Tribology assessment

Tribology has previously been used to model the friction and lubricity of ONSs that would occur between oral surfaces in the mouth (LAIHO *et al.*, 2017). A Stribeck curve (data not shown), which represents the traction coefficient as a function of speed motion, can be typically divided into the boundary regime (< 10 mm/s) and the mixed regime, (10-100

mm/s) which are usually associated with astringency (ROSSETTI *et al.*, 2009) and creamy textures (CHOJNICKA-PASZUN *et al.*, 2012), respectively. Within the boundary regime (<10 mm/s), systems D, A and E exhibited the greatest values of friction coefficient, with values ranging between 0.231 and 0.243, respectively, whereas samples B, C and F displayed the lowest values for friction coefficient with values between 0.217 and 0.204, respectively. According to the results, the ranking of the investigated systems from highest to lowest value at the boundary regimes was as follows: D > A > E > B > C > F (Table 2). This trend can be ascribed to different, interlinked factors, including particle size distribution, viscosity and protein type (i.e., casein and whey) as well as sources (i.e., dairy and plant) (LEY, 2008; HUGHES *et al.*, 2011; ZHAO *et al.*, 2016, VARDHANABHUTI *et al.*, 2011; MALONE *et al.*, 2003; DE WIJK and PRINZ, 2005). Conversely, in the mixed regimes (10-100 $mm \cdot s^{-1}$), lower friction coefficients indicate a creamier texture, which can be associated with sensory attributes such as thickness, smoothness, slipperiness and softness (AKHTAR *et al.*, 2006; CHOJNICKA-PASZUN *et al.*, 2012; BATCHELOR *et al.*, 2015). The ranking in terms of creamy texture, from the lowest to the highest friction coefficient at the boundary regimes, corresponding to the most to least creamy, respectively, was as follows: F > C > E > A > D > B (Table 2). This trend for texture is consistent with the composition of the investigated systems (Table 1) and was predominately ascribed to the fat content, whereby the creamier systems (i.e., F, C and E) had fat content >5% (w/w), and the less creamy systems (i.e., B, D and A) had a fat content <4% (w/w). The work of AKHTAR *et al.* (2005) showed that higher levels of fat in oil-in-water emulsions stabilized with sodium caseinate contributed to an enhanced perception of creaminess. Furthermore, the trend in friction coefficient is aligned with the apparent viscosity at 50 s^{-1} (Table 2), whereby measurement of viscosity at this shear rate is typically related to the perception of thickness (AKHTAR *et al.*, 2006; STOKES *et al.*, 2013; DICKINSON, 2018).

3.3. Sensory evaluation

The sensory profiles of the selected ONSs were mapped by ranking descriptive analysis, which included intensity of beige colour, vanilla aroma, cooked flavour, thickness and astringent after-taste (Table 3). A wide range of beige colour intensity, with values ranging between 3.7 and 7.7 was recorded for the ONS samples and the following rank, from the lowest to the highest, A < E < B < F < C < D was observed. The vanilla aroma intensity ranged between 3.8 and 7.5, with a lowest to highest ranking of B < A < E < D < F < C across the samples. Cooked flavour, which originates from sulphur compounds in thermally processed protein-based beverages, was mildly (3.3-5.1) perceived and the ONSs displayed the following rank, from lowest to highest: B < A < E < D < F < C. A positive correlation (0.847, $P<0.05$) between cooked flavour and the proportion of carbohydrate, one of the prerequisite substrates for the Maillard reaction, was found (MELLEMA and BOT, 2009). A wide range of thickness values, from 2.5 to 7.0 was recorded and the lowest thickness perception was perceived in ONSs having the lowest protein content (4.0 and 4.3% for A and B, respectively). The highest thickness values were recorded in samples C, D and F, which contained relatively high levels of protein (>6%) and carbohydrate (>15%), such as guar gum and carboxy-methyl cellulose. The results displayed a strong positive correlation between thickness perception and instrumental viscosity (0.869, $P<0.05$) as well as thickness perception and mixed regime values (-0.772, $P<0.05$), in agreement with the literature (MALONE *et al.*, 2003; DRESSELHUIS *et al.*, 2008; ROSS *et al.*, 2019).

Table 3. Data for ranking descriptive analysis and sensory hedonic evaluation of the oral nutritional supplements studied A - F.

Sample	Ranking descriptive analysis[1]						Hedonic evaluation[2]				
	Colour (degree of beige)	Vanilla aroma	Cooked flavour	Thickness	Sweet taste	After-taste	Liking of appearance	Liking of aroma	Liking of flavour	Liking of texture	Overall acceptability
A	3.7±0.1^{b}	3.8±0.2^{c}	3.9±0.7^{a}	2.5±0.2^{c}	4.3±0.3^{b}	3.4±0.1^{a}	4.9±0.0^{b}	5.5±0.3^{ab}	4.3±0.3^{b}	5.2±0.3^{b}	4.5±0.0 ^{b}
B	5.4±0.4^{ab}	5.4±0.5^{b}	3.3±0.1^{a}	4.2±0.1^{bc}	7.0±0.0^{a}	2.9±0.1^{a}	6.1±0.0^{a}	6.2±0.2^{a}	5.9±0.2^{a}	6.2±0.1^{b}	5.7±0.1^{a}
C	6.5±0.4^{ab}	4.5±0.3^{bc}	5.1±0.1^{a}	7.0±0.2^{a}	4.9±0.3^{b}	3.8±0.2^{a}	4.7±0.1^{b}	5.3±0.1^{ab}	4.1±0.3^{b}	5.1±0.1^{b}	4.0±0.2^{b}
D	7.7±0.4^{ab}	7.5±0.1^{a}	4.9±0.3^{a}	5.5±0.2^{ab}	7.4±0.8^{a}	3.0±0.5^{a}	3.6±0.0^{c}	4.9±0.1^{bc}	4.2±0.1^{b}	5.7±0.2^{ab}	4.0±0.2^{b}
E	4.0±0.4^{b}	4.4±0.2^{bc}	4.2±0.0^{a}	5.1±0.7^{ab}	5.6±0.0^{ab}	3.1±0.2^{a}	5.9±0.2^{a}	5.2±0.1^{ab}	4.9±0.1^{ab}	5.9±0.1^{ab}	5.2±0.1^{a}
F	5.5±0.1^{ab}	4.1±0.1^{bc}	5.0±0.2^{a}	6.9±0.4^{a}	6.2±0.3^{ab}	3.7±0.5^{a}	5.7±0.2^{a}	4.1±0.2^{c}	4.2±0.1^{b}	5.3±0.0^{ab}	4.0±0.1^{b}

$^{a, b, c}$: means with different letters in the same column are significantly different ($P<0.05$).

[1] corresponds to extremely low descriptor intensity; 10 corresponds to extremely high descriptor intensity.

[2] corresponds to dislike extremely; 10 corresponds to dislike extremely.

Medium sweet intensity, with values ranging between 4.3 and 7.0 was assessed across ONSs and the following order, from highest to lowest sweet intensity, A < C < E < F < B < D was observed. Across the samples, the intensity of after-taste, which is typically perceived in protein-based beverages, ranged between 2 and 3.5 and no significant ($P > 0.05$) differences were observed, probably due to the tailored ingredients balance in the formulations (DE WIJK and PRINZ, 2005; VARDHANABHUTI *et al.*, 2011). The consumer preferences for ONS products were assessed by hedonic testing, which encompasses liking of appearance, aroma, flavour, texture and overall acceptability (Table 3). ONSs displayed a medium liking score in appearance, with values ranging between 4.7 and 6.1. Sample D displayed the lowest liking in appearance (i.e., 3.6), whereas samples B, E and F showed the highest liking scores with values of 6.1, 5.7 and 5.9, respectively. Significant differences ($P<0.05$) in liking in aroma were observed within the selected ONSs and samples F and D obtained the lowest scores (i.e., 4.1 and 4.9), while B displayed the highest liking in aroma (i.e., 6.2). For liking of flavour, significant differences ($P<0.05$) across the samples were found. The liking of texture scores ranged between 5.1 and 6.2 and samples ordered, from lowest to highest as C < A < F < D < E < B. The overall acceptability reported low-medium scores, with values ranging between 4 and 5.7, in agreement with the literature for protein-based beverages (YE *et al.*, 2011; THOMAS *et al,.* 2016; WITHERS *et al.*, 2014). A negative correlation between overall acceptability and cooked flavour (-0.879, $P<0.05$) or astringent after-taste (-0.902, $P<0.05$) was observed. The panellists identified samples, namely B and E, as the most preferred products, with 48% and 23% of the preferences, respectively (Table 3). The high preference for samples B and E may be ascribed to the high sweet intensity, vanilla aroma and thickness values together with low cooked flavour and after-taste intensity recorded in the aforementioned samples.

4. CONCLUSIONS

In this study, colloidal, tribological and sensory properties were investigated in order to better understand the physical and sensory quality attributes of ONSs. Samples characterized by high viscosity and small particle size possessed the highest physical stability. ONSs containing dairy and soy protein blends with lipid content higher that 5% had low coefficient of friction values in both boundary and mixed regimes, which are usually associated with astringent after-taste and creamy texture, respectively. Low-medium liking scores were displayed across all the samples and the highest preference was recorded for samples with high sweet intensity, vanilla aroma, thickness and low values in cooked flavour and after-taste. The detailed information on rheological, colloidal, physical stability, tribological and sensory properties reported in this study will support the development of new ONSs with high physical stability and enhanced sensory acceptability.

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PAPER

CHANGES IN LIPID CONTENT WITH ROASTING TEMPERATURE OF LARGE YELLOW CROAKER (*LARIMICHTHYS CROCEA*) ROE

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ABSTRACT

This study aims to clarify the changes with roasting temperature in the lipid content of ready-to-eat large yellow croaker (*Larimichthys crocea*) roe product. Almost all the lipid class/species showed the same trend with the increasing temperature. Except for some minor differences, the relative amounts of lipids decreased with temperature increase from 0°C (control group, raw roe) to 100°C; increased with further temperature increase to 120°C, at which the amount was maximum; and then decreased with further temperature increase to 180°C. Finally, 120°C was selected as the optimal processing temperature, which may result in a better appearance and a high lipid quality, indicating its potential application value. This study also enhances the understanding of lipid profile in fish roe and demonstrates the applicability of the lipidomic method in aquatic food production.

Keywords: *Larimichthys crocea* roe, phospholipid molecular species, lipid content, roasting temperature

1. INTRODUCTION

Fish roe is known for its high nutritional value. Its products have been consumed as caviar (from sturgeon) and caviar substitutes (from other species); other product forms include whole skeins and formulations with oils and cheese bases, in salted or smoked forms, and the international and domestic markets of these products continue to increase (BLEDSOE *et al*., 2003). In recent years, the valorization of roe has received much attention because of its gigantic yield and health benefits. Some reports have shown that fish roe is available at lower prices. It can be incorporated in various food preparations to combat protein malnutrition and is an interesting source for supplementing the human diet with marine lipids (MAHMOUD *et al*., 2008; BALASWAMY *et al*., 2009; SALIU *et al*., 2019). In addition, the valorization of roe by encouraging professional exploitation (for fillets, caviar, or nutritional supplement production) can also lower the pressure on the aquatic ecosystem (SALIU *et al*., 2017). The large yellow croaker (*Larimichthys crocea*) is a major commercial marine fish in the south of China, with a total production of approximately 180,000 tons in 2017 (CHEN *et al*., 2018). However, its roe is usually large and has an unattractive appearance. During the processing of *L. crocea*, its roe is a major by-product, which is usually discarded as waste. This is an important commercial loss and an environmental problem from the fish industry. The *L. crocea* roe has been verified to contain large amounts of n-3 polyunsaturated fatty acids (PUFAs), mainly eicosapentaenoic acids (EPA, C20:5 n-3) and docosahexaenoic acids (DHA, C22:6 n-3) as known, which can help prevent the incidence of coronary heart diseases, inflammatory and autoimmune disorders, and cancers (WANG *et al.,* 2008; ROSA *et al.,* 2012; OZOGUL *et al.,* 2007). The predominant phospholipids (PLs) in *L. crocea* roe are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as determined in our previous study (LIANG *et al*., 2017a), which corresponds with other reports on fish roe (HAYASHI *et al*., 1999; SHIRAI *et al*., 2006).

Phospholipids are major polar lipid components, and they serve as building blocks for cell membranes and have important physiological and biological functions in almost all known living beings (BURRI *et al*., 2012; SUZUMURA, 2005). The diversity of PL molecule species can be ascribed to the number of carbons and double bonds in the fatty acid moiety and the moiety locations on the glycerol backbone with one headgroup, which are the sn-1 and/or sn-2 position and sn-3 position of PLs. Marine PLs have also been confirmed capable of reducing inflammatory reactions (DEUTSCH, 2007) and preventing colon cancer growth induced by chemicals in vitro (HOSSAIN *et al*., 2009). Moreover, our previous study identified that the PLs with docosahexaenoic acid (DHA) from the *L. crocea* roe had beneficial effects on the lipid metabolism of hyperlipidemic mice (LIANG *et al*., 2017b).

The changes in the PLs and other lipid components can be affected by the storage time and freezing/cooking temperatures, which causes autoxidation, hydrolytic decomposition, lipid browning, lipid-protein copolymerization reactions, and lipolysis or enzymatic degradation in the food products (IGENE *et al*., 1981; LEE *et al*., 1976; WANG *et al*., 2011). Additionally, the PL loss can be due to different mechanisms of heat transfer, which cause cell rupture (MONDY *et al*., 1977). Furthermore, the PL quantity can affect the flavor and nutritional quality in the food matrix (DE LIMA *et al*., 2008). However, the changes in the total lipid content and PL molecular species with high temperatures are still not clear for the *L. crocea* roe.

Recently, the shotgun lipidomic approach was developed to replace the traditional methods (i.e., thin-layer chromatography) for monitoring the molecular compositions and

abundances of individual lipid species from unfractionated lipid extracts more rapidly and with higher sensitivity (WANG *et al*., 2011). For the comprehensive analysis of lipid structures, the developed technology of reversed-phase ultra-performance liquid chromatography (UPLC) coupled with electrospray ionization–quadrupole-time-of-flight–mass spectrometry (UPLC-ESI-Q-TOF-MS) possesses superior separation ability, higher resolution, greater sensitivity, and faster speed (WANG *et al*., 2011; BASCONCILLO *et al*., 2009; LAAKSONEN *et al*., 2006; YAN *et al*., 2010; ZHAO *et al*., 2014). Herein, we assumed that the changes in PLs and other lipid components can be confirmed via shotgun lipidomics.

This study aimed to identify the relative changes in lipid content, especially the PL molecular species, with the roasting temperature in the ready-to-eat *L. crocea* roe product and determine the appropriate roasting temperature that can keep the best lipid compositions of the roe. The study clarifies the value of the *L. crocea* roe products and broadens the comprehensive utilization of the roe. Moreover, the application of lipidomics on the roasting processing of aquatic foods is shown to be beneficial for evaluating the changes in lipid compositions. This study can also promote the lipidomics method application in aquatic food production.

2. MATERIALS AND METHODS

2.1. Materials and reagents

The *L. crocea* roe was provided by Fujian Yuehai Aquatic Food Ltd. (Ningde City, Fujian Province). The roe was mixed and kept under refrigeration (0-4°C) for less than 24 h before analysis in the lab of Aquatic Food Products Processing at Fujian Agriculture and Forestry University.

In total, 10 lipid standards PC (17:0), lysophosphatidylcholine (LPC; 15:0/0:0), phosphatidylglycerol (PG; 15:0/15:0), PC (15:0/15:0), PE (15:0/15:0), sphingomyelin (SM; d18:1/17:0), phosphatidylserine (PS; 17:0/17:0), ceramides (Cer; d18:1/17:0), diacylglycerol (DG; 17:0/0:0/17:0), and triglyceride (TG; 15:0/15:0/15:0) were purchased from Avanti Polar Lipids (Alabaster, Alabama, US). High-performance liquid chromatography (HPLC)-grade isopropanol (IPA) and methanol were purchased from Merck (Darmstadt, Germany). Other HPLC-grade compounds, acetonitrile (ACN), formic acid, ammonium formate, leucine-enkephalin, and sodium formate, were purchased from Thermo Fisher Scientific (Shanghai, China).

2.2. Sample preparation

The fresh *L. crocea* roe was first powderized. This was achieved by drying using a vacuum freeze-dryer (True Ten Industrial Co., Ltd. Taichung, Taiwan) and filtering through an 80-mesh sieve. Then, a certain amount of water (1:2 w/w) was added into the *L. crocea* roe powder. The mixture was evenly stirred and moved onto a plate. A specific shape (1×4×4 cm) of the mixture was cut after extrusion and molding. Afterward, the mixture was moved into a commercial microwave oven (Newsail NS-X4, Henan, China). The roasting temperatures were 100, 120, 140, 160, and 180°C, and the roasting time was 20 min. Finally, the finished ready-to-eat product was prepared. After the product was cooled, it was vacuum-packaged and stored in a freezer (-20°C).

The finished product was added to 1.4 mL of IPA in a 2 mL centrifuge tube, vortex-mixed for 1 min, and sonicated for 10 min. The samples were kept in a freezer (-20°C) for 1 h and then freeze-centrifuged at 14,000 g for 10 min. The supernatant was collected, and 1 mL was filtered into UPLC vials through a 0.22 μm organic filter membrane. The samples were kept in a freezer (-20°C) for later analysis.

2.3. PL molecular species analysis via UPLC-Q-TOF-MS

2.3.1 UPLC parameters

The UPLC system was equipped with a C_{18}CSH column (1 × 50 mm, 1.7 μm; Waters Ltd., Elstree, U.K.). The mass spectrometry (MS) method of the Xevo G2-S Q-TOF (Waters Ltd., Manchester, U.K.) was implemented to improve the isotopic distribution and mass accuracy and to reduce the high ion intensities. In total, 2 μL of the samples was injected onto a C_{18}CSH column at 55°C. The mobile-phase flow rate was set as 400 μL/min. The mobile phases were as follows: (A) ACN/H_2O (60%/40%), including 10 mM ammonium formate and 0.1% formic acid; (B) IPA/ACN (90%/10%), including 10 mM ammonium formate and 0.1% formic acid. The gradient profile was as presented in Table 1.

Table 1. The gradient profile of UPLC.

Time (min)	% A	% B	Curve
Initial	60.0	40.0	Initial
2.0	57.0	43.0	6
2.1	50.0	50.0	1
12.0	46.0	54.0	6
12.1	30.0	70.0	1
18.0	1.0	99.0	6
18.1	60.0	40.0	6
20.0	60.0	40.0	6

Note: "6" means linear increasing, "1" means rapid increasing

2.3.2 Q-TOF-MS parameters

For both positive-ion and negative-ion modes, the MS parameters were as follows: capillary voltage of 3 kV, cone voltage of 25 V, ESI source temperature of 120°C, desolvation temperature of 500°C, desolvation gas flow of 800 L/h, and cone gas flow of 50 L/h. The mass spectra were acquired over m/z 50 to 2000. Leucine enkephalin (m/z 556.2771 in ESI^+, m/z 554.2615 in ESI^-) was continuously infused at 30 μL/min and used as the lock mass.

2.4. Statistical analysis

All analyses were conducted in triplicate, and the results were indicated as mean ± standard deviation. The means and standard deviations were calculated using the SPSS statistical software (version 19.0, SPSS). The software was used to perform a one-way analysis of variance and Tukey's honest significant difference test at a 95% confidence level ($P<0.05$) to identify differences among groups. A statistical *t*-test model was applied for comparative analysis involving different groups.
MassLynx software version 4.1 was used for the MS data acquisition and analysis. All lipid profile data were first standardized and normalized and then subjected to principal component analysis using SIMCA-P 13.0. Heatmaps of lipids data were created using the R software.

3. RESULTS AND DISCUSSION

3.1. Effect of roasting temperature on *L. crocea* roe appearance

Consumers usually assess the quality of a food product by its appearance, which is also important to evaluate how well a product is cooked. Fig. 1 shows the appearances of the prepared *L. crocea* roe product under different roasting temperatures. The appearance changed gradually (yellow-brown-deep brown) with increase in the temperature, from 0°C (control group) to 180°C. A similar finding has been reported for soybeans (YOSHIDA *et al*., 2003). NAKAMURA *et al*. (2011) reported that the color change during grilling consisted of four steps: (1) protein denaturation, (2) water evaporation, (3) browning reaction, and (4) carbonization reaction. According to MATSUDA *et al*. (2013), fish fillets began to darken during grilling when the temperature was close to 150°C under radiant (far-infrared radiation) heating. In the present study, the *L. crocea* roe product started to darken at140°C and exhibited an attractive appearance at 120°C.
Meanwhile, cyclic compounds (e.g., some aldehydes, pyrazines) can be formed to release a fragrant odor during roasting. The brown pigments from the *L. crocea* roe product could also degrade under very high temperatures. The lipids inside may deteriorate through hydrolysis and oxidation (FRITSCH, 1981). Furthermore, PEs have been reported to be related to lipid browning deterioration, which may cause a brown color (LEE *et al*., 1976). The PE in the *L. crocea* roe could be responsible for the browning at high temperatures through hydrolysis and oxidation.

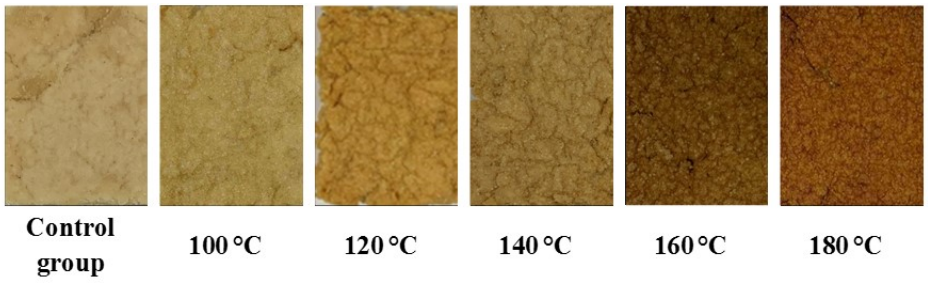

Figure 1. Appearances of the *L. crocea* roe at different roasting temperatures.

3.2. Lipid contents of *L. crocea* roe at different roasting temperatures

The lipid profile was identified and separated using UPLC-ESI-Q-TOF-MS. More analysis details can be seen in our previous paper (LIANG *et al*., 2018).
The species of 167 PCs, 105 PEs, 17 PIs, 26 PGs, 78 PSs, 55 PAs, 12 CLs, 17 SMs, 27 Cers, 10 MGs, 181 DGs, and 248 TGs were detected in the *L. crocea* roe product at different roasting temperatures.

3.2.1 Effects of roasting temperature on relative content of PL

Fig. 2 displays the relative content variations of different lipid classes at different roasting temperatures. Almost all lipid classes exhibited the same trend. Generally, their quantities decreased with temperature increase from 0 to 100°C, increased with further temperature increase to 120°C, at which the amounts were largest; and reduced again with temperature increase from 120°C to 180°C; however, PI, PG, PS, and CL exhibited some minor differences. PI and PG increased with temperature increase from 0 to 100 to 120°C. PI remained constant from 100°C to 160°C, with the largest amount at 160°C, and it decreased slightly from 160°C to 180°C. However, PG decreased gradually with temperature increase from 120°C to 180°C. PS and CL remained stable from 0 to 100°C, and the rest exhibited the same trend.

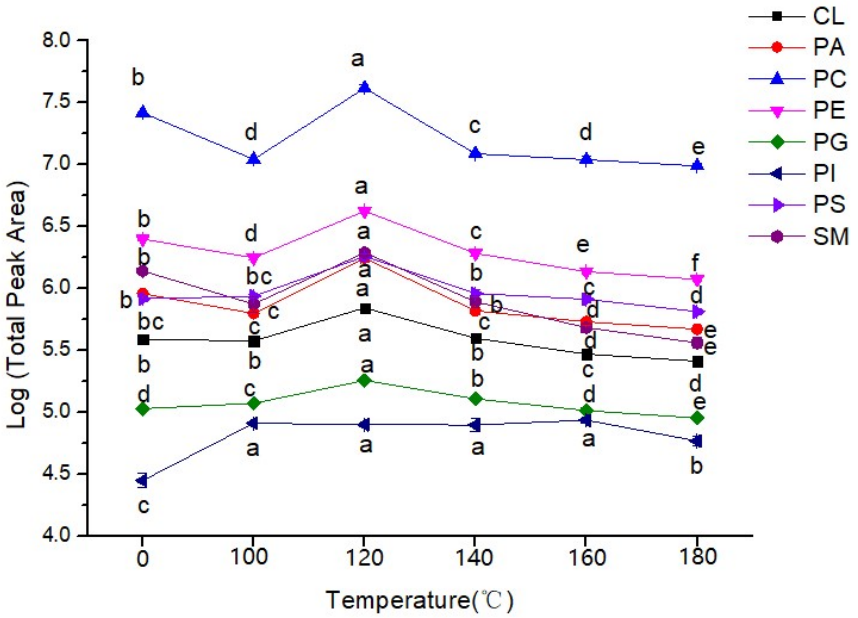

Figure 2. Effects of roasting temperature on the relative content of PLs in *L. crocea* roe.
Note: The same letters in the same column means that no significant difference exists between the amounts at the significance level of $P<0.05$, and vice versa.

The variable importance in projection (VIP) values were adopted for the selected lipid classes that changed significantly. The VIP method is usually used to identify the variables (VIP>1) in the orthogonal projections to latent structures discriminant analysis. According

to their VIP values (VIP>1), 56 PCs, 24 PEs, 17 PSs, 6 CLs, 5SMs, 4 PAs, 3 PGs, and 3 PIs were selected for analysis among the detected lipid molecular species.

All lipid classes except PI showed the highest value at 120°C. VUJASINOVIC *et al*. (2012) determined that the total PL content increased from 0 to 130°C (0, 90, 110, and 130°C) in roasted pumpkin oil. CLARK *et al*. (1991) reported that a high phosphorus content was obtained through the preheating process at 130 °C in crude soybean oil extracted from fine flour. They suggested that PLs had better solubility in the hot oil, and PLs may be released from cell membranes. However, to the best of our knowledge, this study is the first to identify 120 °C as the temperature at which the highest concentration of most lipid classes was found. Further research is needed to obtain the underlying reason for this. Perhaps, this temperature allowed the release of more lipids, which could not be extracted using the abovementioned method.

Apart from the temperature of 120°C, the PL class gradually declined from 0 to 180°C, except for PI, which agrees with the report on safflower seeds (LEE *et al*., 2004), in which temperatures of 0, 140, 160, and 180°C were considered using an electric oven. With increasing temperature, PC, PE, and PA were found to decrease, while PI increased. In the current study, the PL decomposition or formation through a reaction with protein or carbohydrate may explain the PL reduction after the roasting treatment (YOSHIDA *et al*., 2005). ABOU-GHARBIA *et al*. (2000) determined that PC, PI, PE, and PS in sesame oil decreased with roasting treatment (200°C) and steaming (100°C), while PA and LPC increased.

In this study, PI exhibited a distinctive trend, increasing with temperature increase from 0-100°C; this may be because it had the highest saturated fatty acid content among the PLs of the *L. crocea* roe product. The decrease in PE may be related to the PI increase; that is, PE transformed to PI with the increasing temperature (LEE *et al*., 2004). PC can be obtained via subsequent methylation of the amine by S-adenosyl methionine from PE. SM is the only lipid belonging to the sphingolipids class and the PL class. It was formed via the transfer of phosphorylcholine from PC to Cer via SM synthase (MERRILL, 2011). Therefore, it is possible that some PEs were transferred to PCs and that some PCs were transferred to SMs. Similarly, PS is formed when PA reacts with serine, and PE is formed when PA reacts with ethanolamine (AMBROSEWICZ-WALACIK *et al*., 2015). However, it is not clear if the reaction can occur in the *L. crocea* roe product at high temperatures. Further study is needed to explore this in more detail.

However, the compositions of each PL species are different in different food matrices; for example, marine PLs contain more n-3 PUFAs. Normally, the PLs, which contained more unsaturated fatty acids, were easily oxidized. A considerable loss has been detected for the PL molecular species containing more than four double bonds (YOSHIDA *et al*., 2001a). We analyzed the relative content changes in the PCs with VIP of more than four, and the results are illustrated in Fig. 3. The relative contents of these nine PCs displayed the same change trend as the PC total amount in Fig. 2. Almost all the nine analyzed PCs contained unsaturated fatty acids in their sn-2 positions except for one PC (0:0/16:0), and six of them consisted of C20:5 omega-3 and C22:6 omega-3, which influenced the omega-3/omega-6 ratio, meaning that they were easily oxidized in the oven. Similarly, the PEs, PGs, and PSs with the variable of VIP > 1 all contained an unsaturated fatty acid at their sn-1, sn-2 positions, or both.

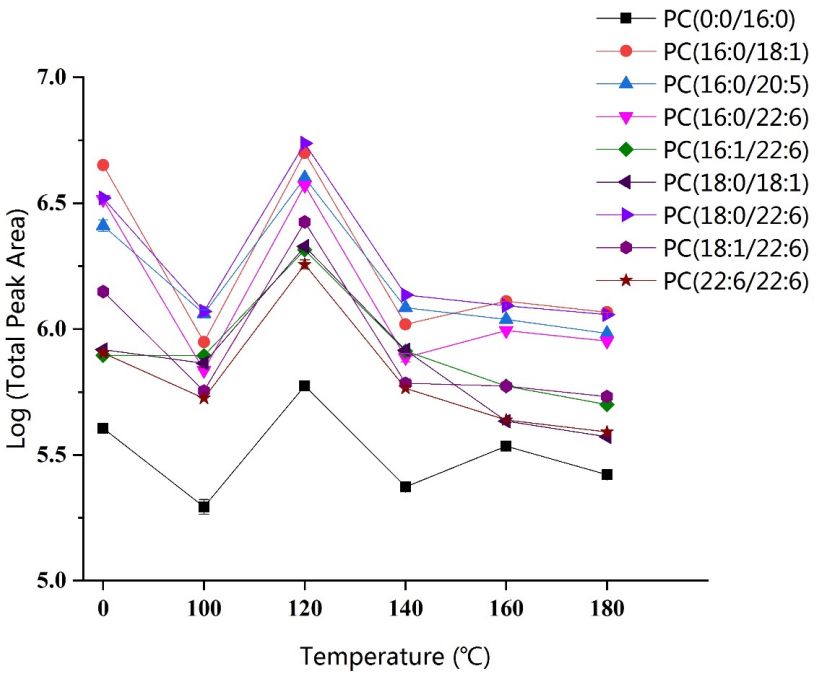

Figure 3. Effects of roasting temperatures on the relative content of PCs in the *L. crocea* roe.
Note: The same letters in the same column means that no significant difference exists between the amounts at the significance level of $P<0.05$, and vice versa.

3.2.2 Effect of roasting temperature on the relative contents of other lipids

A characteristic pattern of TGs exists in almost every type of oil or other food matrices. The pattern is determined by the abundances of different TG molecular species. YOSHIDA *et al*. (2001b) and COSSIGNANI *et al*. (1998) determined that the TG fraction decreased, while DG and MG increased over time in the microwave roasting for sunflower and olive oil.

In this study, 181 DGs, 248 TGs, 10 MGs, and 27 Cers were detected in the *L. crocea* roe at different roasting temperatures. According to Fig. 4, the relative contents of TGs, DGs, and MGs decreased with temperature increase from 0 to 100°C, which corresponds to the findings of previous studies, but the values increased again with temperature increase from 100 to 120°C and reached the highest point at 120°C, which is not mentioned in the other reports. YOSHIDA *et al*. (2001b) only tested the TG loss at 98, 137, 164, and 172°C; they did not know if the TGs changed at 120°C in sunflower oil; however, the TG content at 137°C was less than that at 172 °C, which disagrees with this study results. In this study, the DGs slightly increased with temperature increase from 140 to 180°C, possibly due to the TG decomposition; however, the MGs decreased, which may be because the temperature was still insufficient for the TG and DG decomposition to MGs. This inference needs further verification. Cer remained stable from 0 to 100°C, and the rest exhibited the same trend.

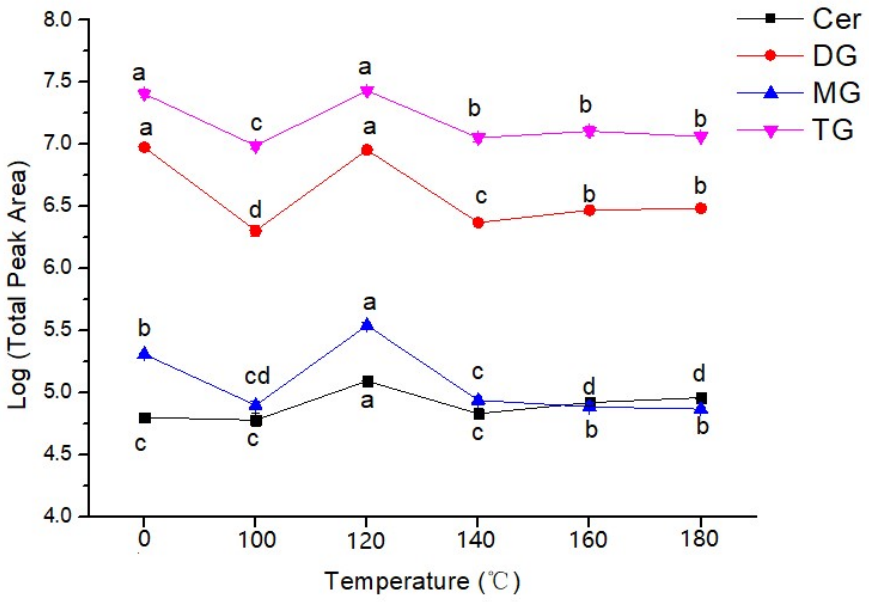

Figure 4. Effects of roasting temperature on the relative amount of glycerol lipids (GLs) and Cer in *L. crocea* roe.
Note: The same letters in the same column means that there is no significant difference between the amounts at the significance level of $P < 0.05$, and vice versa.

According to their VIP values (VIP>1), 4 Cers, 84 TGs, 31 DGs, and 2 MGs were detected in the lipid profile. However, for further analysis, three TGs were selected based on their VIP values (VIP>3); the compounds were TG (16:0/16:0/18:1), TG (16:0/16:1/16:1), and TG (16:0/16:1/18:1) with 53, 51, and 53 carbon numbers (CN), respectively. They all contained an unsaturated fatty acid linked at the sn-3-position. YOSHIDA *et al*. (2001a) reported that an unsaturated fatty acid linked at the sn-2-position of glycerol moiety in TGs helped to keep the moiety more stable than the same unsaturated fatty acid at the sn-1 or sn-3 positions. However, this finding disagrees with the results by LIU *et al*. (2017), who studied TGs with 51–56 CN, which were stable than TGs with 26-48 CN.

3.2.3 Heatmap analysis of the lipid profile data

A heatmap was adopted to better interpret the qualitative information of lipidomics datasets using the R software (Fig. 5). The lipid molecular species in the heatmap were selected according to a combination of multidimensional and one-dimensional analyses. The changes appeared between every two neighboring groups based on the VIP value and *P*-value in the student's *t*-test (VIP>1, $P<0.05$). Green color denotes increase, and red denotes decrease. From Fig. 5, all the selected lipid molecular species followed the same trends in Figs. 2 and 3. PCs were the molecular species that changed the most between two neighboring groups.

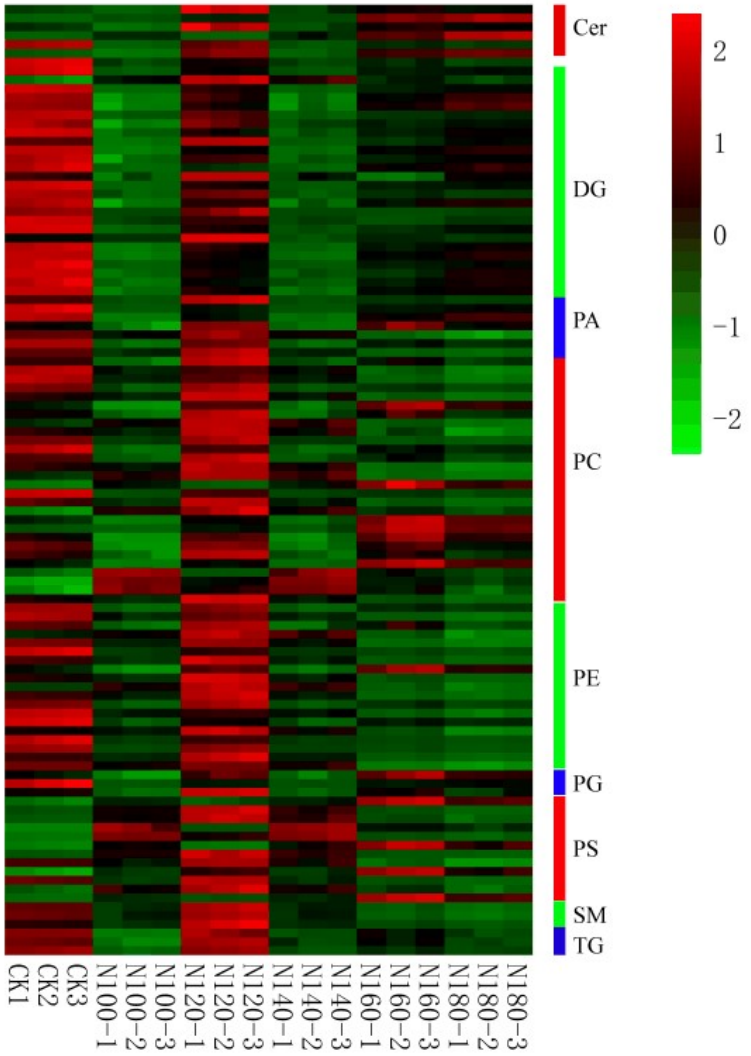

Figure 5. Heatmaps of the lipidomics dataset profiles.
Note: Red color represents an increase, and green represents a decrease. CK-1, CK-2, and CK-3 represent the first, second, and third analyses conducted using the control group, respectively; N100-1, N100-2, and N100-3 represent the first, second, and third analyses conducted using the group at 100 °C, respectively; the rest mark number is similar.

4. CONCLUSIONS

The *L. crocea* roe is a valuable byproduct that contains high amounts of valuable EPA and DHA. However, it is large and has an unattractive appearance. Discarding this roe as waste would be an important commercial loss and an environmental problem from the fish industry. This study investigated the further processing of the roe to make it more acceptable to consumers. The roe was roasted under different temperatures to determine the lipid amount changes. The relative amounts of almost all lipid classes were highest at 120°C, except for PI; meanwhile, the temperature of 120°C allowed to obtain a practically sterilized product, stable at room temperature if well packaged. No previous study has

mentioned this point before. Perhaps, this temperature is the best for processing without a significant loss of valuable PLs, as the obtained product is both of good quality and ready-to-eat. Moreover, this study clarifies the changes in lipid classes, especially PLs molecular species in the *L. crocea* roe, with temperature. Using the lipidomics method for analysis, the study also demonstrates the value of the method in the fish industry.

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ABBREVIATIONS

Phospholipid: PL
Phosphatidylcholine: PC
Phosphatidylethanolamine: PE
Phosphatidylinositol: PI
Phosphatidylglycerol: PG
Phosphatidylserine: PS
Phosphatidic acid: PA
Cardiolipin: CL
Sphingomyelin: SM
Ceramides: Cer
Diacylglycerol: DG
Triglyceride: TG
Monoacylglycerol: MG
Ultra-performance liquid chromatography–electrospray ionization–quadruple-time-of-flight–mass spectrometry: UPLC-ESI-Q-TOF-MS

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PAPER

CHEMICAL CHARACTERIZATION AND BIOACTIVE POTENTIAL OF ESSENTIAL OIL ISOLATED FROM *RHANTERIUM SUAVEOLENS* DESF. SPECIES GROWING IN TUNISIAN ARID ZONE

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ABSTRACT

The purpose of this work is to assess the antioxidant and the antibacterial activities of essential oil from flowers of *Rhanterium suaveolens* Desf. and to investigate its chemical composition. The GC-MS analysis revealed the identification of a total of thirty-one compounds representing 98.4% of the total oil. Spathulenol (18.3%), carvacrol (12.1%), linalool (9.4%), α-terpineol (7.10%), α-terpinolene (6.3%) and pinocarvone (5.6%) were identified as major constituents. The tested oil exhibited weak activities in both DPPH and ABTS radical scavenging assays and ferric reducing power test. However, it showed a good lipid peroxidation activity using the β-carotene/linoleic acid assay with an IC_{50} value of 26.20±1.01 μg/mL. In addition, the highest antibacterial effect was recorded against *Staphylococcus aureus* ((MIC=37.5 μg/mL). These findings show that essential oil of *R. suaveolens* flowers can be used as a promising source of natural food and drug preservatives.

Keywords: antioxidant, antibacterial, essential oil, *Rhanterium suaveolens*, chemical composition

1. INTRODUCTION

Food preservatives are usually used to extend the shelf life of food products and to limit their deterioration caused by oxidation and growth of foodborne pathogens (RUSSELL and GOULD, 2003). As harmful effects caused by the extensive use of chemical preservatives and the increase of microbial resistance to a wide number of antibacterial drugs (SHAN *et al.*, 2007), the search of new bioactive substances with interesting biological activities is required. In this purpose, several studies have been carried out for the prospection of new products derived from plants and their potential use as ingredients in food and pharmaceutical industries (BEN SALAH *et al.*, 2019).

Essential oils are natural products known for their multi-propose applications (DE MARTINO *et al.*, 2015). They have shown a big interest as agents with several healthy-promoting activities such as antibacterial, antioxidant, anti-carcinogenic and antimutagenic properties (GUTIERREZ *et al*., 2009). Therefore, their investigation proves to be a relevant choice in order to limit the use of chemical or synthetic preservatives and minimize their toxic effect (CAILLET and LACROIX, 2007). The use of essential oils can improve food safety and protect our body against bacteria causing food poisoning (ULTEE *et al*., 2000). In fact, many studies have demonstrated the potential use of essential oils as natural antimicrobial agents in cheese-making industry (KHORSHIDIANA *et al*., 2018). As an alternative of specific applications, the essential oils can also be prepared in a large number of formulations, which can be used in food preservation. Recently, GIRARDI *et al.* (2018) reported that the application of microencapsulated *Peumus Boldus* essential oil was useful to prevent peanut deterioration caused by food spoilage microorganisms. This biological potential is mainly attributed to the presence of several constituents such as oxygenated derivatives and terpenoids (ABERRANE *et al.*, 2019; BIDA *et al*., 2019).

Tunisian flora is characterized by a wide variety of aromatic and medicinal species producing several bioactive substances with multiple interests (SALEM *et al.*, 2018). However, only few of these species have been investigated for their antioxidant and antibacterial potential. For example, *Rhanterium suaveolens* Desf. from the *Asteraceae* family is an endemic species from North Africa growing in Algerian Sahara (QUEZEL and SANTA, 1963) and arid zone of Tunisia (CHAIEB and BOUKHRIS, 1998). Three species of the genus *Rhanterium*; namely, *R. epapposum* Oliver, *R. adpressum* Coss. & Durieu and *R. suaveolens* Desf. have been reported in literature. *R. suaveolens* commonly known as "Arfadj" is a forage plant, grazed on by sheep and camel in the desert. It is used by the local population in the production of cheese and in folk medicine as an antidiuretic (HAMIA *et al.*, 2013).

To the best of our knowledge, only few studies have been conducted on the phytochemistry of the *R. suaveolens* essential oil (RSEO) and information on its biological activities, particularly, antioxidant potential, are still scarce in literature. Therefore, the main purpose of this study was to investigate the chemical profile of essential oil collected from the flowers of *R. suaveolens* growing in arid zone of Tunisia and to evaluate its antioxidant and antibacterial activities.

2. MATERIALS AND METHODS

2.1. Plant material

Flowers of *R. suaveolens* were collected during the flowering period in April 2014 from a single population of this species growing in Gorthab from the Tataouine region situated in the South East of Tunisia. Taxonomic identification of the plant material was confirmed by a local botanist at the Institute of Arid Zone Research in Medenine (Tunisia). A voucher specimen (IRABS1865) was prepared and deposited in the Herbarium of the Laboratory of Pastoral Ecology. The collected plant material was cleaned and then air-dried at room temperature for eight to ten days. .The dried flowers were ground to powders and stored in air-tight glass.

2.2. Essential oil extraction

Air dried flowers (100 g) of *R. suaveolens* were subjected to hydrodistillation in a Clevenger-type apparatus for 3h. The obtained oil was dried over anhydrous sodium sulfate (Na_2SO_4) to remove water traces and stored in amber glass vials at 4°C. The oil yield (%) was expressed as volume of essential oil vs. dry weight basis (v/w).

2.3. Gas Chromatography/Mass spectrometry (GC-MS) analysis

The GC-MS analysis of the essential oil was carried out using an Agilent 6890N Network GC system combined with Agilent 5975 B Inert MSD detector (quadrupole) with electron impact ionization (70 eV). AHP-5MS (5% phenyl methyl siloxane) column (30 m×0.25 mm i.d, film thickness 0.25 mm). The analysis was performed using helium (purity > 99.99 vol.%) as a carrier gaz at a flow rate of 1.0 $mL.min^{-1}$. The column temperature was programmed to rise from 50 to 280°C at a rate of 7 °C/min. Injector and detector temperatures were maintained at 220 and 240°C, respectively. Essential oil (1 μL) was injected in a split mode ratio of 1:10. Scan time and mass range were 2.2 s and 50–550 m/z, respectively.

2.4. Identification of the essential oil constituents

Identification of the *R. suaveolens* essential oil (RSEO) components was based on their linear retention indices (RIs) and comparison of their mass spectra with those of the computer library (Wiley 275 library and NIST98 database/ChemStation data system) provided by the instrument software and MS literature data (JOULAIN *et al.*, 2001; ADAMS, 2001). RIs were calculated using n-alkane series (C_6–C_{22}) analysed under the same GC–MS conditions as for the samples.

2.3. Antioxidant assays

2.3.1 Scavenging effect on DPPH (2,2-diphenyl-l-1-picrylhydrazil) radical

The DPPH assay was estimated as described by DHAOUADI *et al*. (2014), with slight modifications. Different concentrations of the RSEO were prepared in pure methanol, then 50 μL of each of them were added to 950 μL of a 40 μmol/L (v/v) DPPH methanolic solution in methanol. After vigorous shaking, the resulting mixtures were left in the dark

at room temperature for 30 min. The absorbance of the resulting solutions was measured at 517 nm. And the radical scavenging ability of RSEO was measured as shown below:

$$DPPH\ scavenging\ effect\ (\%) = \frac{Ao - At}{Ao} \times 100$$

Where $A0$ is the absorption of the control sample after 30 min and At is the sample absorption after 30 min. The antioxidant activity was expressed as IC50 value (mg/mL).

2.3.2 Scavenging effect on ABTS (2,20 azinobis-3-ethylbenzthiazoline-6- sulphonic acid) radical cation

The ABTS+ assay was performed according to a slight modified version of the method described by TUBEROSO *et al*. (2007). The radical cation was produced by mixing the ABTS+ solution (7 mmol/L) with potassium persulfate aqueous solution (2.45 mmol/L). The ABTS+ solution was kept in the dark at room temperature for 12-16 h, then, was diluted with phosphate buffer to the absorbance of 0.7±0.02 at 734 nm. Different concentrations of RSEO were prepared in methanol. To 50 μL of each test concentration, 950 μL of diluted ABTS solution were added. The resulting mixtures were allowed to incubate in the dark for 10 min at room temperature. The absorbance of the mixtures was recorded at 734 nm. The antioxidant activity was calculated as follows:

$$Inhibition\ (\%) = \frac{1 - (\mathrm{A} - \mathrm{B})}{\mathrm{C}} \times 100$$

where, A is the absorbance of the mixture containing the sample, B is the absorbance of the blank reagent and C is the absorbance of the blank sample.The concentration providing 50% of inhibition (IC_{50}) was calculated using a calibration curve in the linear range by plotting the extract concentration.

2.3.3 Reducing power assay

The reducing power of the RSEO was assessed following the method described by SINGH *et al.* (2012). One mL of phosphate buffer (0.2 M 'w/v', pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$], 1% 'w/v' was mixed with 1 mL of different concentrations of RSEO (10, 20, 30, 40 and 50 mg/mL). The obtained mixtures were incubated at 50°C for 20 min. Then 1 ml of trichloroacetic acid (TCA) (10% 'w/v') was added. The resulting mixtures were revolved at 3000 rpm for 10 min. The supernatant was recovered and mixed with 1.5 mL of distilled water and 150 μL of $FeCl_3$ (0.1% 'w/v'). The absorbance was measured at 700 nm and the butylated hydroxyanisole (BHA) was used as standard. The result was expressed as IC_{50} (mg/mL).

2.3.4 Lipid peroxidation activity

The lipid peroxidation activity of RSEO was carried out by β-carotene/linoleic method according to DAPKEVICIUS *et al.* (1998), which is based on the inhibition of the products resulting from the oxidation of linoleic acid. A stock solution of β-carotene/linoleic acid was prepared by mixing 200 mg of Tween 40, 0.5 mg of β-carotene, 25 μL of linoleic acid and 1 mL of chloroform. After chloroform evaporation, under low pressure at 40°C, 100

mL of oxygenated distilled water were added to the mixture with vigorous shaking. An aliquot of the resulting solution (2.5 mL) was dispersed to test tubes and 0.5 mL of prepared sample with different concentrations (5-40 μg/mL) in methanol and water were added. The obtained emulsion was incubated for 2 h at 50°C. Two controls were prepared, one with the standard BHA (positive control) and the other without BHA or extract (blank). The absorbance of each sample was immediately measured at 490 nm after 30 min, 60 min, 90 min and 120 min.
The bleaching rate R of β-carotene was determined according to the following equation:

$$R = \frac{\ln(\frac{A}{B})}{T}$$

Were ln=natural log, A=absorbance at time 0, B=absorbance at time T (30 min, 60 min, 90 min and 120 min). Antioxidant activity was calculated in terms of inhibition percentage using the following equation:

$$Antioxidant\ activity\ (\%) = \frac{(Rcontrol - Rsample)\times 100}{Acontrol}$$

Results were expressed as IC_{50} value (μg/mL).

2.4. *In vitro* evaluation of antibacterial activity

2.4.1 Tested bacterial strains and growth conditions

The antibacterial activity of RSEO was tested against a range of bacterial strains collected from American Type Culture Collection (ATCC, Rockville). Gram-positive: *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19115) and *Bacillus cereus* (ATCC 14579) and Gram-negative: *Eescherichia coli* (ATCC 35218), *Salmonella Typhimurium* (NRLB 4420) and *Pseudomonas aeruginosa* (ATCC 27853). All bacterial strains were cultured at 37°C for 24h in Mueller-Hinon agar (MHA). The cultures were started by adjusting the bacterial suspension in broth to 0.5 Mc Farland turbidity. Then the bacterial suspension was diluted using 10 fold serial dilution method in order to obtain an inoculum of 10^8 colony-forming units (CFU) per plates (DHAOUADI *et al*., 2015).

2.4.2 Disk diffusion method

The *in vitro* antibacterial activity of RSEO was estimated using the disk diffusion method described by DHAOUADI *et al.* (2015) with slight modifications. 10 μL of RSEO were placed onto sterilized paper disc (6 mm ∅), and placed onto the inoculated agar surface. The petri dishes were placed at 4 °C for 1 h and then incubated at 37 °C for 24 h. After incubation, the diameters of the resulting inhibition zones were determined. Tests were performed in triplicate. Gentamicin (10 μg per disk) was used as positive control and sterile water as negative control.

2.4.3 Microdilution method

The antibacterial activity of RSEO was also assessed by the determination of minimum inhibitory and bactericidal concentrations (MIC and MBC) using broth microdilution method. The minimal inhibition concentration (MIC) was determined as described by GULLUCE *et al.* (2007) with slight modifications. RSEO sample previously dissolved in 10% dimethylsulfoxide (DMSO) was first diluted to the highest concentration (3 mg/mL) to be tested. The 96 well plates were prepared by dispensing into each well 95 μL of the nutrient broth and 5 μL of the inoculum. An aliquot from the stock solution of RSEO (100 μL) was added into the first well. Then, 100 μL from the serial dilutions were transferred into eleven consecutive wells. The last well containing 195 μL of nutrient broth without RSEO and 5 μL of the inoculum on each strip were used as the negative control. After that, the plates were incubated at 37°C for 24 h. All samples were screened two times against each microorganism.The MIC is defined as the lowest concentration of the sample that did not allow any visible growth of the tested bacterial strain (BEN SALAH *et al.,* 2019).
To the determination of the MBC value an aliquot (25 μl) was spreaded onto MHA plates and then incubated for 12–16 h at 37°C. The determination of surviving bacterial strains allowed the estimation of the MBC at 99.9 % of bacterial death (FATTOUCH *et al.,* 2007).

2.5. Statistical analysis

All experiments were repeated in triplicate and the results were reported as mean values and standard deviation (mean±SD). Significance differences between the results were performed by analysis of variance (ANOVA) using Tukey's multiple comparison tests at a level of significance set at $P<0.05$. Data analysis was performed using Minitab 18 Statistical Software (Minitab Inc., U.S.A.).

3. RESULTS AND DISCUSSION

3.1. Essential oil composition

The volatile oil extracted from *R. suaveolens* flowers has yellow color with an agreeable intense smell. Its extraction yield was about 0.23%±0.02 (volume/dry weight), which was similar to that reported by BEN SALAH *et al.* (2019) (0.22%), and was slightly, higher than that obtained from the aerial parts of Algerian *R. suaveolens* (0.14%) (CHEMSA *et al.,* 2016).
As depicted in Table 1, thirty-one components have been identified in the RSEO which represent 98.4% of the total composition. This oil contains a complex mixture dominated by oxygenated monoterpenes (46%) followed by oxygenated sesquiterpenes (23.6%) and monoterpenes hydrocarbons (17.5%). The major components of the RSEO were identified as spathulenol (18.3%), carvacrol (12.1%), linalool (09.4%), α-terpineol (7.10%), α-terpinolene (6.3%) and pinocarvone (5.6%). Compared to previous studies, our findings differ from those reported by BEN SALAH *et al.* (2019), with α-pinene (25.84 %), β-pinene (17.57 %), 1-octen-3-ol (16.23 %), camphene (12.28 %), limonene (8.03 %) and β-myrcene (5.13 %) as major compounds. Also, the composition of the Algerian *R. suaveolens* essential oil showed a significant difference in the chemical composition (CHEMSA *et al.,* 2016).

Table 1. Chemical composition of the essential oil from the flowers of *R. suaveolens* analysed by GC-MS.

No.	Compounds[a]	RI_{exp}[b]	RI_{lit}[c]	% Area	Identification methods
1	α-thujene	926	924	0.5±0.02	RI, MS
2	α-pipene	936	939	2.5±0.03	RI, MS
3	Camphene	955	956	1.8±0.01	RI, MS
4	β-pinene	981	979	1.9±0.01	RI, MS
5	β-myrcene	990	993	0.5±0.02	RI, MS
6	α-terpinene	1016	1017	2.4±0.04	RI, MS
7	Limonene	1030	1029	1.2±0.01	RI, MS
8	γ-Terpinene	1060	1059	0.4±0.01	RI, MS
9	α-terpinolene*	1088	1089	6.3±0.10[d]	RI, MS
10	Linalool*	1095	1098	9.4±0.08[c]	RI, MS
11	*Trans*-sabinol*	1140	1142	4.1±0.01[e]	RI, MS
12	p-menth-4(8)-ene	1157	1160	1.6±0.02	RI, MS
13	Pinocarvone*	1160	1164	5.6±0.21[de]	RI, MS
14	α-terpineol*	1190	1192	7.10±0.02[d]	RI, MS
15	*Trans*-carveol	1220	1217	1.5±0.01	RI, MS
16	Carvone	1242	1250	0.5±0.01	RI, MS
17	Geraniol	1255	1252	1.5±0.03	RI, MS
18	α-Thujenol	1287	1290	2.6±0.01	RI, MS
19	Carvacrol*	1298	1299	12.1±0.21[b]	RI, MS
20	*Trans*-caryophyllene	1415	1419	0.6±0.1	RI, MS
21	Aromadendrene	1437	1437	3.3±0.1	RI, MS
22	Alloaromadendrene	1463	1458	0.7±0.2	RI, MS
23	Eremophilene	1511	1512	2.4±0.1	RI, MS
24	ð-cadinene	1522	1523	0.1±0.2	RI, MS
25	α-calacorene	1541	1546	0.4±0.1	RI, MS
26	Spathulenol*	1577	1577	18.3±0.1[a]	RI, MS
27	Caryophyllene oxide*	1581	1582	4.8±0.2[de]	RI, MS
28	α-cadinol	1676	1652	0.5±0.01	RI, MS
29	Myristic acid	1762	1767	0.6±0.1	RI, MS
30	Palmitic acid methyl ester	1908	1909	0.3±0.1	RI, MS
31	Palmitic acid	1970	1968	2.9±0.02	RI, MS
	Monoterpene hydrocarbons			17.5	
	Oxygenated monoterpenes			46.0	
	Sesquiterpene hydrocarbons			7.50	
	Oxygenated sesquiterpenes			23.60	
	Others (%)			3.80	
	Total identified (%)			98.4	

[a]Compounds are listed in order of their elution from a HP-5MS column. [b]Experimental linear retention index on a HP-5MS capillary column using the homologous series of n-alkanes. [c]Linear retention index from literature. [d]Peak area of the essential oil components. [e]Compounds were identified based on their RI on HP-5MS capillary column and GC-MS data. Values are given as mean± S.D. (n=3).
*Values with different letters with in the same column indicate significant difference ($p<0.05$).

The presence of spathulenol, linalool and carvacrol mentioned in this work as major constituents, had never been, already, reported for the *R. suaveolens* species. The differences observed between our findings and those previsouly reported by BEN SALAH *et al.* (2019) and CHEMSA *et al.* (2016) can be attributed to the environmental, agronomic, age and geoclimatic factors (season, location, fertility regime, soil type and climate) as well as the experimental extraction conditions (BOUKHATEM *et al.*, 2014; SINGH *et al.*, 2012).

3.2. Antioxidant activity

As depicted in Figs. 1, 2 and 3, the inhibition of the DPPH and ABTS radicals, the reducing power and the inhibition of lipid peroxidation activities of the RSEO, respectively, are dose dependent.

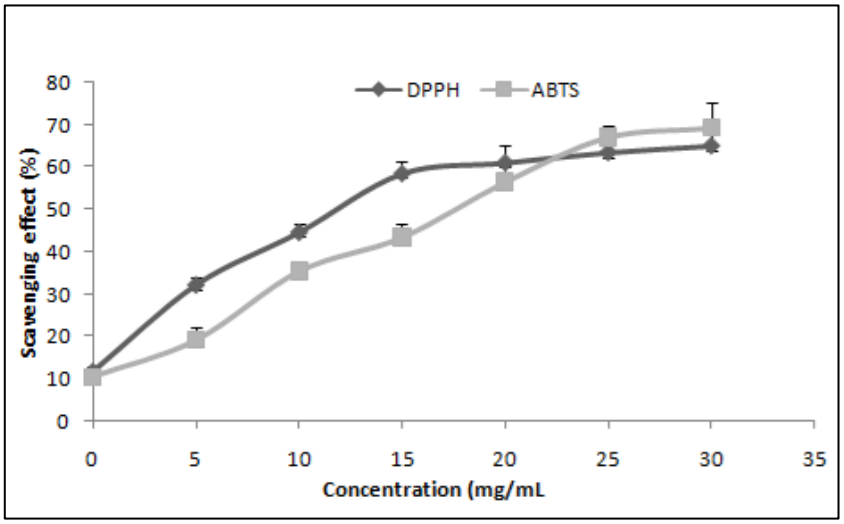

Figure 1. DPPH· and ABTS·· free radical-scavenging properties of the essential oil of the *R suaveolens* flowers. Data were presented as means±SD (n=3).

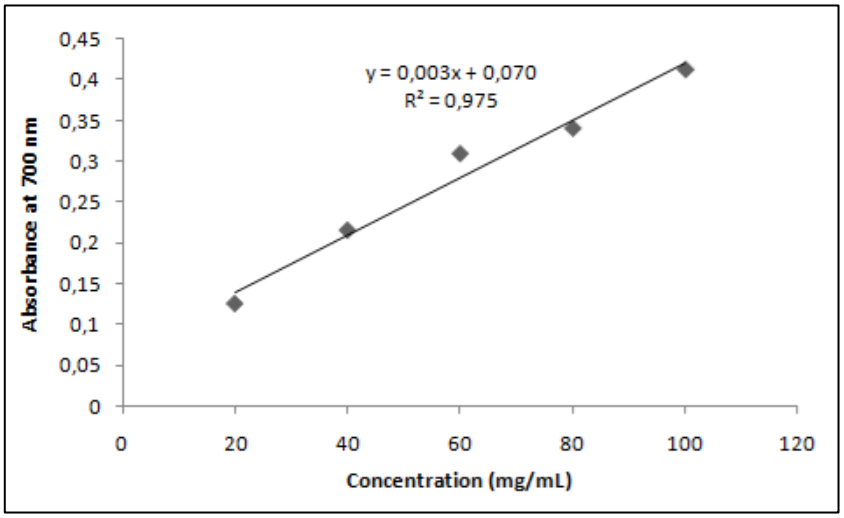

Figure 2. Reducing power of the essential oil of the *R. suaveolens* flowers.

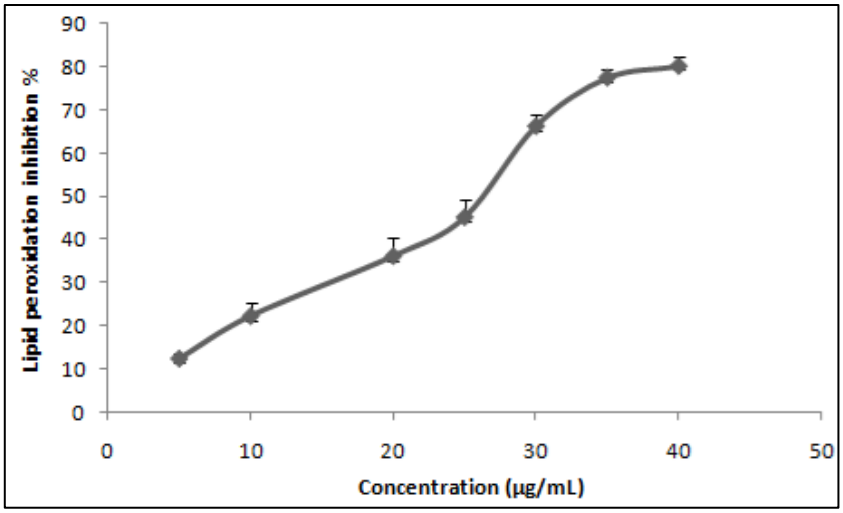

Figure 3. Antioxidant activity (%) of essential oil of the *R. suaveolens* flowers measured by β-carotene–linoleic acid method. Values expressed are means±S.D. (n=3)

As can be seen in Table 2 the IC_{50} values obtained for both DPPH and ABTS assays (11.48±0.11 mg/mL, 18.58±0.39 mg/mL, respectively) are significantly ($p<0.05$) higher than those observed for the tested standard BHA (0.041±0.002 mg/mL, 0.032±0.004 mg/mL, respectively). In other words, the activity of the selected essential oil is lower than that of the BHA standard. Our results are in agreement with the findings of the RSEO isolated from Algeria, which exhibited a weak DPPH scavenging activity (CHEMSA *et al.*, 2016).
As depicted in Table 2, the ferric reducing power of the RSEO (IC_{50}=58.95±1.21 mg/mL) was significantly ($p<0.05$) lower than that of the BHA standard (IC_{50}=0.052±0.001 mg/mL).

Table 2. Antioxidative capacities of the essential oil of the *Rhanterium suaveolens* flowers.

	IC_{50}			
	DPPH (mg/mL)	**ABTS (mg/mL)**	**Reducing power (mg/mL)**	***B*-carotene/linoleic acid (µg/mL)**
Essential oil	11.48±0.11[b]	18.58±0.39[b]	58.95±1.21[b]	26.15±1.01[b]
BHA	0.041±0.002[a]	0.032±0.004[a]	0.052±0.001[a]	5.95±0.82[a]

BHA standard was used as a reference. All the values are means±SD (Standard Deviation) of three parallel measurements. Different letters in the same column indicate a significant difference ($p<0.05$).

The inhibition of the lipid peroxidation activity of the tested essential oil was carried out using the β-carotene bleaching test. As shown in Table 2, the activity of the RSEO was lower (IC_{50}=26.15±1.01 µg/mL) than that of the synthetic standard BHA (IC_{50}=5.95±0.82 µg/mL). Also, this activity is less important than that previously reported for the essential oil of the Algerian *R. suaveolens* aerial parts with an IC_{50} of 17.97±5.40 µg/mL (CHEMSA *et al.*, 2016). Compared to the radical scavenging effects and the reducing power, the RSEO is more active on the inhibition of the lipid peroxidation. This can be, probably, due to the

high specificity of the test to lypophilic molecules (HARKAT-MADOURI *et al.*, 2015). It has been reported that the lipid peroxidation activity may be due to the richness of the tested oil on conjugated sesquiterpenoids. Indeed, these compounds can scavenge the singlet oxygen and consequently, protect the β-carotene color against bleaching, indirectly (CHEMSA *et al.*, 2016). The weak antioxidant activity observed for the RSEO can be related to its chemical composition as well as the abundance of ineffective compounds such as the monohydroxylated compounds which are unable to chelate ferrous ions (HARKAT-MADOURI *et al.*, 2015; AIDI WANNES *et al.*, 2010; DZAMI *et al.*, 2013).
The low antioxidant potential of the tested oil can, also, be attributed to the degradation of bioactive compounds during their extraction. Indeed, during hydrodistillation process, plant material is usually extracted in boiling water for a long period which could cause thermal decomposition of the thermolabile target molecules inducing, therefore, a decrease in the antioxidant capacity of the extract (BAGHERI *et al.*, 2014).

3.3. Antibacterial activity

The antibacterial activity was evaluated against six foodborne pathogens (3 Gram-positive and 3 Gram-negative), using the dilution and disk diffusion methods. As shown in Table 3, the RSEO was sensitive to all tested bacteria and exhibited a variable antibacterial activity dependent on the tested strains. *S. aureus* was the most susceptible bacteria with the largest inhibition zone (IZ=18.25±0.35 mm) followed by *L. monocytogene* (IZ =17.37±0.53 mm) and *B. cereus* (16.0±0.0 mm). However, the highest resistance to the RSEO was observed for the *S. typhimurium* with the lowest ($p<0.05$) inhibition zone (IZ=12.25±0.35 mm). Results showed that the tested essential oil was slightly more active against Gram-positive than Gram-negative bacteria. This can be explained by the complexity of their double membrane containing cell envelope, which can limit the diffusion of hydrophobic compounds through its lipopolysaccharide covering. Generally, the bacteriostatic and/or bactericide action of the plant extracts is attributed to their ability to disrupt cell membrane structures, disturb their permeability barrier and, consequently, to cause the chemiosmotic control loss (BAGAMBOULA *et al.*, 2004). As depicted in Table 3, the MIC and MBC values of the RSEO ranged from 75 to 300 μg/mL for the tested bacterial strains. The highest antibacterial activity was observed against *S. aureus* with the lowest ($p<0.05$) MIC and MBC values (37.5 μg/mL and 75 μg/mL, respectively). There are a few reports on the antibacterial activity of the *R. suaveolens* essential oil for comparison. Recently, BEN SALAH *et al.* (2019), developed the antibacterial activity of Tunisian RSEO against a broad spectrum of bacterial strains. Our findings showed discrepancies between their published data. Larger inhibition zones values were reported against *E. coli* and *P. aeruginosa* (19 mm, 23 mm, respectively). The corresponding MICs were found 230μg/mL for *E.coli* and 46 μg/mL for *P. aeruginosa* (BEN SALAH *et al.*, 2019). In addition, a moderate antibiofilm potential has been reported against six Gram positive bacteria for the essential oil collected from the aerial parts of Algerian *R. suaveolens* (CHEMSA *et al.*, 2016). The differences between our findings and those prevesiouly reported by other authors, may result from different chemical compositions and percentage content of active constituents in the tested essential oils. Factors such as the choice of bacterial strains and their sensitivity, the experimental conditions and the choice of methods used for in vitro antibacterial activity could also be related to the variation in the experimental results (SIDDIQUE *et al.*, 2017).

Table 3. Antibacterial activity of essential oil of the *R. suaveolens* flowers using disc diffusion method and determination of MIC and MBC values.

Bacterial strains	IZ (mm±SD)	Gentamicine	MIC (μg/mL)	MBC (μg/mL)
Gram positive Bacteria				
S. aureus (ATCC 25923)	18.25±0.35^{a}	34.50±0.71^{a}	37.5	75
L. monocytogenes (ATCC19115)	17.37±0.53^{a}	31.00±0.00^{b}	75	150
B. cereus (ATCC14579)	16.00±0.00^{ab}	24.00±0.00^{d}	150	300
Gram negative Bacteria				
E. coli (ATCC35218)	15.62±0.17^{b}	26.00±0.00^{c}	75	150
S. typhimurium (NRLB4420)	12.25±0.35^{d}	21.50±0.71^{e}	150	300
P. aeruginosa (ATCC27853)	14.00±0.00^{c}	32.50±0.71^{b}	150	150

IZ: The diameter of the inhibition zones (mm), including the well diameter (6 mm), are given as mean±SD (n=3). Gentamicine: is used as positive control for bacteria. Different letters in the same column indicate a significant difference (p<0.05).

The appreciable antibacterial potential of the RSEO against some bacterial strains could be related to the presence of a high amount of phytochemicals such as monoterpenes and oxygenated monoterpenes (AGGARWAL *et al.*, 2002). Effectively, many studies have proved the presence of a relationship between the chemical composition of the major components of the essential oils and the antibacterial activity (BEL-HADJ *et al.*, 2017). The major compounds identified in the RSEO such as spathulenol, carvacrol, linalool, α-terpineol, α-terpinonene and pinocarvone have not been tested for their antibacterial activity in the present study. However, some reports have approved their antibacterial properties. Indeed, a number of researchers have shown that carvacrol and linalool are well-known substances with pronounced antimicrobial activity against several pathogenic bacteria (BOZIN *et al.*, 2006). Likewise, it was found that spathulenol and linalool exhibited moderate to strong activities against several microorganisms (MAGIATIS *et al.*, 2002). In addition, it has been revealed that interactions between the constituents of some essential oils may contribute to different effects such as additive, synergistic, or antagonistic (DELAQUIS *et al.*, 2002). A study conducted on the release of the cellular materials test, showed that α-terpineol/linalool combination treatments have shown a strong effect on the release of cell constituents both from Gram-negative and Gram-positive bacteria (ZENGIN and BAYSAL. 2014).

4. CONCLUSIONS

This paper reports the chemical composition and the *in vitro* antioxidant and antibacterial properties of the essential oil collected from the *R. suaveolens* flowers. The GC–MS analysis revealed the identification of 31 constituents. Spathulenol, linalool and carvacrol identified as major compounds, were reported for the first time in the essential oil of this species. Results obtained from the β-carotene/linoleic acid bleaching assay were found to be stronger than those obtained from DPPH, ABTS and CUPRAC systems. Apart from its weak antioxidant activity, the tested essential oil has shown an interesting antibacterial activity against foodborne pathogens, especially, *Staphylococcus aureus* and *Listeria*

monocytogenes. These findings suggest the possible use of RSEO in the food industry as a potential new source of natural additives for functional and nutraceutical food applications.

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PAPER

NMR AND UPLC-QTOF/MS-BASED METABOLOMICS OF DIFFERENT DEVELOPMENTAL STAGES OF *CYNOMORIUM SONGARICUM*

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ABSTRACT

Cynomorium songaricum Rupr., a holoparasitic plant that grows in the desert and has important dietetic and medical value. However, the medicinal efficacy of *C. songaricum* collected in different seasons varies greatly, and the difference in the transformation and accumulation of its major metabolites is still unclear. In this paper, UPLC-QTOF/MS and NMR were used to study the metabolomics of *C. songaricum* in different growth stages, so as to explore the metabolic differences and regularity of *C. songaricum* in different phenological periods during one cycle. The results showed that there were thirty and sixteen compounds with significant differences based on UPLC-QTOF/MS and NMR, respectively, which were distributed in flavonoids, organic acids, sugars and amino acids. Among them, the content of secondary metabolites such as catechins and procyanidins accumulated more in the Unearthing (U), and Maturing (M) stages, while other differential compounds accumulated more in the Tubercle (T), Sprouting (S) and Atrophy (A) stages. The differential metabolic pathways of *C. songaricum* in different stages involved in flavonoids, sugar, amino acid and other pathways. This provides scientific basis for understand of metabolites accumulation, quality evaluation and use as medicinal materials for *C. songaricum*.

Keywords: parasitic plants, desert, comparative metabolomics, partial least squares, multivariate analysis

1. INTRODUCTION

Cynomorium songaricum Rupr. is native to China and parasitizes on the root of Nitraria sp., plant of the Zygophyllaceae family (CHEN *et al*., 2019). There are various bioactive substances of *C. songaricum*, which render *C. songaricum* abilities to enhance adrenocortical secretion, sex and bowel function (LIU *et al*., 2011). Modern pharmacological studies have shown that *C. songaricum* has the functions of promoting human metabolism, enhancing immune regulation, anti-cancer, anti-fatigue, anti-aging, anti-stress, scavenging free radicals, inhibiting the proliferation of HIV and maintaining the balance of trace elements in human body (CUI *et al*., 2013). Traditional medication practice believes that this is due to its unique active ingredients, such as flavonoids, triterpenoids, tannins, organic acids and polysaccharides and glycosides (CUI *et al*., 2018a). However, the contents and types of these bioactive components are greatly influenced by different developmental stages of *C. songaricum*, which is considered to be the main reason for the variation of its quality. Although the Chinese Pharmacopoeia (2015 ver.) recommends that the clinical materials of *C. songaricum* should be collected in spring (Committee for the Pharmacopoeia of PR China, 2015), the ethnic minorities living in the desert believe that *C. songaricum* works best if the plants were collected from the coldest winter (around November). After snowing, they often dig deep in the depths of the ground without snow and obtain high-quality *C. songaricum*. Therefore, it is generally believed that *C. songaricum* collected in other periods except winter and spring, especially at the period after flowering, has poor quality (CHANG *et al*., 2007). In fact, due to the comprehensive influence of gene timing regulation, metabolic transformation of substances and changes of external environment factors, the growth and composition of metabolites of medicinal plants should be different in each developmental period, which form the timeliness characteristics of traditional Chinese medicine (CUI *et al*., 2018b).

In the past, the quality control of *C. songaricum* depends on the contents of several main active ingredients. For example, the evaluation of *C. songaricum* in Chinese Pharmacopoeia depends on the contents of ursolic acid and proline, and in folk practice, polysaccharides, protocatechuate and catechin were also used as evaluation criterion (CUI *et al*., 2018c). However, the metabolites are mutual transformed all the time, and their accumulations vary under different spatial-temporal conditions. For example, it was reported that the contents of protocatechuate, ursolic acid, catechin and other main compounds of *C. songaricum* changed with different growth periods, which could be used as the quality evaluation and control of *C. songaricum* (CUI *et al*., 2018c). However, it should be noted that just detection of several limited ingredients cannot reflect objectively the pharmacological activity of traditional Chinese medicine, especially for *C. songaricum* which always play a role as a whole (MUHAMMAD *et al*., 2016). Because there are thousands of chemical components in *C. songaricum*, and the real quality change should be comprehensively tested for all types of metabolites and their content differences (Johnson *et al*., 2016). Therefore, in order to overcome the drawbacks such as the deficiency of mono component index, non-objective, and methodological insensitivity in traditional methods, the cutting-edge spectroscopy and chromatography-based metabolomics methods combined with multivariate statistics are highly demanded for the high-throughput analysis of metabolites of plant extracts, which can provide a comprehensive and objective evaluation of *C. songaricum* quality (ZAMPIERI *et al*., 2017).

At present, there are several platforms that have been widely used in plant metabolomics research, such as ultrahigh-performance liquid chromatography linked to quadruple time-of-flight mass spectrometry (UPLC-QTOF/MS), gas chromatography-mass spectrometer

(GC-MS), nuclear magnetic resonance (NMR), fourier transform infrared spectrometer (FTIR), capillary electrophoresis (CE) (GHATAK *et al*., 2018). However, each platform has its inherent limitations. For example, FTIR can distinguish differences in compounds, but it only focuses on the identification of chemical group without additional information such as molecular weight; UPLC-QTOF/MS and GC-MS rely solely on retention time and molecular weight to identify compounds without identifying the spectral structure of compounds; NMR is used for chemical analysis based on magnetic resonance spectroscopy but without molecular weight data (RAJU *et al*., 2017). In addition, for the same batch of plant samples, the results of metabolic difference under multivariate statistics are also generally different when the above-mentioned different metabolic platforms are used for metabolic profiling analysis (DESHMUKH *et al*., 2016). However, if two or more platforms are applied to metabolomics analysis for cross validation, the above shortcomings could be greatly reduced, and they have the advantages of mutual complementation, coordination, objectivity, comprehensiveness, accuracy, etc., which will be more of scientific value for the understanding of plant metabolic difference.
In this study, UPLC-QTOF/MS and NMR were simultaneously used to study the metabolic profile of *C. songaricum*, and to explore the differential chemical composition and metabolic pathways of *C. songaricum* in different developmental stages. We aimed at answering the questions: (i) what are the differences of the metabolome analyzed based on the two mentioned platforms? (ii) which specific metabolites are common differential products of *C. songaricum* in different developmental stages? (iii) when is the reasonable harvesting period and what is their chemical characteristic? (iv) what is the metabolic pathway of the differential metabolites in *C. songaricum*? To our knowledge, this study will systematically and objectively study the metabolites and their metabolic traits in different developmental stages of *C. songaricum* for the first time, especially clarify the composition and differences of main secondary metabolites, so as to improve the quality evaluation system of *C. songaricum* and provide scientific basis for the production and drug use of *C. songaricum*.

2. MATERIALS AND METHODS

2.1. Plant sample collection

C. songaricum collected from Xilin-gaole town (39° 05′ 45″ N, 105° 23′ 27″ E, ≈1133.98 m), Alashan League, Inner Mongolia of China from November of 2017 to September of 2018. The developmental stages are divided into five periods in one cycle: Tubercle (T), Sprouting (S), Unearthing (U), Maturing (M) and Atrophy (A) stages in around November (the previous year), March, May, June and September, which was described clearly in our published papers[8]. Ten samples as replicates were randomly collected from each developmental stages. The collected rhizome of *C. songaricum* was cleaned with running water, dried at 35°C, crushed and passed through the No. 4 sieve for compound extraction. They were identified by Dr Jinlong Cui, a professor at the Shanxi University (Shanxi, China). The voucher specimens (CSR20171101-20171110, CSR20180301-20180310, CSR20180501-20180510, CSR20180601-20180610 and CSR20180901-20180910) have been deposited at the biochemical laboratory of Institute of Applied Chemistry of Shanxi University.

2.2. Plant extraction and NMR analysis

200 mg of dry powder of *C. songaricum* was put in 25 mL triangular flask, and 10 mL of 80% methanol was added to flask followed by vortexing for 1 min and ultrasonicating (210 W, 40 kHz) for 25 min. The extraction was centrifuged at 3500 r/min for 25 min, then the supernatant was concentrated with rotary vacuum evaporator and 400 μL buffer (buffer preparation: weigh 1.232 g of KH_2PO_4 dissolved in D_2O, add 50 mg 0.05% w/v Trimethyl silyl propanoic acid (TSP), make up to 10 mL, adjust the pH to 6.0 by 1 mol/L NaOD) to dissolve the sample. After transferred to a 1.5 mL microtube and centrifuged at 13,000 r/min for 10 min, the supernatant (600 mL) was transferred into a 5 mm NMR tube for NMR analysis. Ten repeats were carried out. Analytical grade methanol was purchased from Beijing Chemical Works (Beijing, China). D_2O (99.9 atom % D) and methanol-D_4 (D, 99.8%) were obtained from Qingdao Tenglong Weibo Technology Co., Ltd. (Qingdao, China). TSP was bought from Cambridge Isotope Laboratories Inc. (Andover, MA) and NaOD was purchased from Armar (Dottingen, Switzerland).

^{1}H-NMR was recorded at 25°C on a Bruker 600-MHz AVANCE III NMR spectrometer (600.13 MHz proton frequency). Each ^{1}H-NMR spectrum consisted of 64 scans requiring 5 min acquisition time with the following parameters: relaxation delay = 1.0 s, pulse width = 14.0 μs, and spectral width = 12345.7 Hz. A pre-saturation sequence was used to suppress the residual H_2O signal with low power selective irradiation at the H_2O frequency during the recycle delay. CD_3OD was used for internal lock purposes. The resulting spectra were manually phased and baseline-corrected, and calibrated to TSP at 0.00 ppm for water fractions (ZHI *et al*., 2012).

2.3. Plant extraction and UPLC-QTOF/MS analysis

Two grams of dried powder of *C. songaricum* was weighed into a 50 mL flask, followed by the addition of 20 mL of extraction solution (methanol:water = 4:1) to each sample and extraction with an ultrasonic method (210 w, 40 kHz) for 30 min. The extracted suspension was centrifuged at 12000 rpm at 4 °C for 6 min twice, and their resulting supernatants were combined and dried with rotary vacuum evaporator, and then reconstituted to 10 mL with acetonitrile:water = 1:9. Ten random samples as repeats were performed in each development stage. Quality control (QC) samples were made by equal proportional mixing of all samples. All samples, including the QC samples, were passed through as 0.22 μm syringe filter before MS analysis**.** Methanol (HPLC grade, purity≥99.9%) and acetonitrile (LC/MS grade, purity ≥ 99.9%) were purchased from Thermo Fisher Scientific Co., (Shanghai Pudong New District, Shanghai, China), the water was purified by Ultrapure Water Systems (H_2Opro-VF-T-TOC, Sartorius, Germany).

The metabolites were analyzed and detected using Agilent 6545 UPLC-QTOF/MS (Agilent Technologies, USA) with positive ion mode (+ESI). After the optimization of experimental conditions, a method was established for the non-target metabolomics profiling of secondary metabolites in *C. songaricum*. Samples were analyzed in random order, and one injection of QC sample was inserted for each set of five samples as quality control standards during data acquisition. The samples were separated by SB C18 column (1.8 μm particle size, 4.6 x 50 mm; Agilent Technologies, USA), and maintained at 40 °C. The mobile phase solvent A consisted of 0.1% formic acid in water, and solvent B was pure acetonitrile. The flow rate used for separation was 0.25 mL/min, with an injection volume of 10 μL, and the gradient program used constituted 0-5 min, 10%-20% B; 5-7 min, 20%-50% B; 7-9 min, 50%-55% B; 9-11 min, 55%-60% B; 11-13 min, 60%-65% B; 13-15 min,

65%B. Ion spray voltage was 4.4 kV; nebulizer voltage was 1.5 kV; dry gas rate and temperature were 5 L/min and 300°C; sheet gas flow rate and temperature were 12 L/min and 270°C; collision energy was 140 V. The spectra were collected in full scan mode from 80 to 1200 m/z.

2.4. Data statistics and analysis

The ^{1}H-NMR spectra were processed using MestReNova (version 6.1.0-6224, Mestrelab Research, Santiago de Compostella, Spain). After TSP correction, phase adjustment and baseline adjustment for all ^{1}H-NMR spectra, spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.01 ppm) corresponding to the region of δ 0.60-10.00. The regions of δ 4.80-4.95 and δ 3.32-3.34 were excluded from the analysis because of the residual signals of water and methanol, respectively. The pre-processing of raw data from UPLC-QTOF/MS performed with Masshunter Quality analysis software (Agilent Technologies, USA) to de-convolute and align the spectral peaks, then peaks normalization and retention time correction were done using XCMS online platform (https://xcmsonline.scripps.edu/).
The pre-processed data were separately exported to SIMCA-P 14.1 software (Umetrics UK Ltd, Windsor, UK) for multivariate statistical analysis to elucidate the dynamic accumulation changes of the metabolites of *C. songaricum* at different developmental stages. Principal component analysis (PCA) with unit variance scaling and orthogonal partial least squares-discriminant analysis (OPLS-DA) with Pareto scaling method were used to find class-separating differences, and cross-validation (CV) was used to determine the correctness and prevention over-fitting of the model.
According to the variable importance of projection (VIP>1) of S-plot analysis combining with a t-test (P-value<0.05), the significant differential metabolites were selected. For NMR-metabolomics studies, NMR data from the references (ZHANG *et al*., 2016; JIN *et al*., 2012; MA *et al*., 1999; JIANG *et al*., 2001; ZHANG *et al*., 2007a; ZHANG, 2007; ZHANG *et al*., 2007b; ZHANG *et al*., 1996; MA *et al*., 1993; MA *et al*., 2002; XU *et al*., 1996; WANG *et al*., 2011; HUANG, 1997; ZHANG *et al*., 1990; MENG and MA, 2013) and standard sample from the Human Metabolome Database (HMDB, www.hmdb.ca/) were used for metabolites identification of NMR spectra. For UPLC-QTOF/MS-metabolomics studies, the Pesticides Personal Compound Database and Library (PCDL), HMDB, the Massbank Database (www.massbank.jp/) and the Metlin metabolite database (https://metlin.scripps.edu/) were used for identification of differential metabolites of *C. songaricum*.
The relative contents of differential metabolites in *C. songaricum* (Ten replicates were set in each developmental stage) were analyzed using Origin software (OriginPro 2018C, OriginLab, USA). Finally, in order to further explore the metabolic mechanism of different metabolites of *C. songaricum* at different developmental stages, the metabolic pathways involved by differential metabolites were analyzed through KEGG database (www.kegg.jp/) and KEGG Compound Database (www.genome.jp/kegg/compound/).

3. RESULTS

3.1. Differential marker metabolites in *C. songaricum* based on NMR

A total of 25 metabolites of *C. songaricum* based on NMR were identified, including amino acids, such as cysteine, tyrosine, glutamate, aspartate; sugars such as sucrose, maltose and lactulose; secondary metabolites mainly include flavonoids, such as catechin, epicatechin, epicatechin-3-*O*-gallate, rutin; organic acids including malate, ursolic acid, oleanolic acid and masilinic acid. The chemical shifts and coupling constants of identified metabolites were exhibited in Table 1.

Based on a large number of fingerprint information obtained by NMR, the data were analyzed by means of multivariate statistical methods to accurately reveal the dynamic changes of metabolites in each stage of *C. songaricum*. In order to find potential biomarkers, PCA of *C. songaricum* at each development stage was carried out (Fig. 1), and the results showed distinct distribution of samples based on chemical composition from different developmental stages. It can be seen from Figure 1A**,** the sample from 'T', 'M', and 'A' stages of *C. songaricum* are located at the positive half of the PC2, while the 'S' and 'U' samples are located on the negative half of the PC2. This indicates that the metabolites of the *C. songaricum* in different developmental stages gradually change with the development of *C. songaricum*, which showed that the similar distance became further and further followed by 'T', 'S', 'U' successively, but it return from 'M', and 'A' is the closest to 'T' finally, which form a cycle like the annual development cycle of *C. songaricum* from November (the previous year), March, May, June to September which is the closest to November. The loadings plot (Fig. 1C) was used to find the metabolites that are responsible for the separation between *C. songaricum* at different development stages.

Table 1. Twenty-five identified metabolites including sixteen significant differential metabolites based on NMR from *C. songaricum* at different developmental stages.

No.	Metabolites	Assignment	δ^1H (ppm)	Multiplicity (*J* in Hz)
1	Cysteine*	3-H	3.03	dd (11.1, 6.8)
		2-H	3.98	dd (5.6, 4.2)
2	Tyrosine*	3-H	6.85	d (8.5)
		2-H	7.18	d (8.6)
3	Glutamate*	*β*-CH_2	1.99	m
		β-CH_2	2.08	m
		γ-CH_2	2.34	m
4	Aspartate	*α*-CH	3.75	dd (9.7, 19.2)
		β-CH	2.84	dd (8.0, 17.0)
		β′-CH	2.96	dd (4.0, 17.0)
		α-CH	3.96	dd (7.9, 4.0)
5	Threonine	*γ*-CH_3	1.35	d (6.9)
		α-CH	3.57	d (5.3)
		β-CH	4.27	m
6	Proline	*γ*′-CH_3	1.01	d (7.0)
		γ-CH_3	1.06	d (7.0)
		α-CH	3.61	d (4.0)

7	Alanine*	β-CH_3	1.49	d (7.2)
		α-CH	3.81	q (7.2)
8	α-Glucose	C_1H	5.20	d(3.8)
9	β-Glucose	C_1H	4.60	d (7.9)
10	Sucrose*	Fru-C_1H	4.19	d (8.7)
		Glc-C_1H	5.42	d (3.8)
11	Maltose*	12-H	3.45	t (9.5)
		4-H	5.27	d (3.9)
12	Lactulose	27-H	4.12	dd (7.2, 3.0)
		4-H	4.24	t (4.0)
		11-H	4.44	d (7.8)
		34-H	4.52	d (7.8)
13	Catechin*	4a-H	2.54	dd (16.2, 8.1)
		4b-H	2.82	dd (15.9, 5.8)
		6-H	5.96	d (2.1)
		2′-H	6.90	d (1.9)
14	Epicatechin	4-H	2.91	m
		6-H	6.05	d (2.0)
		5′-H	7.03	d (1.9)
15	Protocatechuate*	5-H	6.88	d (8.1)
		6-H	7.39	dd (2.1, 8.2)
		2-H	7.43	d (2.0)
16	Vanillate*	2′-H	3.89	s
		5-H	6.88	d (8.1)
		6-H	7.39	dd (2.1, 8.2)
		2-H	7.43	d (2.0)
17	Malate*	3a-H	2.46	dd (5.3, 14.3)
		3b-H	2.71	dd (3.4, 15.6)
		2-H	4.30	dd (3.4, 9.1)
18	Masilinic acid*	3-H	3.31	m
		2-H	3.43	m
		12-H	5.16	s
19	Ursolic acid*	29-H	0.91	d (6.8)
		23-H	0.98	s
		18-H	2.24	d (14.3)
		3-H	3.22	dd (6.0, 10.9)
		12-H	5.16	t (5.5)
20	Oleanolic acid*	27-H	0.88	t (12.6)
		17-H	1.22	d (6.5)
		22-H	1.85	d (2.5)
21	Rutin*	1′′′-H	4.44	d (7.8)
		6-H	6.19	d (2.9)
		8-H	6.30	d (1.7)
		5′-H	6.85	d (8.5)
22	Ursane-12-ene-28-acid-3β-malonate monoester	3β-H	4.56	dd (3.6, 10.8)

		10-H	1.94	s
23	3,4-DihydroxyPhenylacetate	7-H	2.54	t (8.0)
		2-H	6.59	d (2.2)
		5-H	1.29	d (6.2)
		3-H	3.45	t (9.5)
24	Vanillin*	4-H	4.10	m
		2-H	4.19	d (8.7)
		4b-H	2.82	dd (15.9, 5.8)
		3-H	5.51	d (2.6)
		6-H	5.98	d (2.3)
		5′-H	6.77	d (8.2)
25	Epicatechin-3-*O*-gallate*	6′-H	6.80	dd (2.0, 8.2)
		2′-H	6.95	d (1.9)
		2″-H, 6″-H	6.99	s

*Sixteen metabolites with significant difference.

Cross-Validation (Fig. 1E) shows that the model has a R^2Y value of 0.98 and a Q^2Y value of 0.90, indicating that the model is valid to use for significant differential metabolites selection. A total of 16 differential metabolites were selected as potential biomarker (Table 1) through the S-plot of OPLS-DA (Fig. 1G).

3.2. Relative content fluctuation of differential metabolites based on NMR

The relative contents of 16 differential compounds were determined by NMR with TSP as internal standards. Comparing to the 'T', 'S', and 'A' stages, the contents of main differential markers were more higher from 'U' and 'M' stages, such as flavonoids, organic acids and amino acids, such as catechins, epicatechin-3-*O*-gallate, protocatechuate, malate, ursolic acid, oleanolic acid, masilinic acid, glutamate and tyrosine. Among them, their relative contents in 'M' stage are higher than those in the 'U' stage. However, the differential primary metabolites such as maltose, sucrose, and alanine have the higher contents in the 'T' and 'A' stages of *C. songaricum*, and were lower in other stages. In addition, the content of vanillate and vanillin are contrary in all stages (Table 2).

3.3. Selection and identification of differential metabolites based on UPLC-QTOF/MS

The differential metabolites were selected with multivariate analysis from UPLC-QTOF/MS data obtained from five developmental stages of *C. songaricum*. The data matrix was 50 × 3374 after the data normalized, and the spectra were subjected to PCA analysis. It can be seen from the constructed PCA score plot (Figure 1B) that the QC samples are concentrated at one point, and the samples from the same developmental stage are relatively concentrated together. The distribution areas are obviously distinguished but also there is overlap from 'T', 'S', 'U', 'M' to 'A', and they gradually change and form a succession cycle. The supervised statistical method, OPLS-DA, was used to analyze the differential metabolites between samples from different developmental stages.

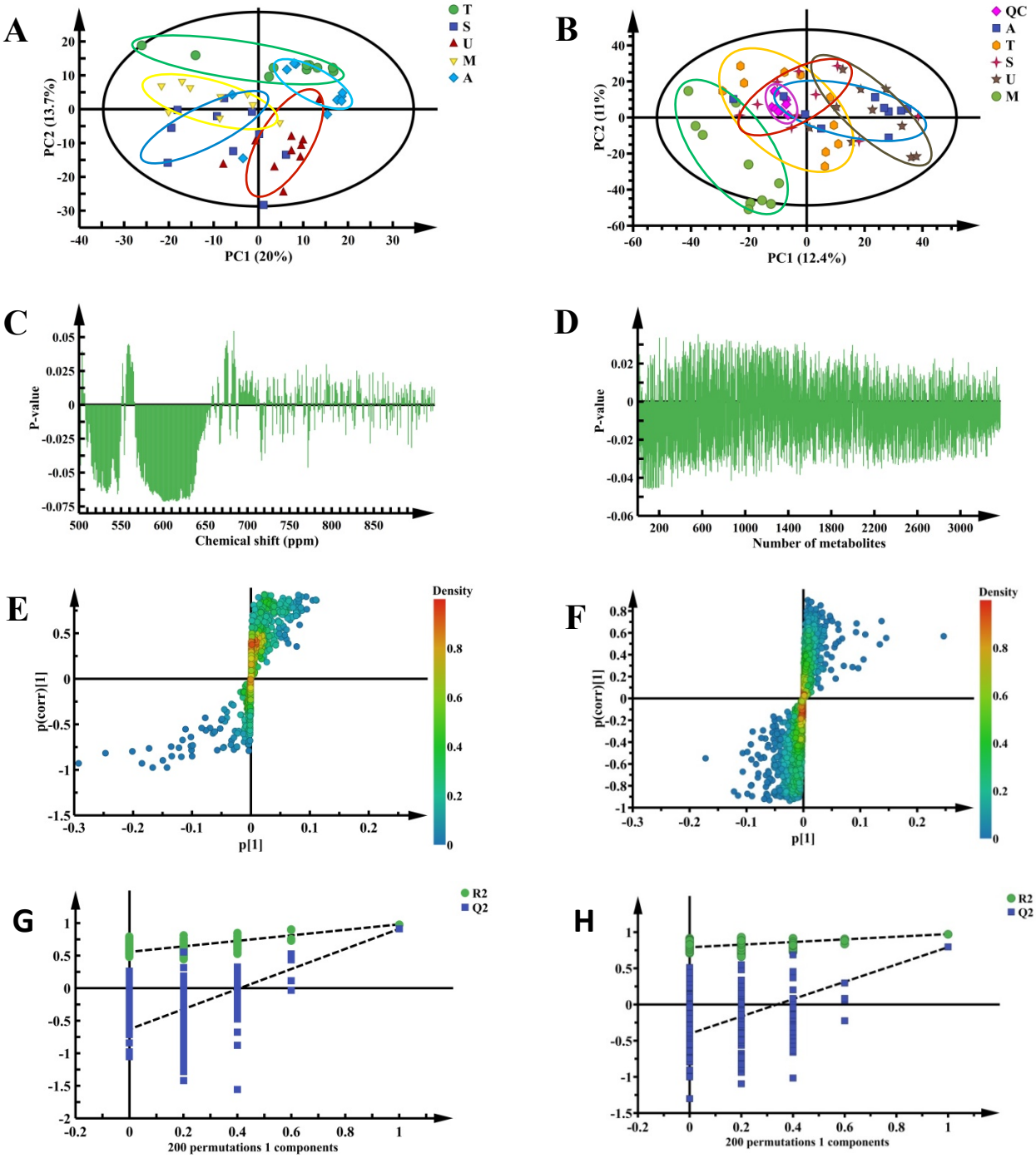

Figure 1. Metabolic analyses of *Cynomorium songaricum* at different developmental stages. (A, B) and (C, D) represent score and loading plot of PCA; (E, F) and (G, H) represent cross validation (CV) of the OPLS-DA model and S-plot, respectively. A, C, E and G for NMR, B, D, F and H for UPLC-QTOF/MS. 'T', 'S', 'U', 'M' and 'A' represent Tubercle, Sprouting, Unearthing, Maturing and Atrophy. CV of the PLS-DA model verifies the high predictability ($Q^2Y > 0.80$) of OPLS-DA at 'T' and 'S' stage for UPLC-QTOF/MS; ($Q^2Y > 0.91$) of OPLS-DA at 'T' and 'U' stage for NMR. The highly significant differential metabolites are included in the ellipse from the S-plot area.

It can be seen from Fig. 1F that the R^2 and Q^2 values ($R^2Y = 0.98$, $Q^2Y = 0.80$) generated by any random arrangement at the left end of the OPLS-DA permutation test (n = 200) were lower than the original value at the right end, indicating the prediction ability of real model is greater than that of any randomly arranged Y variable, which proves that the model has great predictability and goodness of fit. The score loading plot (Fig. 1D) indicated that there were at least 3365 significant difference metabolites for each development stages. Analysis by S-Plot (Fig. 1H), differential metabolites between different developmental stages of *C. songaricum* were determined by standard of VIP > 1 and *P*-value < 0.05. A total of 30 differential metabolites were selected as potential biomarker.

Combining with the m/z, retention time and MS/MS fragment of the mass spectrum in the HMDB and Massbank Database, 30 differential metabolites of *C. songaricum* were identified, including flavonoids (flavonoids and their glycosides are the main chemical constituents of *C. songaricum*), organic acids, esters, polysaccharides and other components. The metabolite M577T8 with m/z 577.1327 and its MS/MS fragmentation information (m/z 109.0290 for $C_6H_5O_2$; m/z 111.0446 for $C_6H_7O_2$; m/z 121.0290 for $C_7H_5O_2$; m/z 123.0446 for $C_7H_7O_2$; m/z 137.0239 for $C_7H_5O_3$; m/z 139.0395 for $C_7H_7O_3$; m/z 253.0501 for $C_{15}H_9O_4$; m/z 271.0606 for $C_{15}H_{11}O_5$; m/z 273.0763 for $C_{15}H_{13}O_5$; m/z 393.0610 for $C_{21}H_{13}O_8$; m/z 395.0767 for $C_{21}H_{15}O_8$; m/z 407.0767 for $C_{22}H_{15}O_8$; m/z 409.0923 for $C_{22}H_{17}O_8$; m/z 419.0403 for $C_{22}H_{11}O_9$; m/z 425.0873 for $C_{22}H_{17}O_9$ and m/z 577.1346 for $C_{30}H_{24}O_{12}$) (Fig. 2) identified as proanthocyanidin A1. Similarly, other compounds have been identified and the results are shown in Table 3.

3.4. The relative variation of metabolites based on UPLC-QTOF/MS

The contents of maltol, N-nitrosothiazolidine-4-carboxylic acid, phenylglyoxal, norartocarpanone, urolithin C, proanthocyanidin A1, procyanidin C1, 1D-myo-inositol 3,4-bisphosphate, HMBA, 2-methylfuran, 3,4-dihydroxyphenacyl caffeate, 3,4-dihydroxybenzaldehyde, DDDB, D-galactose, dimethyl trisulfide, 4-hydroxybenzaldehyde, 3-oxo-valproic acid CoA, 4-*O*-methylgallic acid, BR-xanthone B, aflatoxin G, kaempferol 7-(6''-galloylglucoside), epicatechin 5-*O*-*β*-D-glucopyranoside-3-benzoate is higher in the 'M' stage of *C. songaricum*, lower in the 'S' and 'U' stages, similar in the 'T' and 'A' stages. 5-ethynyl-5'-(1-propynyl)-2,2'-bithiophene was higher in the 'S' and 'U' stages of *C. songaricum*, and lower in the 'M' stage. Meanwhile, the contents of lactulose, cochliophilin A, DPCM, DPOT and 4,5-trihydroxyoxane-2-carboxylic acid are higher in the 'T' and 'A' stages than that in other stages. However, the contents of rotenone are highest in the 'T' stage, low in other stages (Table 4).

3.5. Comparative analysis of differential metabolic pathways based on NMR and UPLC-QTOF/MS metabolomics studies

In order to explore the metabolic differences of *C. songaricum* at different development stages, to reveal the metabolic mechanisms involved among them, metabolic pathways involved 16 and 30 differential metabolites based on NMR (Table 1) and UPLC-QTOF/MS (Table 3) were constructed with bio-information methods. It can be seen from Figs. 3 and 4, seventeen and thirty differential metabolites in NMR and UPLC-QTOF/MS involved in tricarboxylic acid cycle, shikimic acid pathway and other metabolic pathways, respectively.

Table 2. Changes in the accumulation of 16 differential metabolities in C. songaricum across the developmental stages of tubercle (T), sprouting (S), unearthing (U), maturing (M), and atrophy (A) developmental stage using Nuclear Magnetic Resonance (NMR) analysis. Ten samples as replicates were randomly detected from each developmental stages.

Name	mean±SD (n = 10)				
	T	S	U	M	A
Cysteine	0.0035±0.0039**	0.0542±0.0232**	0.0735±0.0303**	0.0135±0.0055	0.0206±0.0243
Tyrosine	0.0765±0.0448**	0.1239±0.0394	0.0745±0.0290**	0.1438±0.0355	0.0520±0.0309**
Glutamate	0.2846±0.0714**	0.4699±0.1570	0.3908±0.1924*	0.6317±0.1914	0.5656±0.2243
Alanine	5.7626±1.8119**	2.1460±0.9384	2.8296±1.0804	2.6147±0.8801	4.8527±2.1000**
Sucrose	2.4065±0.4842**	0.6419±0.4551	0.8578±0.3754**	0.9822±0.4883	1.5574±1.0870**
Maltose	1.3424±0.5816**	0.3284±0.1618**	0.6355±0.2986	0.5638±0.2933	1.0325±0.5912**
Catechin	0.0309±0.0114**	0.0743±0.0227*	0.1152±0.0707	0.1617±0.1158	0.0344±0.0098**
Protocatechuate	0.1341±0.0902**	0.1886±0.0790	0.0951±0.0428**	0.2456±0.0718	0.0818±0.0446**
Vanillate	0.3579±0.0949**	0.7761±0.2635	0.7822±0.2045*	0.5851±0.1227	0.5174±0.2616
Malate	0.2280±0.2030**	0.4925±0.3781	0.1806±0.2586**	0.6693±0.3779	0.1911±0.1366**
Masilinic acid	0.5366±0.3384*	1.0722±0.4503	0.5505±0.3333*	0.9427±0.3147	0.4148±0.1536**
Ursolic acid	0.0635±0.0128**	0.3161±0.1440	0.3475±0.2303	0.2785±0.0838	0.1493±0.0676**
Oleanolic acid	0.0538±0.0103**	0.1442±0.0540*	0.2667±0.0815	0.2192±0.0627	0.1464±0.0727*
Rutin	0.5695±0.4622	1.0686±0.4321*	0.6821±0.4622	0.6294±0.6785	0.4572±0.3862
Vanillin	1.2766±0.4563**	0.7753±0.3315	0.5978±0.2784	0.6088±0.4245	0.9992±0.6224
Epicatechin-3-*O*-gallate	0.0681±0.0436**	0.1048±0.0403	0.0534±0.0283**	0.1425±0.0552	0.0421±0.0267**

$P<0.05$ and $^{**}P<0.01$ represent significant and extremely significant (*t*-test in 'T', 'S', 'U' and 'A' stages compared with 'M' stage) in curve graph of relative content of differential metabolites from different developmental stages. SD: standard deviation values.

Table 3. Identification of 30 differential metabolites based on UPLC-MS/MS from *C. songaricum* at different developmental stages.

No.	Formula	Identification	RT	m/z	Major fragment ion (m/z)	VIP
1	$C_7H_6O_2$	4-Hydroxybenzaldehyde	3.73	123.0440	81.0335, 91.0539, 93.0334	1.93
2	$C_6H_6O_3$	Maltol	6.71	127.0390	95.0497, 109.0282	3.07
3	$C_8H_6O_2$	Phenylglyoxal	3.75	135.0440	109.0282, 107.0488, 91.0539	2.35
4	$C_7H_6O_3$	3,4-Dihydroxybenzaldehyde	7.45	139.0388	109.0284, 121.0283, 81.0335, 107.0487, 123.0440, 111.0074, 91.0539	3.75
5	$C_5H_{10}O_3S$	HMBA[a]	9.20	151.0388	117.0330, 115.0531, 133.0608, 135.0430, 103.0537, 85.0277	4.39
6	C_5H_6O	2-Methylfuran	2.58	158.9638	83.0486	1.68
7	$C_2H_6S_3$	Dimethyl trisulfide	2.11	164.9208	126.9738, 80.0493	3.16
8	$C_8H_8O_5$	4-*O*-Methylgallic acid	2.52	185.0416	123.0438, 97.0280	4.35
9	$C_{13}H_8S_2$	5-Ethynyl-5'-(1-propynyl)-2,2'-bithiophene	2.54	192.9981	229.0489, 121.0281, 109.0025, 147.0436	3.62
10	$C_4H_6N_2O_3S$	N-Nitrosothiazolidine-4-carboxylic acid	2.55	200.9741	163.0376, 111.0429, 117.0686, 114.0902, 145.0632, 111.0066, 129.0682	3.18
11	$C_6H_{12}O_6$	D-Galactose	2.52	203.0524	163.0477, 181.9620, 133.0526	6.56
12	$C_{13}H_8O_5$	Urolithin C	7.46	245.0441	203.0322, 201.0534, 191.0325, 177.0530, 173.0585, 161.0586, 147.0430, 145.0640, 119.0482	3.75
13	$C_{14}H_{10}O_6$	BR-Xanthone B	8.45	275.0544	203.0525, 245.0444	1.62
14	$C_{15}H_{12}O_6$	Norartocarpanone	8.72	289.0701	271.0598, 259.0598, 245.0442, 179.0337, 163.0390, 153.0184, 135.0440, 111.0441	1.09
15	$C_{15}H_{14}O_6$	DDDB[b]	5.71	291.0858	139.0382, 127.0387, 163.0383, 111.0433, 153.0173	1.43
16	$C_{23}H_{22}O_6$	Rotenone	3.46	365.0654	203.0700, 203.0474, 111.0433	6.64
17	$C_{12}H_{22}O_{11}$	Lactulose	3.47	365.1047	181.0494, 147.0439, 119.0492, 105.0700, 93.0336	11.09
18	$C_{16}H_{10}O_5$	Cochliophilin A	3.06	366.1084	181.0494, 151.0391, 139.0387	9.21
19	$C_6H_{14}O_{12}P_2$	1D-Myo-inositol 3,4-bisphosphate	2.52	378.9592	341.0056, 119.0480, 163.0741	1.46

20	$C_{15}H_{18}O_{10}$	DPOT[c]	3.03	381.0787	123.0443, 177.0242	3.74
21	$C_{17}H_{12}O_7$	Aflatoxin G	6.72	407.0756	329.0633, 163.0379, 109.0644	1.36
22	$C_{17}H_{14}O_7$	3,4-Dihydroxyphenacyl caffeate	6.60	409.0910	151.0385, 135.0436, 139.0384, 147.0435, 163.0385, 259.0589	1.50
23	$C_{27}H_{26}O_{11}$	DPCM[d]	2.53	527.1575	123.0441, 527.1574	5.41
24	$C_{30}H_{24}O_{12}$	Proanthocyanidin A1	8.44	577.1327	109.0283, 577.1328, 139.0390, 137.0235, 121.0281	1.60
25	$C_{28}H_{28}O_{12}$	Epicatechin 5-*O*-*β*-D-glucopyranoside-3-benzoate	8.72	579.1483	109.0284, 111.0439, 123.0442, 123.0391, 289.0707	1.49
26	$C_{28}H_{24}O_{15}$	Kaempferol 7-(6''-galloylglucoside)	3.46	601.1306	287.0535, 95.0484, 245.0426, 601.1282	1.26
27	$C_{45}H_{38}O_{18}$	Procyanidin C1	3.46	889.1938	139.0379, 151.0381	1.83
28	$C_{29}H_{48}N_7O_{18}P_3S$	3-Oxo-valproic acid CoA	3.46	890.1967	107.0482, 287.1435	1.08
29	#	Compound 1	2.04	90.9765	122.9245,108.9619,112.8957, 84.9597,80.9670	3.75
30	#	Compound 2	1.97	108.9619	84.9598, 182.9624, 198.9398, 110.9600, 80.9670	2.40

RT = Retention time; VIP = variable importance in projection.

[a]HMBA: (±)-2-Hydroxy-4-(methylthio)butanoic acid.

[b]DDDB: 3-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-4,6,7-triol.

[c]DPOT: 6-{[3-(3,4-dihydroxyphenyl)propanoyl]oxy}-3,4,5-trihydroxyoxane-2-carboxylic acid.

[d]DPCM: 6-(2-{8,8-dimethyl-2-oxo-2H,8H-pyrano[2,3-f]chromen-3-yl}-5-methoxyphenoxy)- 3,4,5-tri-hydroxy oxane-2-carboxylic acid.

Table 4. Changes in the accumulation of 30 differential metabolities in *C. songaricum* across the developmental stages of tubercle (T), sprouting (S), unearthing (U), maturing (M), and atrophy (A) developmental stage using ultrahigh-performance liquid chromatography linked to quadruple time-of-flight mass spectrometry (UPLC-QTOF/MS) analysis. Ten samples as replicates were detected from each developmental stages.

Name	mean±SD (n = 10)				
	T	S	U	M	A
4-Hydroxybenzaldehyde	3052054±974079.8731	2349988±1030667.6990*	1797975±197398.8644**	3696610±1249226.9760	2435452±667730.8568*
Maltol	5466980±1652325.4770*	4527786±1225697.5600**	4186563±1482629.4890**	7522294±1847422.2300	6536740±2041205.6250
Phenylglyoxal	2601581±976131.4279	1767475±966177.5521*	1236669±231346.4457**	3101970±1347034.8430	1797859±563412.0762*
3,4-Dihydroxybenzaldehyde	6692251±2596449.7240	4722502±970534.4062**	4378398±1536202.3430	6001045±702001.9179	4566337±1497013.7270*
HMBA[a]	12863905±568736.0911	11323738±1048794.3430**	10683573±2153598.3930**	13486784±814527.5957	11887307±1412353.9930**
2-Methylfuran	1007852±273519.3367	624210±363458.6314	327635±323745.1858**	825457±227496.2227	483610±385135.2597*
Dimethyl trisulfide	1413753±258615.4354**	565109±393592.7170*	251477±353631.2520**	906374±264866.1238	435924±493473.5895*
4-*O*-Methylgallic acid	2804158±587829.0458*	1121797±702431.9986	601656±403542.9241**	1932974±1036703.8420	1760195±822185.7088
5-Ethynyl-5'-(1-propynyl)-2,2'-bithiophene	13246±19533.5351**	1040725±495288.9971**	959408±568151.3280**	139507±93613.6715	552117±998634.1667
N-Nitrosothiazolidine-4-carboxylic acid	1579760 ±284081.7400*	656162±476466.5605	433136±420160.5731*	1053089±526760.7750	798034±583314.2580
D-Galactose	6146219±1174645.0280*	2499141±1393913.2460	1339882±989245.7267**	3804354±1485630.5010	4015983±1917618.7500
Urolithin C	7604174±912203.2035	5579686±2412457.6690	5144753±1871919.6180**	8435160±1252837.9190	6341496±1973650.1590*
BR-Xanthone B	1657700±424885.8361	1292482±268404.1249**	1211424±313072.5221*	1947122±193752.0798	1565516±396925.8936*
Norartocarpanone	5557428±1495578.7160	5037735±1915316.9250	4160929±766908.5086**	6049982±491056.4182	4737300±870009.3376**
DDDB[b]	5299646±2190820.4470	6080370±4197954.0600	3215349±1418759.5270	4795477±1212686.5750*	3005056±1707066.7080*
Rotenone	4962503±5633469.2440*	324448±433278.3500	617032±1056347.9170	356607±343758.0758	147850±89830.9953
Lactulose	11924149±10368709.2800**	750143±1237427.9060	841598±1222273.3190	816273±1154202.9230	3485684±5532552.6200
Cochliophilin A	6923094±3187667.5540**	630285±334613.5589	344904±237326.7796	604151±492691.2268	2736513±1823162.6500**
1D-Myo-inositol 3,4-bisphosphate	511671±253381.8769	219492±188778.4111	74368±81658.6911**	351300±201820.2842	276995±289767.4383
DPOT[c]	2727458±1150154.5770**	1204665±1108806.8010	929287±593494.2595	487029±347628.8046	4388194±2770220.7160**

Aflatoxin G	1954715±771962.2736	1693389±693790.3924*	1444325±599287.4413**	2469857±455188.4078	2209888±776659.3779
3,4-Dihydroxyphenacyl caffeate	3485423±1567456.9980	2669929±881377.0149**	1803953±1001807.4300**	4764661±1735324.2330	2301070±846909.2224**
DPCM[d]	2709314±499141.3230**	500205±491794.1851	103390±53474.3325**	610276±521630.9730	1258862±848913.9333
Proanthocyanidin A1	1880808±383413.1097**	1470401±728491.4324**	1284322±534560.0540**	2815238±814048.5444	2748300±1844331.6690
Epicatechin 5-*O*-*β*-D-glucopyranoside-3-benzoate	1571741±409796.9876**	1339043±549377.9153**	1182321±330641.1952**	2054982±163447.3481	1309227±455168.3916**
Kaempferol 7-(6''-galloylglucoside)	762077±341965.0748**	616818±311808.6769**	181081±169684.7474**	1144015±193842.6597	374829±361505.8518**
procyanidin C1	1417573±653170.6734**	1144137±668716.5061**	247527±262471.9150**	2183828±425770.2849	664931±733058.3847**
3-Oxo-valproic acid CoA	767809±218778.5229*	553999±326227.0478**	123518±129285.5461**	1038770±210957.2988	326033±360127.6363**
Compound 1	1824992±430660.4145**	643676±430769.4798*	436161±544593.6436**	1241532±233449.9802	631797±517906.8134**
Compound 2	1086532±735713.7158**	1800644±463492.3444**	2526403±969412.1308**	3966564±868224.3731	1494162±835465.2780

[a]HMBA: (±)-2-Hydroxy-4-(methylthio)butanoic acid.

[b]DDDB: 3-(3,4-dihydroxyphenyl)-3,4-Dihydro-2H-1-benzopyran-4,6,7-triol.

[c]DPOT: 6-{[3-(3,4-dihydroxyphenyl)propanoyl]oxy}-3,4,5-trihydroxyoxane-2-carboxylic acid.

[d]DPCM: 6-(2-{8,8-dimethyl-2-oxo-2H,8H-pyrano[2,3-f]chromen-3-yl}-5-methoxyphenoxy)-3,4,5-tri-hydroxyoxane-2-carboxylic acid.

*$P<0.05$ and **$P<0.01$ represent significant and extremely significant (*t*-test in 'T', 'S', 'U' and 'A' stages compared with 'M' stage) in curve graph of relative content of differential metabolites from different developmental stages. SD: standard deviation values.

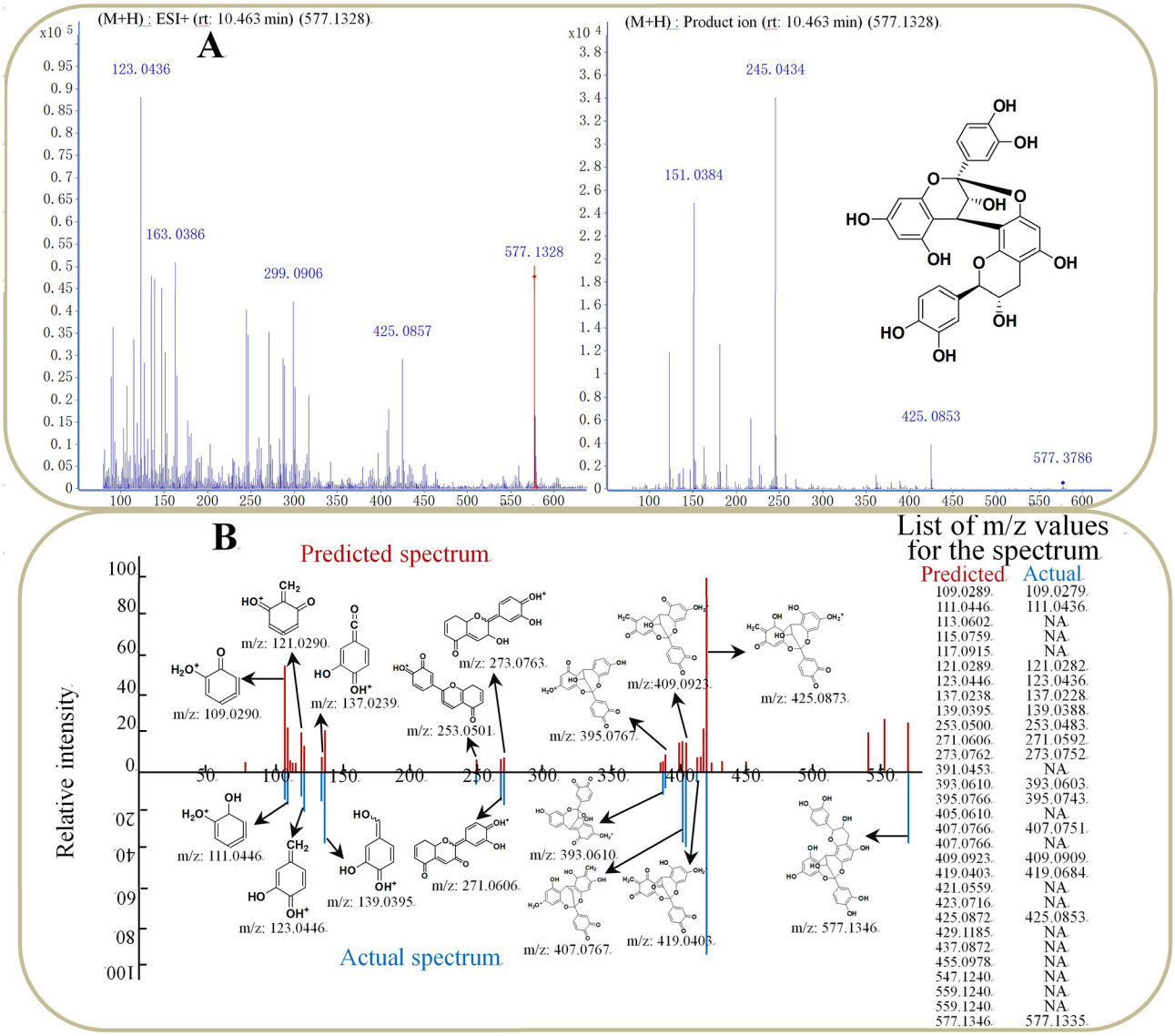

Figure 2. Mass spectrometric identification of the differential metabolite M577T8 as proanthocyanidin A1. Predicted MS/MS match with Agilent Mass Hunter Qualitative analysis in the PCDL database and the structure of the different metabolite (A). Predicted and actual list of m/z values for the differential metabolite spectrum and MS/MS match in the HMDB and METLIN databases (B).

The metabolic pathways are similar involved by differential metabolites between NMR and UPLC-QTOF/MS platforms. In different development stages, there were differences in the primary metabolism of *C. songaricum*, such as sugar metabolism and amino acid (protein) metabolism. In the secondary metabolism, flavonoids were main involvement. But in detail, the differential metabolites involved in sugar metabolism mainly include sucrose, maltose and malate in NMR analysis; but were D-galactose, lactulose and maltol in UPLC-QTOF/MS results. Cysteine, tyrosine, glutamate, alanine were involved in the amino acid metabolism based on NMR analysis while they were 4-hydroxybenzaldehyde,

3,4-dihydroxybenzaldehyde and 3,4-dihydroxyphenacyl caffeate in UPLC-QTOF/MS analysis.

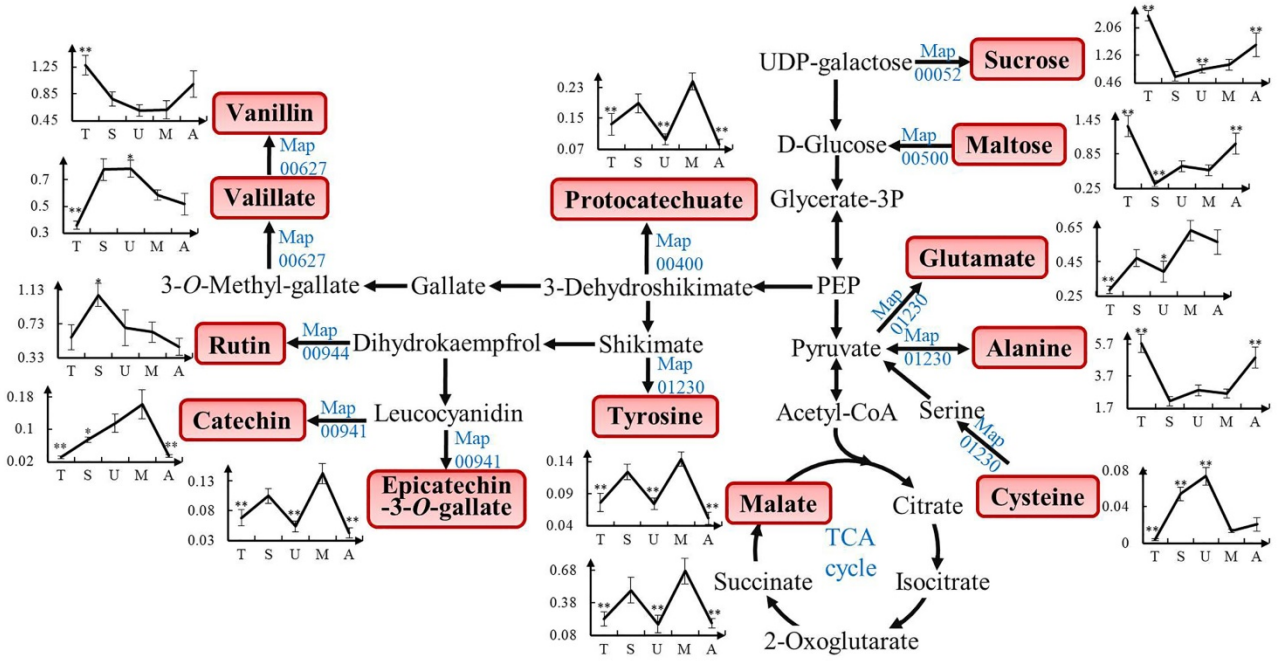

Figure 3. Metabolic pathway map of differential marker metabolites in *Cynomorium songaricum* from different developmental stages based on NMR. The pathways were verified by KEGG database, and the blue words are number of metabolic pathways of differential metabolites. $^{*}P<0.05$ and $^{**}P<0.01$ represent significant and extremely significant (*t*-test in 'T', 'S', 'U' and 'A' stages compared with 'M' stage) in curve graph of relative content of differential metabolites from different developmental stages.

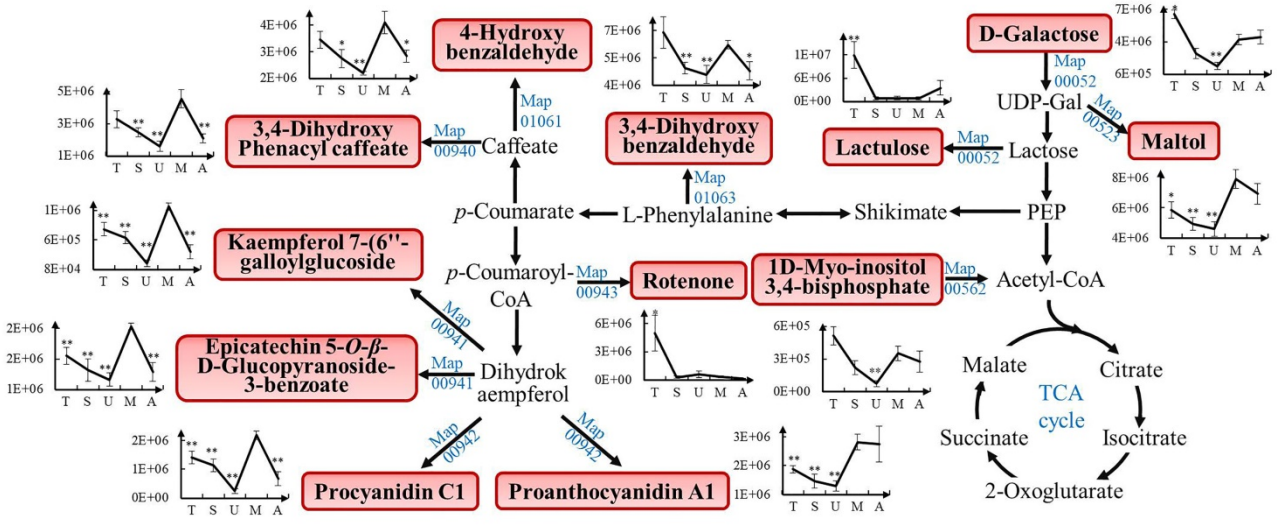

Figure 4. Metabolic pathway map of differential marker metabolites in *Cynomorium songaricum* from different developmental stages based on UPLC-QTOF/MS. The pathways were verified by KEGG database, and the blue words are number of metabolic pathways of differential metabolites. $^{*}P<0.05$ and $^{**}P<0.01$ represent significant and extremely significant (*t*-test in 'T', 'S', 'U' and 'A' stages compared with 'M' stage) in curve graph of relative content of differential metabolites from different developmental stages.

The biosynthesis pathways involved in flavonoids in NMR metabolomics analysis were catechin, epicatechin-3-*O*-gallate and rutin but were BR-xanthan B, proanthocyanidin A1, proanthocyanidin C1, kaempferol 7-(6''-galloylglucoside), epicatechin 5-*O*-*β*-D-glucopyranoside-3-benzoate and rotenone in UPLC-QTOF/MS. NMR analysis showed that protocatechuate was involved in the biosynthesis of antibiotics while maltol and an aflatoxin G may play a role with the UPLC-QTOF/MS analysis. There are other metabolic differences, for example, the NMR analysis showes that vanillin and vanillate were involved in the pathway of aminobenzoate degradation, and the UPLC-QTOF/MS analysis shows that 1D-myo-inositol 3,4-bisphosphate was involved in inositol phosphate metabolism.

4. DISCUSSION

Due to the diversity of metabolites in chemistry structure and type, multi-technological platforms are utilized to study the difference of plant metabolism, which greatly improves the quality evaluation of plant. Dastmalchi *et al*. (2014) demonstrated the advantages of simultaneous use of NMR and UPLC-QTOF/MS to analyze different aspects of the plant metabolome in the same study. NMR technology has merits of excellent reproducibility, fast speed, uncomplicated sample preparation and strong quantitative ability for compounds, but its low sensitivity impedes the detection of low concentration metabolites in samples (WISHART, 2011). In contrast, UPLC-QTOF/MS has higher sensitivity and molecular specificity but lack of lots of standard compounds and comprehensive MS/MS database hinders rapid and reliable identification of a large number of metabolites (Want *et al*., 2010). However, the unique physicochemical principles of NMR and UPLC-QTOF/MS make these two technologies complementary to each other. Both techniques used in one study can achieve the most comprehensive profiling of the entire metabolome (Beltran *et al*., 2012). In this study, the primary metabolites of *C. songaricum*, such as sugar and amino acid (protein) were mainly detected by NMR while the secondary metabolites mainly detected based on UPLC-QTOF/MS. Thus combined use of two or more platforms together to study metabolomics is still significant for systematic and objective metabolomics study.

Although the detected chemicals were not are not exactly the same, the chemical types were consistent between NMR and UPLC-QTOF/MS in this study. They include flavonoids, organic acids, sugars, amino acids and other substances, which are involved in the same metabolic pathways. Differential metabolites from NMR contain more primary metabolites, sugars and amino acids are the highest in 'T' stage, for energy storage of the development of *C. songaricum.* In 'S' and other development stages, the energy was used for germination and growth, then primary metabolites are transformed into secondary metabolites or secondary metabolites transformed each other for specific purpose. For example, the relative contents of the vanillate and vanillin are negatively correlated across all stages (Figure 3), and they might be transformed each other in metabolic process. UPLC-QTOF/MS detected a large proportion of differential secondary metabolites, and their types and contents reach higher level in 'U' and 'M' stages, indicating that the effective components in 'U' and M' stages were much higher than those in other stages, and the bioactive quality of *C. songaricum* was better (MENG *et al*., 2013). Thus this study can provide help in assessing the pharmacological effects of *C. songaricum* collected from different seasons.

The difference of metabolic composition of *C. songaricum* was also influenced by many other factors. Our group have used UPLC-QTOF/MS platform to study the correlation between metabolites and endophytic fungi of *C. songaricum* in the 'U' stage from different locations, and found that metabolites were not only related to environmental factors and gene regulation, but also related to internal environmental biological factors (CUI *et al*., 2018c; CUI *et al*., 2019). In both studies, the differential metabolites and the metabolic pathways involved were similar. However, though the *C. songaricum* samples collected in different years, different locations, even if the collection season and instrument platform are the same, the metabolites are still quite different. Therefore, there is still a long way to go to clearly study the metabolic differences between plants (DELFIN *et al*., 2019).
In summary, for the first time, this study obtained the data of metabolic difference of *C. songaricum* in different developmental stages based on UPLC-QTOF/MS and NMR, which will provide help to understand metabolite accumulations of *C. songaricum*, and will also assist people to establish new ideas and methods for the evaluation of *C. songaricum*.

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PAPER

EFFECT OF HARVESTING TIME ON HEMP (*CANNABIS SATIVA* L.) SEED OIL LIPID COMPOSITION

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ABSTRACT

The most common food using hemp (*Cannabis sativa* L.) is hempseed oil (HSO) because it is a rich source of nutrients with nutritional and functional beneficial effects for human body. Harvesting time can affect the quality of HSO, consequently the aim of this study was to evaluate the composition of lipid fraction, fatty acids, tocopherols and sterols, during ripening. Two cultivars, *Futura 75* and *Carmagnola*, were collected at three ripening stages during August and September 2015 and their lipid composition was determined by analytical techniques. Among the fatty acid identified, the linoleic acid was the preponderant, followed by oleic, α-linolenic and palmitic acid. Linoleic:α-linolenic acid and polyunsaturated:saturated fatty acid ratios decreased and increased, respectively, in both varieties with ripening. γ-tocopherol was the preponderant tocopherol identified, *Futura 75* showed the highest content in the middle of maturation while *Carmagnola* at the beginning. β-sitosterol was the predominant sterol identified in both varieties, followed by campesterol, Δ_5-avenasterol, stigmasterol and Δ_7-stigmasterol. Total sterol content increased and decreased with ripening in *Futura 75* and *Carmagnola*, respectively. The study confirms that ripening stage affects the quality of hempseed oil, important parameter to consider for hemp seed producers.

Keywords: *Cannabis sativa L.*, hempseed oil, fatty acids, tocopherols, sterols

1. INTRODUCTION

Cannabis sativa L., belonged to Cannabaceae family, is an annual plant known by its long, thin flower and spiky leaves (MONTSERRAT-DE LA PAZ *et al*., 2014). *Cannabis sativa* subsp. *sativa* is characterized by a low content of THC (Δ^9-tetrahydrocannabinol), it must be lower than 0.2% on dry basis to be cultivated in most European countries (Official Journal of European Union, 2008). Industrial hemp with a low THC content has no psychoactive effects (SIUDEM *et al*., 2019). The most common food using hemp is hempseed oil (HSO); it is a rich source of nutrients that provide nutritional and functional support for humans (CRESCENTE *et al*., 2018). HSO represents the 25-35% of hemp seed (CALLAWAY, 2004; MONTSERRAT-DE LA PAZ *et al*., 2014; CRIMALDI *et al*., 2017) and it contains more than 80% of polyunsaturated fatty acids (PUFAs) (PETROVIĆ *et al*., 2015; SIUDEM *et al*., 2019) including essential fatty acids usually not contained in oils used for human diet (PETROVIĆ *et al*., 2015); in particular ω-6 linoleic acid (LA) and ω-3 α-linolenic acid (ALA). In addition, LA:ALA ratio is 3:1 which agrees with European Food Safety Agency recommendations (EFSA, 2009). HSO is composed of 1.5-2% unsaponifiable fraction, a source of interesting minor compounds like tocopherols, fat-soluble vitamin D and E (SIUDEM *et al*., 2019) and phytosterols (MONTSERRAT-DE LA PAZ *et al*., 2014). Therefore, HSO has a lot of beneficial effects: cancer and cardiovascular disease prevention, cholesterol level normalization, blood pressure lowering (DEVI *et al*., 2019) and rheumatoid arthritis and dermatitis treatment (OOMAH *et al*., 2002; CHOW, 2008). For all these reasons industries are attracted by HSO for drugs, cosmetics, body care products and dietary supplement production (CALLAWAY, 2004; KOLODZIEJCZYK *et al*., 2012).

To the best of our knowledge, in literature are reported only two studies about the effect of maturation on yield, quality of fiber and oil of industrial hemp. HÖPPNER *et al*. (2007) studied the yield and the quality of fiber and oil of different hemp cultivars in Germany at two different harvest times ("intensive flowering" and "initial seed maturity"). The different harvest stages did not have effect on stem diameter, fiber content and yield; on the other hand, seed yield and seed oil content increased with maturation while the γ-linolenic acid content decreased. BURCZYK *et al*. (2009) studied the effect of sowing density and date of harvest (beginning of panicle forming, full bloom and full seed maturity) on industrial hemp yields. They observed that the maximum yields of biomass, cellulose and fiber can be obtained at 30 kg/ha sowing density at full bloom; while considering hemp for seed or panicles the highest yields were obtained at 10-20 kg/ha of density at full maturity of panicles.

The aim of this study was the characterization of lipid fraction from seeds of two different cultivars of *Cannabis sativa* L., *Futura 75* and *Carmagnola*, harvested at three different harvest stages; in order to investigate the quality of hempseed oil during the maturation, which was never investigated before. To this end, fatty acids, tocopherols and sterols content were determined using fast chromatographic techniques, HPLC and GC equipped with different detectors.

2. MATERIALS AND METHODS

2.1. Fruit harvest and sample preparation

Cannabis sativa L. seeds from two different varieties, *Futura 75* and *Carmagnola*, were picked in Ancona area in Italy approximately 43° 30' 34286" N, 13°15' 33052" E. Fruits were harvested at three different harvesting stages during August and September 2015. At first harvest, *Futura 75* were collected on August 26th (F1) and *Carmagnola* on September 3rd (C1); at the second harvest, *Futura 75* were collected on September 3rd (F2) and *Carmagnola* on September 15th (C2); and at third harvest, *Futura 75* were collected on September 15th (F3) and *Carmagnola* on September 23rd (C3). At each sampling time 20 g of seeds were picked from different field's area; all of them were grounded before analyses.

2.2. Oil extraction and moisture content determination

The lipid content was obtained by grounding the samples of *Cannabis sativa* L. seeds (5 g) and the oil was extracted with *n*-hexane in a Soxhlet apparatus according to ISO method 659:1998. The remaining solvent was removed under vacuum and the oil was taken up with *n*-hexane/isopropanol (4:1 v/v) solution and stored at −18°C until use. Each extraction was carried out two times for each cultivar.
Moisture content (%) was evaluated on hemp seeds samples in an oven at 105°C until constant weight was receached. For each samples, 3 replicates of 3 g weighted for each extraction were dried (n=6) (AOAC, 1995).

2.3. Fatty acid analysis

The fatty acid composition was determined as fatty acid methyl esters (FAMEs) by capillary gas chromatography analysis after alkaline treatment (CHRISTIE, 1982). Methyl tridecanoate (C13:0, 2 mg/mL) was used as internal standard and FAMEs were measured on a GC 2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation), at the same conditions reported in MARZOCCHI *et al.* (2018). Peak identification was accomplished by comparing peak retention time with GLC-463 standard mixture from Nu-Check (Elysian, MN, USA) and FAME 189-19 standard mixtures from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and expressed as weight percentage of total FAMEs. FAMEs composition was measured in 3 replicates for each lipid extract (n = 6) and each analysis lasted 7 minutes.

2.4. Tocopherols analysis

Approximately 50 mg of hempseed oil was dissolved in 0.5 ml of *n*-hexane. After homogenization, the solution was filtered through a 0.2 μm nylon filter and 2.5 μl was injected in a HPLC 1200 series (Agilent Technologies, Palo Alto, California, USA) equipped with a fluorimeter detector (Agilent, Palo Alto, CA, USA). The excitation wavelength was 290 nm and the emission wavelength was 325 nm. The separation of tocopherols was performed by a HILIC Poroshell 120 column (100 mm × 3 mm and 2.7μm particle size; Agilent Technologies, USA), in isocratic conditions, using an *n*-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) mobile phase. The flow rate was 0.8 ml/min. Tocopherols were identified by co-elution with the respective standards. The calibration

curve used for quantification was constructed with α-tocopherol standard solutions. Tocopherols composition was measured in 3 replicates for each lipid extract (n = 6) and expressed in mg/100g of oil; each analysis lasted 8 minutes.

2.5. Sterols determination

In order to determine the sterols content, 0.5 ml of dihydro-cholesterol (2 mg/mL) was added to 250 mg of oil and saponification was conducted at room temperature (SANDER *et al.*, 1989). After about 20 h, the organic fraction was washed with 10 ml of diethyl ether and 10 ml of water. The unsaponifiable fraction was further extracted twice with 10 ml of diethyl ether, 10 ml of 0.5 N aqueous KOH and 10 ml of distilled water, respectively. The organic solvent was removed under vacuum and the unsaponifiable fraction was used for the sterols analysis. Before injection, samples were silylated according to SWEELEY *et al.* (1963) and the sterol separation was performed by GC/MS (GCMS-QP2010 Plus, Shimadzu, Tokyo, Japan) in the same chromatographic conditions reported by CARDENIA *et al.* (2012). Sterols identification was achieved by comparing peak mass spectra with peaks of standard mixture and by comparing them to the GC–MS data reported by PELILLO *et al.* (2003). An internal standard was used to quantify all the sterols identified in seed samples. Sterols composition was measured in 3 replicates for each lipid extract (n = 6) and expressed in mg/100g of oil.

2.6. Statistical analysis

Relative standard deviation was obtained, where appropriate, for all data collected. One-way analysis of variance (ANOVA) was evaluated using Statistica 10 software (StatSoft, Tulsa, OK, USA). The differences between the means of data for the two different cultivars at the three different harvesting stages were compared at the 5% level of significance (p<0.05) using Tukey honest significant difference (HSD) test.

3. RESULTS AND DISCUSSION

3.1. Oil and moisture content

Table 1 shows oil content of *Futura 75* and *Carmagnola* at three different harvest stages expressed on dry basis. *Futura 75* showed a significantly higher (p<0.05) oil content during the second harvest stage (29.9%) rather than the other two harvestings, 22.9% and 26.4% for the first and the third harvest, respectively. This trend should be related to a non homogeneous maturation, in fact *Cannabis sativa* L. shows a maturation from the bottom to the top of the plant and for this reason is not suitable wait the complete seeds maturation because they tend to fall from the plant. This can be solved operating a constant plant selection to make a more uniform maturation and by adopting appropriate cultivation techniques. In addition, also climatic conditions should be responsible of this trend, in fact *Cannabis sativa* L. shows higher yield in raining condition (MADIA et al., 1998). On the other hand, *Carmagnola* did not show any significant differences (p<0.05) in oil content during maturation, in fact it was 24.5%, 24.8% and 24.9% at first, second and third harvest, respectively. Our results are in line, or slightly lower, with literature, LATIF *et al.* (2009), DA PORTO *et al.*, (2012) and KOSTIĆ *et al.* (2014) that reported an oil content of 26-32%, 30% and 25-29%, respectively. The lower oil content may be attributed to cultivation

techniques and climatic conditions; in addition, seeds were completely covered by a green skin that certainly influenced the oil content, it took up space which was reflected in the overall oil content.
As regard moisture (Table 1), an expected reduction in percentage of moisture was recorded in both varieties with the increasing of maturation; it is well known that immature seeds contain a higher water content rather than mature seeds (MATTHÄUS *et al.*, 2006). *Futura 75* starting from a moisture value of 50% at F1, that decreased significantly ($p<0.05$) at F2 (32%) reaching a final value of 20% at full maturity. Moisture content in *Carmagnola* had the same trend, starting from a value of 31% at C1 and decreasing significantly ($p<0.05$) at C2 (17%) and finally at C3 (10%). Moisture value reported in various studies, OOMAH *et al.* (2002), TANG *et al.*, (2006) and DA PORTO *et al.* (2012), are much lower than ours; in fact, they reported values of 7.7, 6.7 and 7.8%, respectively. This could be due to the green skin, as previously mentioned, that covered the seeds that certainly had a high impact on moisture content. SACILIK *et al.* (2006) conducted a study on physical properties of hemp seeds (size, density and porosity) as a function of moisture content and found out values more similar to ours (ranging from 8.6 to 20.9%).

Table 1. Oil (%) and moisture content (%) of the two different cultivars of hemp at three harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
Oil content	22.9±0.6 ^{c}	29.9±0.6 ^{a}	26.4±0.4 ^{b}	24.5±0.2 ^{a}	24.8±0.2 ^{a}	24.9±1.1 ^{a}
Moisture	50.0±1.0 ^{a}	32.0±0.4 ^{b}	20.7±1.3 ^{c}	31.1±2,1 ^{a}	17.5±0.2 ^{b}	10.0±0.1 ^{c}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: $p<0.05$, lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

3.2. Fatty acid profile

A total of 17 and 15 fatty acids in *Futura 75* and in *Carmagnola*, respectively, were identified and quantified by fast GC-FID analysis, within a run time less than 7 minutes. As showed in Table 2, the predominant fatty acid in all samples was linoleic acid (C18:2, ω-6), ranging from about 49 to 54%. Oleic acid (C18:1 *cis9*) was the second major fatty acid detected (~15-16%), followed by α-linolenic acid (C18:3, ω-3, ~ 12-15%), palmitic acid (C16:0, ~ 7-9 %), stearic acid (C18:0, ~ 2-3%) and γ-linolenic acid (C18:3, ω-6, ~2-3%).
In *Futura 75*, the saturated fatty acids (SFA) were present in significantly ($p<0.05$) greater amount at the first harvesting time (16.7%), whereas mono-unsaturated fatty acids (MUFA) did not showed significant differences among the three different harvest stages (18.7%, 18.3% and 18.1% for F1, F2 and F3, respectively). Polyunsaturated fatty acids (PUFA), on the other hand, increased significantly ($p<0.05$) their concentration with hemp maturation, accounting for 64.6%, 69.6% and 69.6% for F1, F2 and F3, respectively. These trends reflect those of the main fatty acid: in fact, PUFA was the most abundant class because of linoleic acid concentration. In *Carmagnola*, SFA had a significantly higher ($p<0.05$) concentration at first harvest stage (12.2%), than the other two (11.8% and 11.6%

in C2 and C3, respectively); while MUFA did not show significant differences between first and third harvest stages (18.2% and 18.0%, respectively), with a slightly decrease in the middle stage (16.7%). PUFA, on the contrary, showed a significant increase (p<0.05) at the second harvest stage (71.6%), between first and third harvestings (69.7% and 70.5%). Comparing our results with literature, SFA content is generally higher than data already reported for hemp native from Italy (7%, DA PORTO *et al.*, 2012) and from other countries like Croatia (9-11%, PETROVIĆ *et al.*, 2015), Spain (11%, MONTSERRAT-DE LA PAZ *et al.*, 2014) and Turkey (9-10%, KIRALAN *et al.*, 2010); this because of palmitic and stearic acid content that is higher than reported literature. Only in a study conducted by DEVI *et al.* (2019) is reported a similar concentration of palmitic acid compared to our results, 10% referred to hemp cultivated in India. MUFA content was higher in our study than literature; 12-16% (KIRALAN *et al.*, 2010), 11% (DA PORTO *et al.*, 2012), 13% (MONTSERRAT-DE LA PAZ *et al.*, 2014) and 10-14% (PETROVIĆ *et al.*, 2015). This because of oleic acid, that represent almost the entire MUFA content. On the contrary, PUFA determined in this study was lower than the already cited literature; in fact, KIRALAN *et al.* (2010) showed a 73-78%; DA PORTO *et al.* (2012) a 80-81%, MONTSERRAT-DE LA PAZ *et al.* (2014) a 75% and Petrović *et al.* (2015) a 74-80%. For this reason, the ratio between unsaturated and saturated fatty acids reported in our study, 3-5 in *Futura 75* and 5-6 in *Carmagnola* is slightly lower than that reported in literature (6,7 showed by MONTSERRAT-DE LA PAZ *et al.*, 2014). In general, this characteristic high ratio between unsaturated and saturated fatty acids can reduce serum cholesterol and atherosclerosis; and prevent heart diseases (REENA *et al.*, 2007). On the other hand, because of its high unsaturation level and susceptibility to oxidation, hemp seeds oil has a short shelf life (KIRALAN *et al.*, 2010) and it is not suitable for hot uses (DA PORTO *et al.*, 2012).

As regard to the ratio between the two essential polyunsaturated fatty acids, linoleic and α-linolenic acid, both varieties, *Futura 75* and *Carmagnola*, showed the highest value at the first harvest, 4.05 and 3.90, respectively (Table 2). This even though linoleic and α-linolenic acids did not have the same trend in the two different varieties. In *Futura 75* linoleic acid showed a significant (p<0.05) increase from the beginning to the end of maturation (49.2%, 52.3% and 52.5% for F1, F2 and F3); in *Carmagnola*, instead, its concentration increased significantly (p<0.05) from first (53.7%) to second harvest (54.7%) and then decreased again at the third harvest stage (53.9%). α-linolenic acid, instead, in *Futura 75* increased significantly (p<0.05) between first (12.1%) and second harvest (14.3%), before decreased again at third harvest stage (13.9%); in *Carmagnola* its concentration increased significantly during all the maturation; 13.8%, 14.6% and 14.9% for C1, C2 and C3, respectively. The high quantity of α-linolenic acid improves hemp oil quality for its positive nutritional implications and beneficial effects against coronary disease and cancer (ARSHAD *et al.*, 2011; FRETTS *et al.*, 2013). In general, our results about these two fatty acids are slightly lower than results in literature. In fact linoleic acid content is reported in a range between 48 and 59% and α-linolenic acid in a range between 16 and 26% (MONTSERRAT-DE LA PAZ *et al.*, 2014; ORSANOVA *et al.*, 2015; PETROVIĆ *et al.*, 2015; MIKULCOVÁ *et al.*, 2017; DEVI *et al.*, 2019; SIUDEM *et al.*, 2019).

3.3. Tocopherols

The individual tocopherols identified are showed in Table 3; a total of 49.8, 86.9 and 41.2 mg/100g of oil were quantified in F1, F2 and F3, respectively; while a total of 94.8, 87.7 and 77.8 mg/100 g of oil were quantified in C1, C2 and C3, respectively. It is recognized

that tocopherols are the most important natural antioxidants because of their free radical scavenge activity, involving a tocopherol-tocopheryl semiquinone redox system (MONTSERRAT-DE LA PAZ *et al.*, 2014). Tocopherols, as well as phenols compounds, have shown different beneficial effects, on degenerative diseases such as atherosclerosis, cardiovascular disease, Alzheimer's disease and certain type of cancer (FROMM *et al.*, 2012).

Table 2. Fatty acids composition (mg/100 mg FAMEs) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			**CARMAGNOLA**		
	F1	F2	F3	C1	C2	C3
C14:0	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}	n.d.	n.d.	n.d.
C16:0	9.3±0.8 ^{a}	7.7±0.1 ^{b}	7.5±0.2 ^{b}	7.4±0.1 ^{a}	7.3±0.1 ^{c}	7.3±0.1 ^{b}
C16:1 t	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}	n.d.	n.d.	n.d.
C16:1 c	0.2±0.0 ^{a}	n.d.	n.d.	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}
C17:0	n.d.	0.05±0.0 ^{ab}	0.0±0.1 ^{a}	0.04±0.3 ^{a}	0.06±0.0 ^{a}	0.07±0.0 ^{a}
C18:0	3.4±0.5 ^{a}	2.7±0.1 ^{b}	2.9±0.1 ^{ab}	3.3±0.1 ^{a}	3.0±0.0 ^{b}	3.0±0.2 ^{c}
C18:1 t	n.d.	n.d	0.03±0.0 ^{a}	0.2±0.1 ^{a}	0.1±0.0 ^{a}	0.12±0.0 ^{a}
C18:1 c9	16.7±0.6 ^{a}	16.7±0.3 ^{a}	16.3±0.9 ^{a}	16.5±0.2 ^{a}	15.1±0.1 ^{b}	16.4±0.1 ^{a}
C18:1 c11	1.1±0.1 ^{a}	1.0±0.1 ^{a}	1.0±0.2 ^{a}	0.9±0.1 ^{a}	0.9±0.1 ^{a}	0.8±0.1 ^{a}
C18:2, ω-6	49.2±1.4 ^{b}	52.3±0.2 ^{a}	52.5±0.6 ^{a}	53.7±0.3 ^{b}	54.7±0.1 ^{a}	53.9±0.1 ^{b}
C18:3, ω-6	2.9±0.1 ^{a}	2.9±0.1 ^{a}	3.0±0.1 ^{a}	2.2±0.1 ^{a}	2.2±0.1 ^{a}	1.5±0.1 ^{b}
C18:3, ω-3	12.2±0.4 ^{b}	14.3±0.1 ^{a}	13.9±0.2 ^{a}	13.8±0.1 ^{c}	14.6±0.1 ^{b}	14.9±0.1 ^{a}
C20:0	1.3±0.1 ^{a}	0.9±0.2 ^{b}	1.1±0.1 ^{a}	1.0±0.2 ^{a}	0.9±0.1 ^{b}	0.9±0.1 ^{c}
C20:1 c	0.7±0.3 ^{a}	0.5±0.0 ^{a}	0.7±0.1 ^{a}	0.4±0.1 ^{b}	0.5±0.1 ^{a}	0.4±0.0 ^{b}
C20:2, ω-6	n.d.	0.03±0.0 ^{a}	0.04±0.0 ^{a}	0.02±0.0 ^{a}	0.02±0.0 ^{a}	0.05±0.0 ^{a}
C20:3, ω-6	0.4±0.1 ^{a}	0.1±0.0 ^{b}	0.2±0.0 ^{ab}	0.02±0.0 ^{b}	0.1±0.0 ^{ab}	0.1±0.0 ^{a}
C22:0	1.9±0.7 ^{a}	0.6±0.1 ^{b}	0.7±0.1 ^{b}	0.5±0.0 ^{a}	0.5±0.0 ^{a}	0.4±0.0 ^{b}
SFA	16.7±1.6 ^{a}	12.1±0.2 ^{b}	12.3±0.2 ^{b}	12.2±0.1 ^{a}	11.8±0.1 ^{b}	11.6±0.1 ^{c}
MUFA	18.7±0.5 ^{a}	18.3±0.2 ^{a}	18.1±0.7 ^{a}	18.2±0.3 ^{a}	16.7±0.1 ^{b}	18.0±0.1 ^{a}
PUFA	64.6±2.0 ^{b}	69.6±0.2 ^{a}	69.6±0.8 ^{a}	69.7±0.4 ^{c}	71.6±0.1 ^{a}	70.5±0.1 ^{b}
Omega-6	52.5±2.4	55.3±3.2	55.7±2.9	55.9±1.8	57.0±1.3	55.6±2.4
LA/ALA	4.05	3.66	3.76	3.90	3.75	3.61
PUFA/SFA	3.87	5.66	5.65	5.70	6.08	6.06

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05, lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

As expected, considering the literature, in all samples γ-tocopherol was the predominant compound followed by β-tocopherol, α-tocopherol and α-tocotrienol. In *Futura 75* γ-tocopherol showed a significative increase (p<0.05) between first and second harvest (40.8 and 82 mg/100 g of oil in F1 and F2, respectively) and then significantly (p<0.05) decrease

at last maturation time (F3, 37.1 mg/100 g of oil). The other tocopherols were present in very low concentration; in fact, β-tocopherol was present only at the first harvesting stage (9.0 mg/100 g of oil) and α-tocotrienol just in the last one (3.3 mg/100 g of oil). α-tocopherol was not detected at first maturation stage, but only in the other two, with a significative (p<0.05) decrease between them, 4.9 and 0.8 mg/100 g of oil for F2 and F3, respectively. For this reason, the total tocopherols content had the same trend of γ-tocopherol, increasing significantly (p<0.05) the total concentration at the second harvest stage (F2) reaching a value about 87 mg/100 g of oil (Table 3). This trend is related to oil content trend, in fact it increased at the second maturation stage, increasing the total tocopherols content. Also in *Carmagnola* γ-tocopherol was the predominant tocopherol with a significative decrease (p<0.05) during maturation, 90.2, 81.8 and 72.9 mg/100 g of oil for F1, F2 and F3, respectively. β-tocopherol, on the contrary, was not detected during the entire maturation. α-tocopherol was present in every maturity stage with a significant increase (p<0.05) in the middle of maturity; 3.5, 5.9 and 4.9 mg/100 g of oil in C1, C2 and C3, respectively. Finally, α-tocotrienol was present just at the beginning of maturity (C1) with a concentration of 1.1 mg/100 g of oil. Total tocopherol concentration follows γ-tocopherol trend, so a constant significative decrease during maturation was recorded; 94.8, 87.7 and 77.8 mg/100 g of oil in C1, C2 and C3, respectively.
The total tocopherols content showed a different trend in the two cultivars analyzed and considering they were cultivated in the same field this is related to the cultivars different origin and their gene pool.
Our results agree with literature where γ-tocopherol represents the 90% of the total tocopherols content (OOMAH *et al.*, 2002; ANWAR *et al.*, 2006; LATIF *et al.*, 2009; MONTSERRAT-DE LA PAZ *et al.* 2014). In a study conducted by KRIESE *et al.* (2004), where hempseed oil was extracted by supercritical fluids, the general tocopherol content was much lower than ours, so, in addition to botanical characteristics, climatic and cultivation conditions, also the extraction method affects the tocopherols content.

Table 3. Tocopherols content (mg/100 g of oil) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
α-tocopherol	n.d.	4.9±0.9^{a}	0.8±0.1 ^{b}	3.5±1.0 ^{b}	5.9±0.1 ^{a}	4.9±0.2 ^{b}
α-tocotrienol	n.d	n.d	3.3±0.1 ^{a}	1.1±0.1 ^{a}	n.d.	n.d.
β-tocopherol	9.0±0.3	n.d	n.d	n.d	n.d	n.d
γ-tocopherol	40.8±1.8^{b}	82.0±4.9 ^{a}	37.1±8.2 ^{b}	90.2±4.8 ^{a}	81.8±3.8 ^{ab}	72.9±2.0 ^{b}
Total	49.8±0.1 ^{b}	86.9±5.7 ^{a}	41.2±1.0 ^{b}	94.8±6.7 ^{a}	87.7±3.8 ^{ab}	77.8±0.9 ^{b}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05. lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

3.4. Sterols

Analysis of the trimethylsilyl derivatives of phytosterols led to identify nine compounds in both varieties of *Cannabis sativa* L. such campesterol, campestanol, stigmasterol,

clerosterol, β-sitosterol, sitostanol, Δ_5- avenasterol, Δ_5-24-stigmastadienol and Δ_7-stigmasterol (Table 4). The total sterols content showed different trend in the two varieties of *Cannabis sativa* L. considered; in fact, it significantly increased (p <0.05) in *Futura 75* during maturation (642.8, 679.1 and 913.4 mg/100 g of oil in F1, F2 and F3, respectively) and, on the other hand, significantly decreased in *Carmagnola* (532.7, 518.7 and 456.1 mg/100 g of oil in C1, C2 and C3, respectively). These trends reflect the β-sitosterol trends, the predominant sterol detected. In fact, it represented the 63% of the total content in both varieties, but its concentration significantly increased and decreased in *Futura 75* and *Carmagnola*, respectively, with maturation (Table 4). In *Futura 75* it had an initial increase about 5% from F1 to F2 and about 36% between F2 and F3; in *Carmagnola*, on the other hand, β-sitosterol decrease about 6% from first to second harvest stage and then, again about 10%, between second and third harvest.
In *Futura 75* campesterol, campestanol, stigmasterol and clerosterol had the same trend reported for β-sitosterol; instead the other sterols detected decreased between the first and the second harvest stage and then increased at full maturity (Table 4). In *Carmagnola*, instead, campesterol and clerosterol decreased significantly (p<0.05) during the maturation; campestanol and stigmasterol did not show significant differences during the three harvesting stages.

Table 4. Sterols content (mg/100 mg of oil) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
Campesterol	74.5±2.5 ^{c}	88.6±4.5 b	123.6±17.0 ^{a}	66.6±4.6 ^{ab}	69.8±1.3 ^{a}	61.8±0.4 ^{a}
Campestanol	4.5±0.6 ^{b}	6.9±2.9 a	8.2±1.4 ^{a}	2.4±0.8 ^{a}	3.7±0.7 ^{a}	3.3±0.3 ^{a}
Stigmasterol	34.1±1.3 ^{b}	33.7±3.5 b	41.8±1.9 ^{a}	16.1±2.7 ^{a}	16.2±1.5 ^{a}	15.7±1.6 ^{a}
Clerosterol	6.1±0.5^{c}	6.3±0.3 b	8.6±0.4 ^{a}	4.3±0.7 ^{a}	3.9±0.8 ^{a}	3.4±0.3 ^{a}
β-sitosterol	402.1±4.3 ^{c}	423.7±6.6 ^{b}	575.9±6.1 ^{a}	344.1±3.9 ^{a}	324.2±4.7 ^{b}	293.6±1.4 ^{c}
Sitostanol	30.3±1.7 ^{a}	27.5±3.6 a	33.1±5.3 ^{a}	14.8±0.7 ^{a}	11.3±0.4 ^{b}	14.2±0.2 ^{a}
Δ_5-avenasterol	50.6±2.3 ^{c}	54.6±1.2 b	71.8±19.0 ^{a}	47.3±2.1 ^{a}	52.8±8.5 ^{a}	38.8±0.8 ^{b}
Δ_5-24-stigmastadienol	8.7±0.3 ^{a}	8.4±0.6 a	9.0±0.8 ^{a}	7.2±0.7 ^{a}	6.4±0.3 ^{a}	4.8±0.1 ^{b}
Δ_7-stigmasterol	31.9±0.9 ^{b}	29.4±0.3 b	41.5±1.8 ^{a}	29.8±1.3 ^{a}	30.4±6.1 ^{a}	23.1±0.2 ^{b}
Total	642.8±14.4 ^{b}	679.1±21.5 b	913.4±35.7 ^{a}	532.7±17.5 ^{a}	518.7±34.3 ^{a}	456.1±0.8 ^{b}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05. Lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages

To the best of our knowledge, only MONTSERRAT-DE LA PAZ *et al.* (2014) reported a study of sterols in hemp seed oil. Our results were higher than what is reported by these authors for Spanish hempseed oil (total content of 279.4 mg/100 g of oil) but β-sitosterol (190.5 mg/100 g of oil) and campesterol (50.6 mg/100 g of oil) were the same predominant sterols. Comparing sterols composition of HSO with other oils it is possible to see some differences. Considering other vegetable oils, olive oil, linseed oil and hazelnut oil contain

an higher amount of campesterol, β-sitosterol and Δ_5-avenasterol than HSO. Campesterol reachs values of 40, 50-95, 785 mg/kg; stigmasterol values of 20, 10-18, 343 mg/kg; β-sitosterol values of 750, 1050-1700, 1600 mg/kg and Δ_5-avenasterol value of 40-140, 20-80, 369 mg/kg in olive oil, hazelnut oil (AZADMARD-DAMIRCHI *et al.*, 2015) and linseed oil (MATTHÄUS *et al.*, 2017), respectively. In addition, linseed oil contains cholesterol, brassicasterol and 5,24-stigmasterol that are not present in HSO. On the other hand, HSO is richer in sterols that sunflower oil, in fact campesterol, stigmasterol, β-sitosterol and Δ_5-avenasterol report value of 20, 28, 186 and 20 mg/100g, respectively (YILMAZ *et al.*, 2019). Corn oil has the same stigmasterol content of HSO (about 33 mg/100g); is poorer in β-sitosterol than HSO (266 mg/100 g) and, on the other hand, is richer in campesterol (191 mg/100 g) (YANG *et al.*, 2018).
In general, even if sterols are minor constituents of vegetable oils and are present in the unsaponifiable fraction (GUSAKOVA *et al.*, 1998), it is well known that they have a lot of beneficial effects on human health. In fact, they can reduce the serum level of cholesterol concentration, atherosclerotic risk (NTANIOS *et al.*, 2003; PATEL *et al.*, 2006), low-density lipoprotein cholesterol and they are related to a lower risk of myocardial infarction (KLINGBERG *et al.*, 2013).

4. CONCLUSION

This study confirms that ripening stage affects the quality of hempseed oil extracted from *Cannabis sativa* L.; oil content was constant during the maturity only in cultivar *Carmagnola* and moisture content decreased constantly for both cultivars; essential fatty acids (LA and ALA) increased with ripening while tocopherols decreased. Sterols showed a different trend in the two varieties considered, in *Futura 75* the total concentration increased and in *Carmagnola* decreased. Knowledge of the influence of ripening stage on hempseed oil quality has important consequences for industrial output, as harvest could be programmed when oil, tocopherols and sterols are most abundant. *Futura 75* seemed to have a higher concentration of oil, sterols and LA/ALA ratio; while *Carmagnola* a higher concentration of tocopherols and PUFA/SFA ratio. The comparison between the two varieties and the results obtained are very important for hemp seeds producers since they can choose the best variety to plant from a production and quality point of view

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