Grasas y aceites

International Journal of Fats and Oils

Volumen 72 | Nº 2 | April-June 2021 | Sevilla (España) | ISSN-L: 0017-3495

CSIC
INSTITUTO DE LA GRASA

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Grasas y aceites

International Journal of Fats and Oils

Volume 72 • N.º 2 **April-June 2021** Sevilla (España) ISSN-L: 0017-3495

MINISTERIO DE CIENCIA E INNOVACIÓN

CSIC

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

Volume 72 • N.º 2 **April-June 2021** Sevilla (España) ISSN-L: 0017-3495

GRASAS Y ACEITES
REVISTA PUBLICADA POR EL INSTITUTO DE LA GRASA, CSIC

La revista *Grasas y Aceites (Grasas Aceites)*, de periodicidad trimestral, es una publicación dedicada a la información científica y técnica sobre grasas comestibles y sus derivados.
Publica trabajos de investigación originales, artículos de información, notas de laboratorio, trabajos de revisión así como bibliografía sobre revistas, patentes o libros. El campo que cubre se refiere fundamentalmente a frutos y semillas oleaginosas, materias grasas, productos afines o derivados y aceitunas de mesa. Igualmente, incluye trabajos relacionados con subproductos de todas las materias anteriores y el tratamiento de las aguas residuales de las industrias correspondientes. Los originales recibidos son evaluados por el Consejo de Redacción y por evaluadores externos.
Se publica desde 1.991 en edición electrónica, modelo acceso abierto en:
http://grasasyaceites.revistas.csic.es.

Grasas y Aceites (Grasas Aceites) is a quarterly published journal devoted to scientific and technological information on the field of edible fat and oils and their derivatives.
Grasas y Aceites publishes full research articles, research notes, reviews as well as information on references, patents, and books. Grasas y Aceites covers the following fields: oleaginous fruit and seeds, edible fatty materials as well as related products and their derivatives, including table olives. It also accept works related to by-products from all the previous materials and the handling and treatment of the wastewaters from the corresponding industries. Originals are always reviewed by the Editorial Board and by qualified experts.
There is since 1.991 an electronic edition, open access model, at:
http://grasasyaceites.revistas.csic.es

REDACCIÓN E INFORMACIÓN

Revista Grasas y Aceites
INSTITUTO DE LA GRASA, CSIC
Universidad Pablo de Olavide - Edificio 46
Ctra. de Utrera, km. 1 - 41013, Sevilla (Spain)
Tel.: +34 954 611 550
e-mail: grasasyaceites@ig.csic.es
http://grasasyaceites.revistas.csic.es

DISTRIBUTION, SALES and SUBSCRIPTIONS

Editorial CSIC / CSIC Press
c/ Vitruvio 8
28006 Madrid
Tel: +34 915 681 402
Email: publ@csic.es
editorial.csic.es

Librería Científica del CSIC
Calle de Serrano, 123 28006 Madrid
Telf.: +34 915 680 051
E-mail: libreria@csic.es

Sale of Printed edition available until 2013 at:
http://editorial.csic.es/publicaciones/revista/17/1/10/grasas-y-aceites.html:
Subscription to the Digital Collection as PDF available since 2011 through the following associated plataforms: Casalini Libri s.p.a., Digitalia US, e-Libro.net and e-Libro.com

SERVICIO DE INFORMACIÓN

Los artículos publicados por Grasas y Aceites son recogidos, entre otras, por las siguientes bases de datos: C.A.S. Chemical Abstracts Services (USA), I.A.L.I.N.E. (Francia), C.A.B.S. Current Awareness in Biological Sciences (Gran Bretaña), F.S.T.A. Food Science and Technology Abstracts (USA-Gran Bretaña), BIOSIS Biological Abstracts (USA), SCISEARCH Science Citation Index Search (USA), SWETS (Holanda), CAB Commowealth Agricultural Bureaux (Gran Bretaña), OSTI Office of Scientific and Technological Information (USA), FOODS-ADLIBRA (USA), PASCAL (Francia), A.A. Analytical Abstract (Gran Bretaña), Cambridge Scientific Abstracts (USA), Current Contents (USA), Deutsche Gessellscharft für Fettiwissenschaft (Alemania), ICYT Índice Español de Ciencia y Tecnología España) y en el CINDOC.

Cada autor es responsable del contenido de sus respectivos trabajos.
El Consejo Superior de Investigaciones Científicas no se hace responsable, en ningún caso, de la credibilidad y autenticidad de los trabajos.
Los originales de la revista Grasas y Aceites, publicados en papel y en versión electrónica, son de propiedad del Consejo Superior de Investigaciones Científicas, siendo necesario citar la procedencia en cualquier reproducción parcial o total.

Authors take full responsibility for all statements or opinions included in their papers.
CSIC does not assume any liability with respect to the credibility or authenticity of the contributions.
Articles appeared in Grasas y Aceites, both in the printed and the electronic versions, are property of the CSIC and it is needed to cite the origin of the article in any total or partial reproduction.

ISSN-L: 0017-3495
NIPO (en línea): 833-20-022-X
Impreso en España. Printed in Spain.
Maquetación: Editorial MIC

Volume 72 • N.º 2 **April-June 2021** Sevilla (España) ISSN-L: 0017-3495

Grasas y aceites

CONTENIDO

Investigación

CONTENTS

Research

GRASAS Y ACEITES 72 (2)
April-June 2021, e401
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1261192

The impact of different levels of nisin as a biopreservative agent on the chemical, sensory and microbiological quality of vacuum-packed sea bass (*Dicentrarchus labrax*) fillets stored at 4 ± 2 °C

Y. Ucar[a, ✉], Y. Ozogul[b], F. Ozogul[b], M. Durmus[b], A.R. Kösker[b] and E. Küley Boga[b]

[a]Fatsa Faculty of Marine Science, Ordu University, Ordu, Turkey

[b]Faculty of Fisheries, Cukurova University, Balcalı, 011330 Adana, Turkey
✉Corresponding author: yucar@cu.edu.tr

Submitted: 25 December 2019; Accepted: 10 March 2020; Published online: 03 June 2021

SUMMARY: Nisin is produced by *Lactococcus lactis* subsp. *lactis* and is also known as an antimicrobial agent especially effective against gram-positive bacteria. It has long been used as a preservative in foods and beverages and is generally regarded as safe (GRAS). In the present work, the effects of different concentrations of nisin (0.2, 0.4 and 0.8%) on the sensory, chemical and microbiological quality and shelf-life of vacuum-packed sea bass (*Dicentrarchus labrax*) fillets were investigated during chilled (4 ± 2 °C) storage. The sensory points for raw and cooked fillets increased with time during the storage period ($p < 0.05$). The control group, with scores of 9.08, was rejected by panelists on day 12; whereas nisin-treated groups were rejected on day 14 with scores ranging from 9.00-9.17 score. As a result of chemical analyses, lower values ($p < 0.05$) were obtained from the nisin groups with low oxidative rancidity. Moreover, nisin inhibited microbial growth, which shows antimicrobial activity. Consequently, it was concluded that the application of nisin (especially 0.8%) preserved the organoleptic quality and extended the shelf-life of sea bass fillets.

***KEYWORDS*:** *Antimicrobial activity; Dicentrarchus labrax; Nisin; Quality changes; Shelf life*

RESUMEN: ***Impacto de diferentes niveles de nisina como agente bioconservador en la calidad química, sensorial y microbiológica de filetes de lubina (*Dicentrarchus labrax*) envasados al vacío y almacenados a 4 ± 2 °C.*** La nisina es producida por *Lactococcus lactis* subsp. lactis y conocida como agente antimicrobiano, especialmente contra las bacterias grampositivas. Se ha utilizado como conservante en alimentos y bebidas durante mucho tiempo y generalmente se considera seguro (GRAS). En el presente trabajo, se investigaron los efectos de diferentes concentraciones de nisina (0,2, 0,4 y 0,8%) sobre la calidad sensorial, química y microbiológica y la vida útil de los filetes de lubina (*Dicentrarchus labrax*) envasados al vacío durante el enfriamiento y almacenamiento (4 ± 2 °C). La puntuación sensorial de los filetes crudos y cocidos aumentó con el tiempo durante el período de almacenamiento ($p < 0,05$). El grupo de control con puntuación de 9,08 fue rechazado por los panelistas el día 12, mientras que los grupos de tratamiento con nisina fueron rechazados el día 14 con un rango de puntuación de 9,00-9,17. Como resultado de los análisis químicos, se obtuvieron valores más bajos ($p < 0,05$) de los grupos de nisina con baja rancidez oxidativa. Además, la nisina inhibió el crecimiento microbiano que muestra actividad antimicrobiana. En consecuencia, se evaluó que la aplicación de nisina (especialmente 0,8%) conservó la calidad organoléptica y prolongó la vida útil de la lubina.

PALABRAS CLAVE: *Actividad antimicrobiana; Cambios de calidad; Dicentrarchus labrax; Nisina; Vida útil*

Citation/Cómo citar este artículo: Ucar Y, Ozogul Y, Ozogul F, Durmuş M, Köşker AR, Küley Boğa E. 2021. The impact of different levels of nisin as a biopreservative agent on the chemical, sensory and microbiological quality of vacuum-packed sea bass (*Dicentrarchus labrax*) fillets stored at 4 ± 2 °C. *Grasas Aceites* **72** (2), e401. https://doi.org/10.3989/gya.1261192

1. INTRODUCTION

Food preservation has been seen as a serious problem throughout human history not only of fish meat but also of food products in general. This has led to the development of many traditional conservation methods in different regions of the world in order to keep the foods at their consumable levels for a long time. Today, due to the development of communication technologies and urbanization, the food industry is growing day by day. Despite this growth in the food industry, microbiological spoilage and food poisoning due to microorganisms is still an issue. According to recent information from Centers for Disease Control and Prevention in the United States, 76 million food-borne diseases occur in the United States every year and this leads to the death of approximately 5000 people (Mead *et al.,* 1999). This has led the food industry, the government and the public to examine the efficacy of current food preservation techniques (Anonymous, 2000). The negative effects of synthetic preservatives on food and human health have raised the demand for more minimally-processed or natural foods due to the development of antibiotic-resistant strains and negative perceptions on the part of consumers against synthetic preservatives. Thus, there has been a considerable amount of interest in natural antimicrobial agents.

Natural or synthetic preservatives such as antioxidants and antimicrobials are widely used to prevent or control the growth of microorganisms and undesirable compounds. One of the most prominent methods in recent years is to preserve the freshness of foods by using bacterial products. Food safety has become an increasingly crucial problem worldwide, so it is of great interest to apply antimicrobial peptides from lactic acid bacteria (LAB) which target food pathogens without causing toxicity and other adverse effects. Bacteriocins are widely used in commercial applications in nearly 50 countries and generally accepted as safe (GRAS) as approved by the Food and Agriculture Organization/World Health Organization and the European Union. LAB is well known for its bacteriocin production capacity.

Nisin, one of the emerging interesting bacteriocins, is a peptide with antimicrobial activity produced by *Lactococcus lactis* subsp. *lactis*, and generally considered safe and used to control pathogens in foods (Juneja *et al.,* 2012; Meral *et al.,* 2019; Ceylan *et al.,* 2018; Ucar *et al.,* 2020). Nisin is defined as an antimicrobial agent discovered before penicillin which showed an antimicrobial activity against a wide variety of gram-positive bacteria (vegetative cells and spores). Nisin has been included in the European Food Additives list as a biopreservative component with the code E234 (EFSA, 2006).

There are some studies showing the synergistic effects when nisin was applied to food without the interaction of nisin with some antioxidant extracts or sensory changes (Sallam, 2007; Behnama *et al.,* 2015; Ghomi *et al.,* 2011). Sea bass (*Dicentrarchus labrax*) is heavily consumed among commercial marine fish species due to its flavor and taste. To our knowledge, there is little information on the efficacy of nisin on the quality and safety of sea bass. Thus, in the current study, the effects of different concentrations of nisin on the sensory, chemical and microbiological quality and shelf-life of vacuum-packed sea bass fillets were investigated during refrigerated storage (4 ± 2 °C) conditions.

2. MATERIALS AND METHODS

2.1. Preparation of nisin

Nisin (in powder form) obtained from *Lactococcus lactis* (2.5% balance between sodium chloride and denatured milk solids, EC No 215-807-5, Sigma N5764) was purchased from a local company in Turkey (Tutar Lab. Chemicals, Adana, Turkey). A nisin solution was prepared according to Ceylan (2014) with minor modifications. Stock solutions (0.2%, 0.4% and 0.8% w/v) of nisin were prepared by dissolving in pure, sterile water. The chemical properties of the nisin used in this study are presented in Table 1.

2.2. Sample preparation

Sea bass (*Dicentrarchus labrax*) was provided from a local fish farm in Mersin, Turkey and they were killed by immersing them in ice-cold water (hypothermia). The samples were moved to the laboratory packed in ice within 1 hour from harvesting and death. The average weight of the sea bass was 312.06 ± 26.85g; while the length was 29.77 ± 1.02 cm. The samples were divided into four lots and then immediately gutted and filleted with the skin on. One lot was stacked onto plates (6 fillets per plate) and the rest were treated with nisin solutions.

Table 1. Physical and chemical properties of nisin

CAS-Number	1414-45-5
Formula	$C_{143}H_{230}N_{42}O_{37}S_7$ (polypeptid)
Molekular weight	3.354,07 g/mol
Solubility	Soluble in water, insoluble in non-polar solvents
Functional use	Antimicrobial preservative
E number	E-234
GRAS	Yes
ADI (acceptable daily intake)	0-2 mg/kg bw
Boiling temperature	2967ºC
Flash point	110 °C (230°F)
Storage temperature	2-8 °C
pH	for 0,2% nisin 4.40 for 0,4% nisin 4.20 for 0,8% nisin 4.12
Color	Light brown micronized white powder
Color parameters of nisin	*L** :73.26±0.02 *a**:1.36±0,01 *b**:8,38±0,03
Total phenolic content of nisin	23.56 (mg GAE/g)

GAE: equivalence of gallic acid

The fish fillets were placed in the formulated nisin solutions at various concentrations for 10 minutes. After that, all the groups were wrapped in pouches of 90 µm thick polyamide film (Polinas, Manisa, Turkey) by using a vacuum packing machine (Reepack RV50; Reepack, Seriate, Italy). Water and oxygen permeability were 8.5 g/m^2 per 24 h and 160 cm^3·m^2 per 24 h, respectively. Then, all of the samples were stored in a chilled room (4 ± 2 °C). 3 plates (total of 12 fillets) were randomly chosen from each group for each analysis daily. Analyses were carried out on days 0, 3, 6, 8, 10, 12, 14, 16 and 18.

2.3. Proximate analysis

The protein contents in the samples were adjusted using the Kjeldahl procedure (AOAC, 1984) and a Buchi Digestion System, Model K-424 (BÜCHI Labortechnic, Flawil, Switzerland) and a Kjeltec Distillation Unit B-324 (BÜCHI Labortechnic). A Kjeldahl conversion factor of N x 6.25 was used to calculate the percent protein. In addition, lipid content was determined according to the method described by Bligh and Dyer (1959). Furthermore, ash and moisture analyses were carried out according to the methods of AOAC 920.153 (2002) and 950.46 (2002), respectively**.**

2.4. Sensory analysis

For sensory analysis, the Quality Index Method (QIM) scheme improved by Bonilla *et al.,* (2007) was used for raw fish with minor modifications. The scheme comprised of quality parameters (e.g., skin brightness, skin mucus, flesh texture, flesh-blood, odor, color, brightness, gaping). The corresponding procedure is mentioned in our previous study (Özogul *et al.,* 2016).

The measurement of the freshness of cooked fish (odor, taste, and texture) was assessed according to Torry Scheme (Howgate, 1982). A hedonic scale from 10 to ≤ 3 was used to evaluate the fish. A score of 10 represents very fresh fish, while ≤ 3 represents putrid or spoiled fish. Three random fillets from each group were cooked on top of a glass plate in a microwave (Model: Siemens HF24G241, Munich, Germany) at 600 W for 2 min.

2.5. Chemical analyses

Total volatile basic nitrogen (TVB-N) content in muscle was determined according to the method of Antonocopoulos (1973); the thiobarbituric acid reactive substances (TBARS) analysis was performed according to the method of Tarladgis *et al.,* (1960); the free fatty acid (FFA) analysis was carried out according to AOCS method Ca 5a-40 (1997) and peroxide value (PV) was determined according to AOCS method Ja 8-87 (1994). All details pertaining to the analytical methods are given in our previous study (Özogul *et al.,* 2016).

A pH meter (315i/SET, Weilheim, Germany)

was used to determine the pH of the fish fillets. The samples (n=3) were homogenised with a homogenizer (Ika-Werke Ultra-turrax, model T25, Staufen, Germany) at a ratio of 1:10 (w/v) in distilled water at its highest setting (12000 rpm) for 3 min.

2.6. Microbiological analysis

To estimate the mesophilic aerobic bacteria and psychrophilic viable counts, triplicate samples (one per storage plate) with duplicate measurements from each group (n=6) were taken from each of the different treatments. Violet Red Bile Agar (VRBA, Oxoid, CM0107, Hampshire, England) was carried out and prepared according to the instructions of the manufacturer for total *Enterobacteriaceae*. The detailed measurement was described in our previous study (Özogul *et al.,* 2016).

2.7. Statistical analysis

All of the experiments in the study were performed in triplicate. Therefore, the results were presented as the mean and their standard deviation of the measurements. Significant differences in the results were determined by applying a one-way analysis of variance (ANOVA) using the SPSS version 22 software (SPSS, Chicago, Illinois, USA) and the Duncan's Multiple Range Test comparisons at p-value of < 0.05.

3. RESULTS AND DISCUSSION

3.1. Proximate analysis

Proximate analyses (crude protein, lipid, moisture, and ash) of the sea bass fillets were determined as 19.52, 3.90, 74.44, and 1.18%, respectively. Yazgan *et al.,* (2017) found similar values for crude protein (19.36%), lipid (5.14%), moisture (73.85%) and ash contents (%1.36) in sea bass. The average crude protein content (N × 6.25) values ranged from 17.9 to 21.5% for sea bass as reported by Ballestrazzi and Lanari (1996), Kyrana and Lougovois (2002), Alasalvar *et al.,* (2002), which coincide with the results of the current study. It was reported that the differences were caused by environmental conditions such as region, season and cultural factors (Alasalvar *et al.,* 2002).

3.2. Sensory analyses

Sensory changes (smell, taste, and texture) during the storage of vacuum packaged raw sea bass fillets are depicted in Figure 1. The sensory points for raw fillets increased during the storage period and there were statistical differences among the control group and the treatment groups. Treated groups were found to have a longer shelf-life than those of the control group in terms of sensory parameters. Firstly, the control group with 9.08 points (d12) was rejected by panelists. 0.2% nisin group (9.17), 0.4% (9.00) and 0.8% nisin groups (9.00) were rejected later (d14). The application of nisin reduced fish odor and maintained the appearance of the fish. The use of nisin groups prolonged the shelf-life by 2 days when compared to the control group. Some researchers reported that nisin has a synergistic effect when used with different preservation techniques (Lu *et al.,* 2010). Ceylan (2014) reported that the sensory values for raw sea bass treated with nisin in combination with the irradiation method were higher than those of the group with only nisin and the irradiated group. The combination group of nisin and irradiation extended the shelf-life of the fish by 6 days, showing beneficial effects in terms of sensory quality, similar to our study.

Changes in the sensory quality of cooked sea bass are shown in Figure 1. The acceptability scores for the odor, taste, and texture of the fish decreased with storage time. Sea bass with nisin received better scores from the panelists compared to the control group during storage. Increasing nisin concentration had a positive effect on the sensory quality of the fish fillets especially for protecting the color of fillets and removing odor. The odor, taste, and texture scores for the control were 4.57, 4.71 and 4.86, respectively, at the time of rejection (d 12). The nisin-treated groups were rejected on the 14th day of storage. The applications of nisin to sea bass fillets produced a notable development ($p < 0.05$) in the odor, texture, and taste of the fish. However, among the treatment groups, the best values for odor (4.67), taste (4.75) and texture (4.83) were observed in the 0.8% nisin-treated group at the end of the storage time. Ceylan (2014) reported that during the storage period, the groups treated with nisin received higher scores for odor, texture, color, and taste in cooked sea bass fillets compared to the control group.

3.3. Chemical analyses

3.3.1. Changes in peroxide values

There are high levels of unsaturated fatty acids in seafood and these oils are very susceptible to

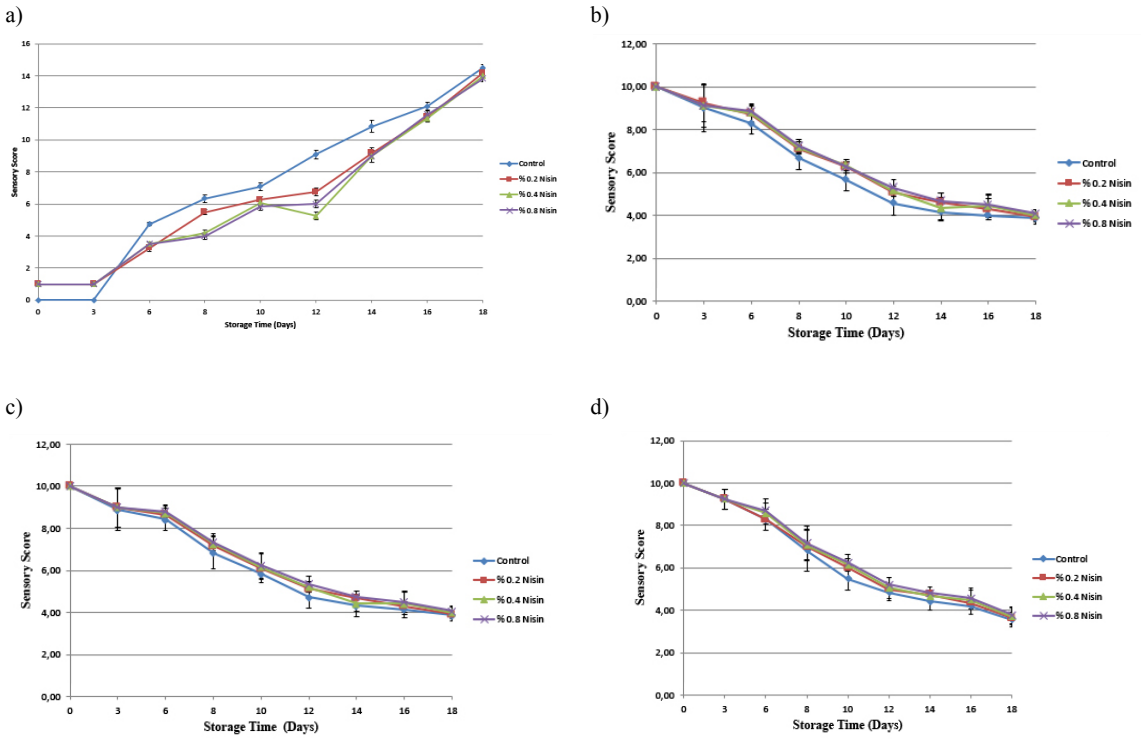


FIGURE 1. The sensory evaluation of sea bass treated with nisin. a) Sensory scores for raw sea bass; b) odor scores for cooked sea bass; c) taste scores for cooked sea bass; and d) texture scores for cooked sea bass.

oxidation. As mentioned above, PV is an indicator of the beginning of lipid oxidation and is the most common index of lipid hydroperoxides. The changes in PV of the control and nisin-treated groups are presented in Table 2. The initial PV was in the range of 2.18 and 2.65 meq O_2/kg. The PV of all treatment and the control groups fluctuated during the storage period and the PV of the nisin-treated groups was lower than the control group during storage. There were statistically significant differences for all groups ($p < 0.05$). A PV value below 5 meq O_2/kg demonstrates that the lipids are fresh due to the hydroperoxides having degraded into ketones. A PV value between 5 and 10 meq O_2/kg showed that the lipids are rancid (Gracey *et al.,* 1999). The upper limit for PV is 20 meq O_2/kg oil (Connell, 1995). The PV did not reach the upper limit in any of the groups during storage, which means the values were below the maximum recommended value for human consumption. Among the nisin groups, the lowest PV was observed in the 0.8% nisin group (2.73 meq O_2/kg) at end of the trial followed by the 0.4% nisin group (3.00 meq O_2/kg) and the 0.2% nisin group (3.84 meq O_2/kg) groups. The nisin application delayed lipid oxidation, depending on its concentration. The decrease in PV in the treatment groups was thought to be due to the influence of vacuum packaging and nisin on the antioxidant effect of lipolytic bacteria (Nykanen *et al.,* 2000) and also its antimicrobial effect on some bacteria such as *L. monocytogenes* and Pseudomonas sp. Similar results were obtained by Ghomi *et al.,* (2011) for grass carp slices and by Behnama *et al.,* (2015) for vacuum-packed rainbow trout (*O. mykiss*) treated with sodium and nisin.

3.3.2. Changes in thiobarbituric acid (TBARs)

Peroxides are formed in the primary stage of the oxidation of lipids found in seafood. In the advanced stage of oxidation, TBARs appear as a freshness parameter that measures the degree of secondary lipid oxidation. The TBAR concentration of freshly-caught fish changes from 3 to 5 mg of malondialdehyde (MA) equivalents per kilogram of flesh (Kuley *et al.,* 2012). Table 2 shows the TBAR changes in the sea bass fillets treated with or without nisin stored at 4 ± 2 °C. While the initial TBARs of the asea bass fillets were found in the range of

Table 2. The changes in PV, TBARs, FFA, TVB-N, and pH contents of sea bass fillets during storage

Strorage days	Control	0.2%	0.4%	0.8%
PV values (meq O_2/kg)				
0	2.65 ± 0.14^{Ea}	2.18 ± 0.08^{Fb}	2.32 ± 0.11^{Gb}	2.22 ± 0.09^{Fb}
3	2.85 ± 0.05^{Ea}	2.32 ± 0.07^{Fbc}	2.42 ± 0.06^{Gb}	2.19 ± 0.10^{Fc}
6	5.20 ± 0.15^{Ca}	4.53 ± 0.23^{Cbc}	4.63 ± 0.25^{Cb}	4.25 ± 0.08^{Cc}
8	6.26 ± 0.13^{Ba}	4.08 ± 0.15^{DEbc}	4.33 ± 0.17^{Db}	4.03 ± 0.16^{Cc}
10	4.58 ± 0.17^{Dc}	5.01 ± 0.28^{Bab}	5.13 ± 0.16^{Ba}	4.70 ± 0.08^{Bbc}
12	5.38 ± 0.53^{Ca}	4.41 ± 0.20^{CDb}	3.96 ± 0.22^{Ebc}	3.70 ± 0.14^{Dc}
14	6.92 ± 0.37^{Aa}	6.53 ± 0.37^{Aa}	5.63 ± 0.22^{Ab}	5.74 ± 0.22^{Ab}
16	7.29 ± 0.22^{Aa}	4.21 ± 0.24^{CDEb}	4.25 ± 0.07^{Db}	4.13 ± 0.06^{Cb}
18	4.46 ± 0.14^{Da}	3.84 ± 0.18^{Eb}	3.00 ± 0.12^{Fc}	2.73 ± 0.13^{Ec}
TBARs content (mg malonaldehyde (MA)/kg fillet)				
0	0.27 ± 0.01^{Ea}	0.26 ± 0.01^{Eab}	0.24 ± 0.01^{Gc}	0.25 ± 0.01^{Ebc}
3	0.28 ± 0.01^{Ea}	0.27 ± 0.01^{Ea}	0.25 ± 0.01^{FGb}	0.27 ± 0.02^{Ea}
6	0.43 ± 0.04^{CDa}	0.36 ± 0.02^{Cb}	0.36 ± 0.02^{Db}	0.37 ± 0.02^{BCDb}
8	0.32 ± 0.04^{Ea}	0.27 ± 0.01^{Eb}	0.27 ± 0.00^{Fb}	0.27 ± 0.00^{Eb}
10	0.46 ± 0.03^{Ca}	0.36 ± 0.01^{Ca}	0.39 ± 0.01^{Ca}	0.35 ± 0.17^{CDa}
12	0.52 ± 0.04^{Ba}	0.42 ± 0.01^{Bb}	0.42 ± 0.01^{Bb}	0.40 ± 0.05^{BCb}
14	0.62 ± 0.06^{Aa}	0.53 ± 0.03^{Ab}	0.47 ± 0.01^{Ac}	0.44 ± 0.03^{ABc}
16	0.39 ± 0.06^{Da}	0.31 ± 0.00^{Db}	0.31 ± 0.00^{Eb}	0.31 ± 0.00^{DEb}
18	0.59 ± 0.06^{Aa}	0.52 ± 0.04^{Ab}	0.45 ± 0.04^{Ac}	0.51 ± 0.01^{Ab}
FFA content (% FFA as oleic acid)				
0	1.72 ± 0.08^{Ea}	1.54 ± 0.07^{Fb}	0.91 ± 0.03^{Ed}	1.18 ± 0.06^{Ec}
3	1.85 ± 0.04^{Ea}	1.57 ± 0.03^{Fb}	0.96 ± 0.06^{Ed}	1.22 ± 0.07^{Ec}
6	3.67 ± 0.18^{Da}	2.98 ± 0.11^{DEb}	3.01 ± 0.16^{Cb}	3.17 ± 0.15^{Cb}
8	3.95 ± 0.05^{CDa}	2.86 ± 0.16^{Eb}	2.63 ± 0.11^{Dc}	2.89 ± 0.13^{Db}
10	3.66 ± 0.12^{Da}	3.03 ± 0.14^{DEb}	3.08 ± 0.11^{Cb}	3.26 ± 0.19^{Cb}
12	5.25 ± 0.31^{Ba}	3.82 ± 0.16^{Cb}	3.62 ± 0.05^{Bbc}	3.32 ± 0.14^{Cc}
14	5.14 ± 0.28^{Ba}	4.22 ± 0.09^{Bb}	3.68 ± 0.21^{Bc}	4.04 ± 0.08^{Bb}
16	6.56 ± 0.35^{Aa}	5.50 ± 0.20^{Ab}	4.96 ± 0.25^{Ac}	4.51 ± 0.21^{Ac}
18	4.23 ± 0.34^{Cb}	3.10 ± 0.07^{Dc}	4.95 ± 0.25^{Aa}	4.25 ± 0.03^{Bb}
TVB-N content (mg/100g fillet)				
0	19.70 ± 0.47^{Ea}	19.57 ± 0.03^{Da}	18.83 ± 0.96^{Ea}	19.78 ± 0.34^{Ea}
3	20.04 ± 0.99^{Ea}	20.61 ± 0.55^{Da}	19.84 ± 0.47^{Ea}	20.82 ± 0.16^{Ea}
6	28.96 ± 0.02^{Da}	26.42 ± 0.65^{Cb}	23.87 ± 0.67^{Dc}	24.93 ± 0.23^{Dc}
8	16.99 ± 0.33^{Fa}	16.17 ± 0.36^{Eab}	15.19 ± 1.11^{Fb}	15.87 ± 0.14^{Fab}
10	31.48 ± 1.12^{Ca}	27.11 ± 0.82^{Cb}	26.76 ± 0.60^{Cb}	25.83 ± 0.02^{Db}
12	38.47 ± 0.41^{Aa}	32.36 ± 0.04^{Bb}	31.50 ± 1.37^{Bb}	28.40 ± 1.20^{Cc}
14	35.36 ± 1.95^{Ba}	35.95 ± 1.03^{Aa}	34.66 ± 1.70^{Aa}	32.98 ± 0.23^{Ba}
16	38.50 ± 0.52^{Aa}	37.03 ± 0.07^{Aab}	35.68 ± 0.53^{Abc}	34.67 ± 1.08^{Ac}
18	39.31 ± 1.33^{Aa}	36.78 ± 1.89^{Aab}	34.66 ± 0.52^{Ab}	35.29 ± 0.33^{Ab}
pH values				
0	6.60 ± 0.04^{CDa}	6.59 ± 0.00^{Ca}	6.53 ± 0.05^{Ca}	6.48 ± 0.19^{BCa}
3	6.62 ± 0.03^{CDa}	6.60 ± 0.01^{BCab}	6.58 ± 0.02^{Bb}	6.60 ± 0.02^{Aab}
6	6.78 ± 0.04^{Ba}	6.64 ± 0.00^{Bb}	6.66 ± 0.01^{Ab}	6.64 ± 0.03^{Ab}

Strorage days	Control	0.2%	0.4%	0.8%
8	6.56±0.02^{Da}	6.47±0.01^{Db}	6.49±0.01^{Db}	6.48±0.01^{BCb}
10	6.64±0.03^{Ca}	6.51±0.02^{Dc}	6.58±0.02^{Bb}	6.57±0.03^{ABb}
12	6.78±0.07^{Ba}	6.50±0.01^{Db}	6.50±0.01^{CDb}	6.38±0.01^{CDc}
14	6.92±0.07^{Aa}	6.60±0.04^{BCb}	6.48±0.02^{Dc}	6.48±0.01^{BCc}
16	6.70±0.07^{Ba}	6.42±0.01^{Eb}	6.36±0.04^{Ebc}	6.34±0.03^{Dc}
18	6.88±0.03^{Aa}	6.75±0.08^{Ab}	6.60±0.05^{Bc}	6.57±0.02^{ABc}

Values represent mean ± SD of 3 replicates in duplicate. Means sharing the same letter in the same column (a–f) and in the same row (A-F) are not significantly different ($P < 0.05$) using Duncan's multiple range test.

0.24 - 0.27 mg MA/kg, at the end of the trial it was between 0.45 mg MA/kg and 0.59 mg MA/kg. The TBAR value remained below the limit value. TBAR values were found statistically significant for both the control and nisin groups during the storage periods ($p < 0.05$). The highest TBAR value was found for the control group (0.62 mg MA/kg) on day 14, compared to those of all groups. These results demonstrated low oxidative rancidity because of the nisin and also the effects of vacuum packaging. Mendes and Golcalves (2008) reported that TBAR values in vacuum packed sea bream and sea bass were lower since the removal of oxygen from the package prevented oxidation. Ceylan (2014) found that the TBAR values of sea bass fillets at the beginning of storage were 0.81 and 1.32 mg MA/kg for the control and nisin groups, respectively, and 4.70 mg MA/kg on the first day of storage in the nisin + irradiation group.

3.3.3. Changes in free fatty acids (FFA)

Free fatty acids are known to result from the enzymatic hydrolysis of esterified lipids. The changes in FFA values of sea bass fillets treated with or without nisin and stored at 4 ± 2 °C are given in Table 2. The initial FFA value of raw fillets was determined as 1.72 (oleic acid %) for the control group and 1.54, 0.91 and 1.18 for 0.2%, 0.4% and 0.8% nisin-treated groups, respectively. Fluctuations were observed in FFA values in all groups during storage and FFA values among the groups were found to be statistically significant ($p < 0.05$). FFA values ranged from 0.91 to 6.56 (oleic acid %) for all groups during the storage period. Although the minimum FFA values for sea bass fillets treated with nisin (0.4% group) were determined to be 0.91% at the beginning of storage, the maximum FFA values for sea bass fillets treated with nisin (0.2% group) were found to be 5.50% on day 16 of storage. The highest FFA value was found as 6.56% in the control group on the 16th day of storage, while the lowest FFA value was found to be 0.91% in the 0.4% nisin-treated group at the beginning of storage. FFA and their oxidation products interact with myofibrillar proteins and promote protein aggregation. Therefore, they affect the muscle texture and functionality (Gracey *et al.,* 1999). In the current study, it was determined that nisin-treated groups had lower FFA values compared to the control group. It was determined that nisin application depends on the concentration used and vacuum packaging delayed lipid hydrolysis. Similar results for the FFA values were reported by Kyrana and Lougovois (2002) for sea bass. Durmuş *et al.,* (2019) reported that the FFA value fluctuated with the storage period of sea bass fillets (*D. labrax*) stored in cold and vacuum-packed nanoemulsions using vegetable oil.

3.3.4. Changes in total volatile basic nitrojen (TVB-N)

TVB-N is one of the most important chemical parameters used to determine fish quality during storage. Generally, volatile bases are obtained from the microbial degradation of protein and non-protein nitrogenous compounds such as trimethylamine, dimethylamine, ammonia and other volatile basic nitrogen compounds. The TVB-N contents in sea bass fillets stored at 4 ± 2 °C are shown in Table 2. While the TVB-N value was determined as 19.70 mg/100g for the control group, this parameter ranged between 18.83 and 19.78 mg/100g for the nisin-treated groups at the beginning of storage. Mendes and Gonçalves (2008) found that the initial TVB-N value in the sea bass was 21.9 mg/100g. Castro *et al.,* (2006) reported the initial TVB-N value of sea bass to be 19-22 mg/100g. The non-protein nitrogen content in fish, depending on the type of fish feeding, season of catching, fish size, various environmental factors as well as the initial microbiological quality of the fish

tissue may cause these differences in TVB-N values (Connell, 1995). In addition, the TVB-N content was reported to be related to the initial microbial activity in fish meat (Connell, 1995).

The increase in TVB-N values in sea bass by the time of storage was also reported in previous studies (Ceylan, 2014; Durmus *et al.,* 2019). Unlike the results of these studies, TVB-N values showed fluctuations in the early stages of storage and then increased regularly after 8 days of storage and significant differences ($p < 0.05$) were observed between the control and treated groups throughout the storage period. The TVB-N level exceeded the upper acceptability limit (35 mg N/100g of fish flesh) set by the Commission of the European Communities (CEC, 1995) for all groups at the end of the storage period. At the end of the trial, TVB-N values were in the range of 34.66 and 39.31 mg/100 gr. The lowest TVB-N value ($p < 0.05$) was obtained from the 0.4% nisin group (34.66 mg TVB-N/100 g) followed by the 0.8% nisin group (35.29 mg TVB-N/100 g) at end of the storage period, but the highest TVB-N value was obtained from the control (39.31 mg TVB-N/100 g) followed by the 0.2% nisin group (36.78 mg TVB-N/100 g). The fish fillets treated with nisin were observed to have better quality than the control group, depending on its concentrations. Similar findings were observed for Atlantic salmon and rainbow trout fillets treated with nisin together with vacuum packaging (Han *et al.,* 2016; Behnama *et al.,* 2015). During storage, decomposition of the nitrogenous compounds caused an increase in the pH of the fish meat, which may be partly related to the production of alkali compounds.

3.3.5. Changes in pH

As one of the most important factors, pH affects microbial growth and the deterioration of foods, especially seafood. The pH value of fish meat changes due to microbial and enzymatic activities, giving information on the freshness and quality of the seafood. The typical pH of live fish muscle is ≈ 7.0 (Abbas *et al.,* 2008). However, post-mortem pH can oscilate from 6.0 to 7.1, based on the season, species, and the other factors (Simeonidou *et al.,* 1998). The pH changes in vacuum-packed sea bass fillets treated with nisin in cold storage are shown in Table 2. pH was measured at 6.48 and 6.60 at the beginning of storage and fluctuations were observed throughout the storage period. Statistical differences ($p < 0.05$) were observed between nisin-treated groups and the control group during the storage period. The pH changed from 6.34 to 6.92 (Table 2). Among the nisin groups, lower pH values were determined for the 0.8% nisin group (6.34) at end of the storage period, followed by the 0.4% and 0.2% nisin groups, but the control group (6.92) gave the highest pH value. This may be due to the inhibitory effect of nisin on microbial growth. Similar findings were observed for farmed sea bass (Durmus *et al.,* 2019). Ceylan (2014) reported that the pH values of sea bass fillets stored in the cold increased at the end of storage. Behnama et al. (2015) measured the pH values of rainbow trout fillets for the control and the groups treated with nisin at 6.37 and 6.36, on day 0 of storage, respectively.

3.4. Microbiological changes

The degradation of most seafood products is mainly caused by microorganisms. Figure 2 shows microbiological changes in vacuum-packed sea bass fillets treated with different concentrations of nisin.

3.4.1. Total mesophilic bacteria

Figure 2(a) shows total mesophilic bacteria changes in vacuum-packed sea bass fillets treated with different concentrations of nisin. The initial total mesophilic bacterial count was 2.55 log cfu/g and reached a maximum level of 7.89 log cfu/g for the control group at the end of the trial. The lowest bacterial count was obtained in fish fillets treated with nisin. When fish was unacceptable by panelists, total mesophilic counts were above 7.39 log cfu/g for the control and about 6.65 log cfu/g for the other nisin-treated groups. Meral *et al.,* (2019) reported that total mesophilic bacteria in nisin-curcumin- treated rainbow trout fillets were 6.12 log cfu/g after 12 days of storage.

Durmuş *et al.,* (2019) reported that the control group exceeded the limit on the 12th day of storage (day of sensory rejection) with 7.38 log cfu/g in sea bass. Ceylan (2014) reported that the control group exceeded the limit value (6 log cfu/g) on the 9th day and the nisin group exceeded the limit value at 11th days of storage for sea bass. Mesophilic bacteria counts were reported to be above 8 log cfu/g at the time of sea bass rejection (Ozogul *et al.,* 2016). With a combination of nisin and vacuum packaging, total aerobic mesophilic bacteria counts were found to be lower than those of the control, extending the shelf-life

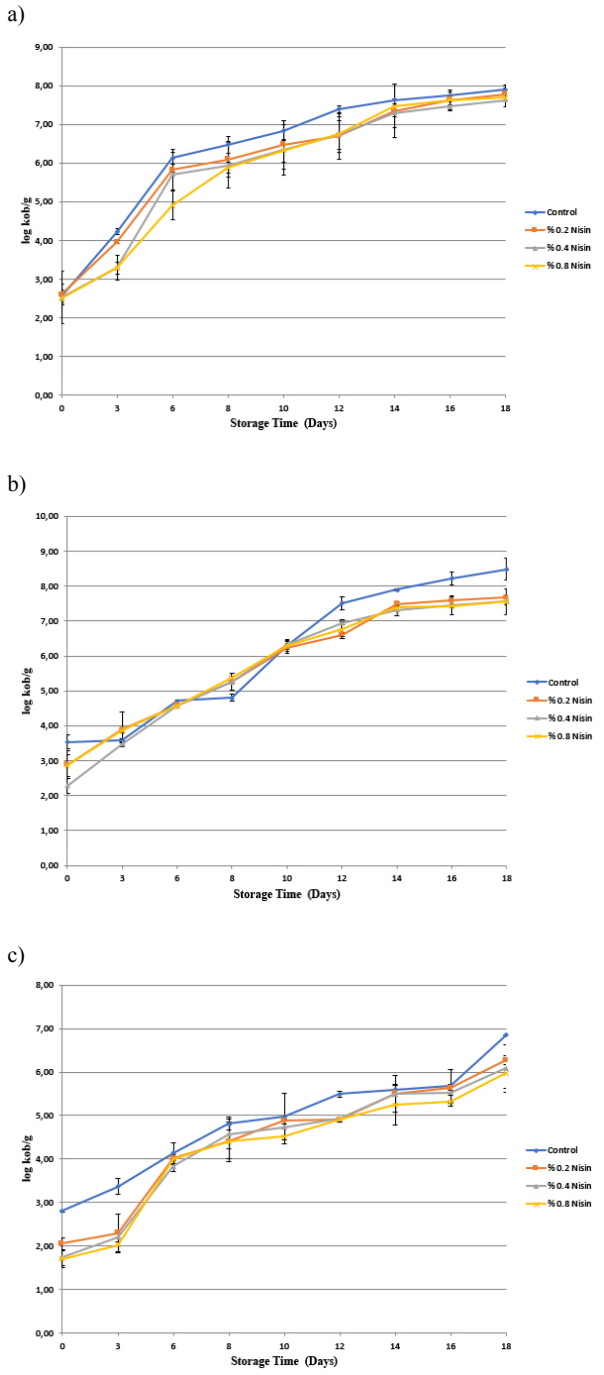


FIGURE 2. Microbiological changes in sea bass treated with nisin. (a) Total mesophilic bacteria, (b) total psychrotrophic bacteria, (c) total *Enterobacteriaceae* counts

of sea bass fillets. Thus, the total aerobic mesophilic bacteria count correlated with the sensory analysis.

3.4.2. Total psychrotrophic bacteria

Psychrotrophic bacteria in the seafood stored in cold causes the deterioration of products. Figure 2(b) shows the total psychrotrophic bacteria counts in vacuum-packed sea bass fillets treated with different concentrations of nisin. The initial total psychrotrophic bacterial count was 3.53 log cfu/g and reached the maximum level of 8.48 log cfu/g for the control group at the end of the trial. At the beginning of storage, total psychrotrophic bacteria was found to be within the range of 2.28 and 2.88 log cfu/g for nisin-treated groups and differences were observed in the total psychrotrophic bacteria during storage. In contrast to the control group, the application of nisin and vacuum packaging decreased total psychrotrophic bacteria growth. It was reported that the growth of psychrotrophic bacteria was delayed in fish treated with nisin (Behnama *et al.,* 2015). Similarly, in the current study, the treatment of fish fillets with nisin and vacuum packaging resulted in lower bacterial growth. When fish was unacceptable by panelists, total psychrophilic counts were above 7.52 log cfu/g for the control (day 12) and about 6.90 log cfu/g for all nisin-treated groups (day 14). Durmus *et al.,* (2019) reported that total psychrotrophic bacteria for the control group of vacuum-packed seabass fillets exceeded the limit value with 7.09 log cfu/g on the 16th day of storage. Psychrotrophic bacteria count was reported to be above 7.2 log cfu/g at the time of sea bass rejection (Ozogul *et al.,* 2016).

3.4.3. Total Enterobacteriaceae counts

Paleologos *et al.,* (2004) found that *Enterobacteriaceae* were part of the spoilage microflora of whole gutted and filleted sea bass stored in ice. In this study, gradual increases in total *Enterobacteriaceae* counts were observed during the storage period. Nisin applications combined with vacuum packaging generally showed the lowest bacterial load. At the beginning of storage, total *Enterobacteriaceae* counts were found to be 2.80, 2.06, 1.74 and 1.71 log cfu/g for the control, 0.2%, 0.4% and 0.8% nisin-treated groups, respectively and statistically crucial divergences were obtained between the control group and the nisin-treated groups during the storage period. The total *Enterobacteriaceae* number increased in direct proportion to the storage period and the highest increase was observed in the control group. At the rejection time of fish by panellists, total *Enterobacteriaceae* counts were 5.49 log

cfu/g (12 d), 5.49 and 5.51, 5.50 ve 5.25 log cfu/g for for the control, 0.2%, 0.4% and 0.8% nisin-treated groups (14 d), respectively. Although total *Enterobacteriaceae* counts were below 6.85 log cfu/g for all groups at the end of the trial, the maximum load of *Enterobacteriaceae* was observed in the control group throughout the storage period. Durmuş *et al.,* (2019) reported that the amount of *Enterobacteriaceae* increased with the duration of storage of sea bass (*D. labrax*) fillets and the highest value of 6.20 log cfu/g on the last day of storage (day 18). Nisin combined with vacuum packaging was effective in the deceleration of the growth rate of *Enterobacteriaceae* in refrigerated sea bass fillets.

4. CONCLUSIONS

The combined use of nisin with vacuum packaging in the cold storage of sea bass fillets led to the extention of shelf-life. Nisin inhibited microbial growth and lowered the incidence of oxidation in sea bass fillets, with antimicrobial and antioxidant activity. As a result, it was concluded that the application of nisin, especially 0.8% nisin, preserves the nutrient and organoleptic quality and extends the shelf-life of sea bass (2 days) at 4 ± 2 °C.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare there are no conflicts of interest.

ACKNOWLEDGEMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or non profit sectors.

REFERENCES

Abbas KA, Mohamed A, Jamilah B, Ebrahimian M. 2008. A review on correlations between fish freshness and pH during cold storage. *Am. J. Biochem. Biotechnol.* **4,** 416-421.

Alasalvar C, Taylor KDA, Zubcov E, Shahidi F, Alexis M. 2002. Differentiation of cultured and wild sea bass (*Dicentrarchus labrax*): total lipid content, fatty acid and trace mineral composition. *Food Chem*. **79**, 145-150. https://doi.org/10.1016/S0308-8146(02)00122-X

Anonymous. 2000. "http:rraids.medscape.comrreutersrprofr1999r10r10.28rpb10289b.html", 2000. Marathon Enterprises Recalls Hotdogs Due to Possible Listeria Contamination.

Antonocopoulus N. 1973. Bestimmung des flüchhtigen basensticktoofs. *Fische und Fischerzeugnisse* 224-225.

AOAC. 1984. Official Methods of Analysis of the Association of the Official Analysis Chemists. Washington DC: Association of Official Analytical Chemists.

AOAC 2002. Official Method 920.153. Ash content. In: Official methods of analysis, 17th Ed, Association of Official Analytical Chemists, Gaithersburg, Maryland, USA.

AOAC 2002. Official Method 950.46. Moisture content in meat. In: Official methods of analysis, 17th Ed, Association of Official Analytical Chemists, Gaithersburg, Maryland, USA.

AOCS 1994. Method Ja 8-87, Peroxide value. In: D. E. Firestone (Ed.), Official Methods and Recommended Practices of the American Oil Chemists' Society, 4th ed., Champaign, Illinois, USA: AOCS Press.

AOCS 1997. Official Method Ca 5a-40. Free fatty acids. In: D.E. Firestone (Ed.), Official Methods and Recommended Practices of the American Oil Chemists' Society. [5th ed.,] Champaign, Illinois, USA: AOCS Press.

Ballestrazzi R, Lanari D. 1996. Growth, body composition and nutrient retention efficiency of growing sea bass (*Dicentrarchus labrax*) fed fish oil or fatty acid Ca salts. *Aquac*. **139**, 101-108. https://doi.org/10.1016/0044-8486(95)01146-3

Behnama S, Anvari M, Rezaei M, Soltanian S, Safari R. 2015. Effect of nisin as a biopreservative agent on quality and shelf life of vacuum packaged rainbow trout (*Oncorhynchus mykiss*) stored at 4 °C. *J. Food Sci. Technol*. **52**, 2184-2192. https://doi.org/10.1007/s13197-013-1241-2

Bligh EC, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 913–917. https://doi.org/10.1139/o59-099

Bonilla AC, Sveinsdottir K, Martinsdottir E. 2007. Development of quality index method (QIM) scheme for fresh cod (*Gadus morhua*) fillets and application in shelf life study. *Food Control* **18**, 352–358. https://doi.org/10.1016/j.foodcont.2005.10.019

Castro P, Padron JCP, Cansino MJC, Velázquez ES, De Larriva RM. 2006. Total volatile base nitrogen and its use to assess freshness in European sea bass stored in ice. *Food Control* **17** (4), 245-248. https://doi.org/10.1016/j.foodcont.2004.10.015

Ceylan Z. 2014. Nisin ve ışınlama uygulamalarının birlikte kullanılmasının soğukta depolanan balığın raf ömrüne etkisi. İstanbul Üniversitesi, Fen Bilimleri Enstitüsü, Su Ürünleri Avlama ve İşleme Teknolojisi Anabilim Dalı, Yükseklisans Tezi. 104.

Ceylan Z, Meral R, Cavidoglu I, Yagmur Karakas C, Tahsin Yilmaz M. 2018. A new application on fatty acid stability of fish fillets: Coating with probiotic bacteria-loaded polymer-based characterized nanofibers. *J. Food Safety* **38** (6), e12547. https://doi.org/10.1111/jfs.12547

Connell JJ 1995. Control of fish quality, 4th Edition. Fishing News Books Limited, London.

Durmus M, Ozogul Y, Boga EK, Uçar Y, Kosker AR, Balikci E, Gökdogan S. 2019. The effects of edible oil nanoemulsions on the chemical, sensory, and microbiological changes of vacuum packed and refrigerated sea bass fillets during storage period at 2 ± 2 °C. *J. Food Process. Pres*. **43** (12), e14282. https://doi.org/10.1111/jfpp.14282

EFSA 2006. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food on a request from the commission related to the use of nisin (E 234) as a food additive. *EFSA J*. **314**, 1–16.

Ghomi MR, Nikoo M, Heshmatipour Z, Jannati AA, Ovissipour M, Benjakul S, Hashemi M, Faghani Langroudi H, Hasandoost M, Jadiddokhan D. 2011. Effect of sodium acetate and nisin on microbiologicaland chemical changes of cultured grass carp (*Ctenopharyngodon idella*) during refrigerated storage. *J. Food Safety* **31**, 169–175. https://doi.org/10.1111/j.1745-4565.2010.00281.x

Gracey J, Collims DS, Huey R. 1999. Harcourt brace and company. *Meat Hyg*. **10**, 407.

Han D, Han I, Dawson P 2016. Combining modified atmosphere packaging and nisin to preserve Atlantic salmon. *J. Food Res.* **6** (1), 22. https://doi.org/10.5539/jfr.v6n1p22

Howgate, PF. 1982. Quality assessment and quality control. *Fish Handling and Processing* 177-186.

Juneja VK, Dwivedi HP, Yan X. 2012. Novel natural food antimicrobials. *Annu. Rev. Food Sci. T.* **3** (1), 381e403. https://doi.org/10.1146/annurev-food-022811-101241

Kuley E, Ozogul F, Durmus M, Gokdogan S, Kacar C, Ozogul Y, Ucar Y. 2012. The impact of applying natural clinoptilolite (zeolite) on the chemical, sensory and microbiological changes of vacuum packed sardine fillets. *Int. J. Food Sci. Tech*. **47** (9), 1977-1985. https://doi.org/10.1111/j.1365-2621.2012.03060.x

Kyrana VR, Lougovois VP. 2002. Sensory, chemical and microbiological assessment of farm-raised European sea bass (*Dicentrarchus labrax*) stored in melting ice. *Int. J. Food Sci. Tech*. **37** (3), 319-328. https://doi.org/10.1046/j.1365-2621.2002.00572.x

Lu F, Ding Y, Ye X, Liu D. 2010. Cinnamon and nisin in alginate–calcium coating maintain quality of fresh northern snakehead fish fillets. *LWT-Food Sci. Technol.* **43** (9), 1331-1335. https://doi.org/10.1016/j.lwt.2010.05.003

Mead PS, Slutsker L, Dietz V, Mc Caig LF, Bresee JF, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5** (5), 607–625. https://doi.org/10.3201/eid0505.990502

Mendes R, Goncalves A. 2008. Effect of soluble CO_2 stabilisation and vacuum packaging in the shelf life of farmed sea bream and sea bass fillets. *Int. J. Food Sci. Tech*. **43**, 1678–1687. https://doi.org/10.1111/j.1365-2621.2008.01737.x

Meral R, Alav A, Karakas C, Dertli E, Yilmaz MT, Ceylan Z. 2019. Effect of electrospun nisin and curcumin loaded nanomats on the microbial quality, hardness and sensory characteristics of rainbow trout fillet. *LWT- Food Sci. Technol*. **113**, 108292. https://doi.org/10.1016/j.lwt.2019.108292

Nykanen A, Weckman K, Lapveteläinen A. 2000. Synergistic inhibition of *Listeria monocytogenes* on cold-smoked rainbow trout by nisin and sodium lactate. *Int. J. Food Microbiol.* **61** (1), 63-72. https://doi.org/10.1016/S0168-1605(00)00368-8

Ozogul Y, Durmus M, Ucar Y, Özogul F, Regenstein JM. 2016. Comparative study of nanoemulsions based on commercial oils (sunflower, canola, corn, olive, soybean, and hazelnut oils): Effect on microbial, sensory, and chemical qualities of refrigerated farmed sea bass. *Innov. Food Sci. Emerg*. **33**, 422-430. https://doi.org/10.1016/j.ifset.2015.12.018

Paleologos EK, Savvaidis IN, Kontominas MG. 2004. Biogenic amines formation and its relation to microbiological and sensory attributes in ice-stored whole, gutted and filleted Mediterranean sea bass

(*Dicentrarchus labrax*). *Food Microbiol*. **21** (5), 549-557. https://doi.org/10.1016/j.fm.2003.11.009

Sallam KI. 2007. Antimicrobial and antioxidant effects of sodium acetate, sodium lactate and sodium citrate in refrigerated sliced salmon. *Food Control* **18**, 566–575. https://doi.org/10.1016/j.foodcont.2006.02.002

Simeonidou S, Govaris A, Vareltzis K. 1998. Quality assessment of seven Mediterranean fish during storage on ice. *Food Res. Int*. **30**, 479–484. https://doi.org/10.1016/S0963-9969(98)00008-8

Tarladgis B, Watts BM, Yonathan M. 1960. A distillation method for determination of malonaldehyde in rancid food. *J. Am. Oil Chem. Soc.* **37**, 44-48. https://doi.org/10.1007/BF02630824

Ucar Y, Özogul Y, Özogul F, Durmuş M, Köşker AR. 2020. Effect of nisin on the shelf life of sea bass (*Dicentrarchus labrax* L.) fillets stored at chilled temperature (4 ± 2 °C). *Aquacult. Int*. 1-13. https://doi.org/10.1007/s10499-020-00512-5

Yazgan H, Ozogul Y, Durmuş M, Balikçi E, Gökdoğan S, Uçar Y, Aksun ET. 2017. Effects of oil-in-water nanoemulsion based on sunflower oil on the quality of farmed sea bass and gilthead sea bream stored at chilled temperature (2 ± 2 °C). *J. Aquat. Food Prod. T.* **26**, 979-992. https://doi.org/10.1080/10498850.2017.1366610

GRASAS Y ACEITES 72 (2)
April-June 2021, e402
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1256192

Lipid composition of different parts of Cape gooseberry (*Physalis peruviana* L.) fruit and valorization of seed and peel waste

V. Popova[a, ✉], Z. Petkova[b], T. Ivanova[a], M. Stoyanova[c], N. Mazova[a] and A. Stoyanova[a]

[a]Department of Tobacco, Sugar, Vegetable and Essential Oils, University of Food Technologies, 4002 Plovdiv, Bulgaria.

[b]Department of Chemical Technology, University of Plovdiv "Paisii Hilendarski", 4000 Plovdiv, Bulgaria.

[c]Department of Analytical Chemistry and Physicochemistry, University of Food Technologies, 4002 Plovdiv, Bulgaria.

[✉]Corresponding author: vpopova2000@abv.bg

Submitted: 12 December 2019; Accepted: 03 April 2020; Published online: 03 June 2021

SUMMARY: The consumption of Cape gooseberry (*Physalis peruviana* L.) fruit (CG), fresh or processed, is gaining popularity worldwide, due to its nutritional and medicinal benefits. This study was based on the analysis of the lipid fraction of different parts of CG fruit and on further valorization of the resulting CG waste. The content of glyceride oil in CG seeds, peels and seed/peel waste, as well as the individual fatty acid, sterol and tocopherol composition of the oils was determined. CG seeds and seed/peel waste were a rich source of oil (up to 22.93%), which is suitable for nutritional application, due to its high proportions of unsaturated fatty acids (up to 83.77%), sterols (campesterol, Δ^5-avenasterol, β-sitosterol) and tocopherols (β-, δ- and γ-tocopherols). Seed/peel waste and the extracted seed cakes contained macro- and microminerals (K, Mg, Na, Fe, Zn, Mn, Cu) which are important for human and animal nutrition. Seed cakes had relatively high protein (24.32%) and cellulose (42.94%) contents, and an interesting amino acid profile. The results from the study contribute to a deeper understanding of the composition of CG fruit, and might be of practical relevance in the development of functional foods and feeds.

KEYWORDS*: Amino acids; Fatty acids; Minerals; Physalis peruviana L.; Sterols; Tocopherols*

RESUMEN: ***Composición lipídica de diferentes partes del fruto del aguaymanto (*Physalis peruviana *L.) y valorización de residuos de semillas y cáscaras*.** El consumo del aguaymanto (*Physalis peruviana* L.), fresco o procesado, está ganando popularidad en todo el mundo debido a sus beneficios nutricionales y medicinales. Este estudio se basó en el análisis de la fracción lipídica de diferentes partes de la fruta y en una mayor valorización de los desechos resultantes. Se determinó el contenido de la fracción glicerídica en semillas, cáscaras y residuos de semillas/cáscaras, así como la composición individual de ácidos grasos, esteroles y tocoferoles de los aceites. Las semillas de aguaymanto y los residuos de semillas/cáscaras fueron una rica fuente de aceite (hasta 22,93%), adecuados para un uso nutricional, debido a las altas proporciones de ácidos grasos insaturados (hasta 83,77%), esteroles (campesterol, Δ5-avenasterol, β-sitosterol) y tocoferoles (β-, δ- y γ-tocoferol). Los residuos de semillas/cáscaras y los residuos desengrasados de semillas extraídos (tortas) contenían macro y microminerales (K, Mg, Na, Fe, Zn, Mn, Cu) importantes para la nutrición humana y animal. Las tortas de semillas tenían un contenido relativamente alto de proteínas (24,32%) y celulosa (42,94%), y un perfil de aminoácidos interesante. Los resultados del estudio contribuyen a una comprensión más profunda de la composición del aguaymanto y pueden ser de relevancia práctica en el desarrollo de alimentos y alimentos funcionales.

PALABRAS CLAVE*: Ácidos grasos; Aminoácidos; Esteroles; Minerales; Physalis peruviana L; Tocoferoles*

Citation/Cómo citar este artículo: Popova V, Petkova Z, Ivanova T, Stoyanova M, Mazova N, Stoyanova A. 2021. Lipid composition of different parts of Cape gooseberry (*Physalis peruviana* L.) fruit and valorization of seed and peel waste. *Grasas Aceites* **72** (2), e402. https://doi.org/10.3989/gya.1256192

1. INTRODUCTION

Cape gooseberry (*Physalis peruviana* L.), also known as goldenberry, Inca berry or Peruvian groundcherry, is the most extensively cultivated *Physalis* species, constituting an important cash crop in many countries of the tropical and subtropical regions (Puente *et al.*, 2011). Colombia is the biggest producer and exporter of fresh and dehydrated Cape gooseberry (CG) fruit worldwide, with an annual export volume of about 6000 tons, directed mainly to the European Union (the Netherlands, Germany and Belgium being the largest markets) (Olivares-Tenorio *et al.*, 2016). The fruit of CG, a berry enveloped in a protective calyx, is bright yellow to orange in color, ovoid-shaped, small and shiny (with a diameter between 1.25 and 2.50 cm and weight between 4 and 10 g), and contains about 100-300 seeds. The berries are rich in flavor, sweet and sour, resembling those of tomato, strawberry, kiwi and citrus, with a tender and juicy texture (Puente *et al.*, 2011). Ripe berries are consumed mostly fresh, but like other exotic fruits, CG is an excellent ingredient in many low calorie and dietetic products (beverages, jellies, jams, juices, yoghourts, dressings, etc.) (Ramadan, 2011; Kalugina *et al.*, 2017). Several comprehensive reviews have been published recently, which summarize the data about the chemical composition, biological activities and uses of CG fruit or whole plants (Puente *et al.*, 2011; Ramadan, 2011; Zhang *et al.*, 2013; Sharma *et al.*, 2015). The nutritional and medicinal values of CG fruit were related to the high levels of beneficial compounds such as vitamins, minerals, carotenoids, polyphenols, alkaloids, fatty acids, phytosterols, polysaccharides and others, as well as to their biological activities, such as anti-inflammatory, immunomodulatory, antioxidant, cytotoxic, antimicrobial, hepatoprotective, antiglycemic, anticholesterolemic, etc. (Ramadan and Mörsel, 2003; Ramadan *et al.*, 2008; Rodrigues *et al.*, 2009; Puente *et al.*, 2011; Ramadan 2011, 2012; Zhang *et al.*, 2013; Sharma *et al.*, 2015; Yıldız *et al.*, 2015; Kupska *et al.*, 2016; Ertürk *et al.*, 2017; Ramadan *et al.*, 2017; Mokhtar *et al.*, 2018). CG fruit was evaluated as a promising source of vegetable oil (Puente *et al.*, 2011; Ramadan, 2011), with a total oil content of 2.0, 1.8, and 0.2% (on a fresh weight basis (FW)), respectively, in the whole berries, seeds, and pulp/peel fraction of fruit of Colombian origin (Ramadan and Mörsel, 2003). Some other studies investigated the triacylglycerol, tocopherol and phytosterol composition of CG pomace oil extracted from the seed and peel waste resulting from juice processing (Ramadan *et al.*, 2008; Ramadan, 2012; Mokhtar *et al.*, 2018). The seed/peel pomace, as outlined by Ramadan, (2011), constituted the largest portion of the waste generated from juice production, 27.4% of fruit weight, and contained 19.3% oil, 17.8% protein, 3.10% ash, 28.7% crude fiber, and 24.5% carbohydrates. Therefore, the seed/peel waste remaining after juice extraction, as well as the seed cakes remaining after oil extraction, can be considered an integral element in the sustainable CG usability, as they constitute a significant amount of fruit weight and contain compounds which are important for human and animal nutrition. These considerations create reasonable grounds for targeted analysis of these by-products, e.g. the determination of macro- and microminerals, fibers, protein, amino acids, vitamins, and other constituents with nutritional value. However, to the best of our knowledge, research data based on fractionization by fruit structural parts and processing by-products (seeds, peels, seed/peel waste, and seed cakes) are far from exhaustive, despite the intensive marketing promotion of CG fruit worldwide and the growing awareness of CG's nutritional benefits.

Therefore, the objective of this work was to study the lipid composition of the different parts of CG fruit and the valorization of the resulting CG waste. We hypothesized that the results from the analysis of the lipid fraction and other micro- and macronutrients (in terms of oil content; sterol, tocopherol, and fatty acid composition; protein, cellulose, amino acids, minerals) of CG seeds, peels, seed/peel waste and extracted seed cakes, respectively, would reveal the individual contribution of each element to the properties of the whole fruit, as well as provide further arguments in favor of CG waste assessment and its potential for wider use. Therefore, the outcomes from this study may contribute to the better understanding and evaluation of this exotic tropical fruit worldwide, and could be of interest to the food industry and nutrition.

2. MATERIALS AND METHODS

2.1. Plant material

CG fruit (*P. peruviana* L.) of Colombian origin was purchased from a local supermarket in January 2019.

Undamaged fruit of uniform ripeness were selected, then two separate sub-samples were formed, seeds and berry peels. Those were obtained by careful manual isolation of peels and seeds from individual berries in order to make distinguishable samples of berry structural elements. Fruit samples then were kept in the refrigerator at a temperature of –18 °C until analysis. Another sample constituted air-dried seed and peel mixture (seed/peel waste, seed/peel pomace) remaining after a high-speed vacuum separation of fruit juice. Seed cakes, in turn, were taken after the Soxhlet extraction of seed oil with n-hexane and air dried. The moisture content of the samples was determined by oven-drying at 103 ± 2 °C to constant weight.

2.2. Fatty acids (FAs), sterols and tocopherols in CG seed and peel oils

The isolation of the oil (%, v/w) from fruit peels and seeds was carried out by extraction with n-hexane in a Soxhlet apparatus for 8 h. The solvent was then completely evaporated in a rotary vacuum evaporator at a temperature of 40 °C (ISO 659:2014). The FA composition of the oils was determined by GC analysis after transmethylation of the sample with 2% H_2SO_4 in CH_3OH at 50 °C (ISO 12966-1:2014, ISO 12966-2:2011). Determination was performed on a Hewlett Packard 5890 A gas chromatograph equipped with a capillary Supelco 2560 column, 75 m × 0.25 mm × 18 μm (i.d.), and a flame ionization detector (FID). The column temperature was programmed from 130 °C (4 min) to increase at 15 °C/min to 240 °C (5 min); injector and detector temperatures were 250 °C; hydrogen was the carrier gas at a 0.8 mL/min flow rate; the split ratio was 50:1. The identification of FAs was performed by comparison of the retention times with those of a standard mixture of fatty acid methyl esters (FAME) (37 component FAME mix, Supelco, USA), according to ISO 12228-1:2014.

Tocopherols were determined directly by HPLC analysis using a Merck-Hitachi unit equipped with a 250 mm × 4 mm Nucleosil Si 50-5 column and a fluorescent Merck-Hitachi F 1000 detector. The operating conditions were as follows: mobile phase n-hexane:dioxan (96:4, v/v); flow rate of 1.0 mL/min; detector excitation at 295 nm, emission at 330 nm; injected sample volume of 20 μL (1 g/100 mL solution of crude oil in n-hexane). Tocopherols were identified by comparison with the retention times of reference tocopherol standards (DL-α-, DL-β-, DL-γ- and DL-δ-tocopherols with 98% purity, purchased from Merck, Darmstadt, Germany), according to ISO 9936:2016.

For the determination of sterols, the unsaponifiable fraction was isolated after saponification of the oil and extraction with n-hexane, according to ISO 18609:2000. Sterols were identified by comparison of the retention times with those of a standard sterol mixture containing cholesterol (stabilized, purity 95%, Across Organics, New Jersey, USA), stigmasterol (purity 95%, Sigma-Aldrich, St. Louis, MO, USA) and β-sitosterol (with ca 10% campesterol, ca 75% β-sitosterol, Across Organics, New Jersey, USA), according to ISO 12228-1:2014.

The phospholipid content was determined by spectrophotometry, measuring the content in phosphorus at 700 nm after mineralization of the oil with a solution of $HClO_4$ and H_2SO_4 (1:1, v/v) (ISO 10540-1:2014).

2.3. Mineral elements in seed/peel waste and seed cakes

The seed/peel fraction and seed cakes were mineralized at 450 °C; the residue was first dissolved in concentrated HCl, evaporated to dryness and then dissolved again in 0.1 mol/L HNO_3. The concentrations of mineral elements were determined by using an atomic absorption spectrophotometer (AAS) Perkin Elmer/HGA 500 (Norwalk, USA). The respective wavelengths for the AAS detection were: Na - 589.6 nm, K - 766.5 nm, Mg - 285.2 nm, Ca - 317.0 nm, Zn - 213.9 nm, Cu - 324.7 nm, Fe - 238.3 nm, and Mn - 257.6 nm. Elemental identification was carried out by comparing with a standard solution of metal salts, and the calculation of the respective metal concentrations were based on a standard solution of 1 μg/mL, using a calibration curve.

2.4. Protein, amino acids and cellulose in seed cakes

The total protein content in CG seed cakes was determined by the Kjeldahl method, as described in AOAC Method 976.06 (2016), on a UDK 152 System (Velp Scientifica, Italy).

The hydrolysis of protein to free aminoacids was completed by reacting 30 mg dried seed cake with 3

mL 6N HCl in a sealed glass ampule at 105 °C for 24 h. The solvent was evaporated in a vacuum chamber at 40 °C and the residue was fully diluted in 20 mM HCl (2 mL). After filtration, 20 µL of the solution were derivatized with AccQ-Fluor kit (WATO52880, Waters Corporation, USA). Finally, the solution was heated to 55 °C and 20 µL were injected into an ELITE LaChrome HPLC chromatograph (Hitachi) equipped with a diode array detector (DAD) and a reverse phase C 18 AccQ-Tag (3.9 mm × 150 mm) column. The mobile phases in the gradient elution were WATO52890 buffer (Waters Corporation, USA) and 60% acetonitrile. The detection wavelength was 254 nm and column temperature was 37 °C.

The content of cellulose (crude fiber) in the CG seed cakes was determined by a modification of the method of Brendel *et al.*, (2000). The hydrolysis of cellulose and hemicellulose was carried out by boiling 1 g of seed cakes in 16.5 mL of 80% CH_3COOH and 1.5 mL concentrated HNO_3 for 1.5 h. After filtration of the suspension, the solid residue was dried at 105 °C for 24 h and weighed.

2.5. Statistics

The measurements were made in triplicate (n = 3), and the results were presented as mean values of the individual measurements with the corresponding standard deviation (mean ± SD). Statistical tools, such as ANOVA and Tukey's multiple comparison test, were used to determine significant differences ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Lipid fraction of different CG fruit elements (seed, peel and seed/peel waste)

As stated above, CG seeds and seed/peel waste have been reported as promising sources for obtaining edible oil (Ramadan and Mörsel, 2003; Ramadan, 2012). In turn, the shiny surface of the CG fruit is also associated with the presence of lipid fractions in the peel, so in this study the two oil-containing fruit elements – seeds and peels, were evaluated separately in order to trace their individual contributions. The pulp (juice) was not analyzed alone, as previous studies showed that it contained considerably smaller amounts of oil (0.2% FW) (Ramadan, 2011). Results on the total oil content and the general composition of the oil isolated from the respective fruit elements are presented in Table 1.

As anticipated, the data revealed considerable numerical differences in the lipid fractions of CG fruit elements. The oil content in the peels was about seven times lower than that in the seeds and seed/peel waste; the latter two showing insignificant variation. The content of the oil extracted from CG seeds and seed/peel waste (22.93% and 21.03%, respectively) was higher than that of some common oilseed crops, e.g. corn (3-5%), cotton (16%) and soybean (18%) (Popov and Ilinov, 1986), or some prospective fruit seeds, such as grape seeds (8-20%) (Heuzé and Tran, 2017). The phospholipid concentration in the peel oil was about twice as high as that in the seed/peel oil, and about four times higher than that in seed oil. The contents in phospholipids in the three CG oils were higher than the respective data for sunflower, corn germ and safflower oil (0.4-0.9%) or for soybean oil (1.0-3.0%) (Popov and Ilinov, 1986). The contents in sterols, a known group of biologically active dietary cholesterol-reducing agents, were similar in the three fruit fractions, and higher than many seed oils, such as sunfower, soybean, cotton, safflower, and others (0.24-0.64%) (Popov and Ilinov, 1986; FAO/WHO, 1999). The total amount of biologically active tocopherols in the two seed-containing CG fruit samples (5096 and 5634 mg/kg, respectively) was twice as high as that in the peel oil (2648 mg/kg). These results were comparable to the tocopherol

Table 1. Contents in glyceride oil and sterols, tocopherols and phospholipids in Cape gooseberry oil

Index	**Seed/peel waste** [a]	**Peels**	**Seeds**
Oil (%)	21.03 ± 0.20 [b]	3.21 ± 0.03 [c]	22.93 ± 0.21 [b]
Phospholipids (%)	4.38 ± 0.04 [b]	10.72 ± 0.09 [c]	2.69 ± 0.02 [d]
Sterols (%)	1.42 ± 0.01 [b]	1.29 ± 0.01 [b]	1.31 ± 0.01 [b]
Tocopherols (mg/kg)	5634.00 ± 54.00 [b]	2648.00 ± 20.00 [c]	5096.00 ± 50.00 [d]

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3).

[b – d] Values with different superscripts within a row differed significantly (by Tukey's test at 5% probability).

values for common oils, such as soybean (600-3370 mg/kg), maize (330-3720 mg/kg) and rapeseed (430-2680 mg/kg) (FAO/WHO, 1999).

The FA composition of the analyzed GC oil is presented in Table 2. The results revealed significant differences in the FA composition of CG peel oil compared to the seed and seed/peel oils. The most abundant FAs in the CG peel oil, out of 14 identified, were capric, palmitic and oleic acids. The ratio between saturated (SFA) and unsaturated (UFA) FAs was 67.7:32.3, and the ratio between monounsaturated (MUFA) and polyunsaturated (PUFA) FAs was 23.7:8.6. Linoleic, oleic and palmitic acids were the dominating FAs in the seed and seed/peel waste oils. These results suggested that both CG wastes from juice production could be used as alternative sources of vegetable oils rich in linoleic (63.19-67.89%) and oleic (14.69-16.56%) acids, which are important for the prevention of cardiovascular diseases – as are the seed oils of grapes, melon, tobacco, poppy, and others (Popov and Ilinov, 1986). The ratios of SFA to UFA were similar in the two oils, 17.7:82.3 and 16.2:83.8, respectively, as well as the PUFA-to-MUFA ratios (63.6:18.7 and 68.3:15.5, respectively).

The comparative analysis of our results and literature data about CG fruit oil revealed that there were some differences in oil yield and FA composition. It should be noted that most of the available studies on CG oil and its FAs were carried out either on whole (intact) fruit, seeds or pomace (the combined seed and peel waste from juice separation), so it was difficult to compare our results for CG peel alone. In the study by Yıldız *et al.*, (2015) the n-hexane extracted oil from whole fruits harvested at Bursa, Turkey, was 0.18% (on a fresh weight basis, FW), while the yield reported by Ramadan and Mörsel, (2003) was 2.0% oil of berry weight, in which seed oil comprised 1.8% and pulp/peel oil, 0.2%. In another study, the n-hexane extracted oil from seed/peel pomace was 19.3% (Ramadan, 2012), which was very close to our results.

In terms of FA profiles, our results differed only numerically from the data provided by previous studies.

Table 2. Fatty acid (FA) composition of Cape gooseberry oil

Fatty acids		Contents (% of the oil)		
		Seed/peel waste oil [a]	Peel oil	Seed oil
$C_{8:0}$	Caprylic	nd [b]	nd	0.11 ± 0.00
$C_{10:0}$	Capric	0.11 ± 0.00	32.17 ± 0.28	nd
$C_{12:0}$	Lauric	0.12 ± 0.00	6.21 ± 0.05	nd
$C_{14:0}$	Myristic	0.11 ± 0.00	1.26 ± 0.01	0.62 ± 0.00
$C_{14:1}$	Myristoleic	0.09 ± 0.00	0.52 ± 0.00	nd
$C_{15:0}$	Pentadecanoic	nd	0.44 ± 0.00	nd
$C_{16:0}$	Palmitic	12.48 ± 0.11	24.51 ± 0.23	11.81 ± 0.10
$C_{16:1}$	Palmitoleic	0.64 ± 0.00	0.79 ± 0.00	0.78 ± 0.00
$C_{17:0}$	Margaric	0.18 ± 0.00	0.28 ± 0.00	0.21 ± 0.00
$C_{17:1}$	Heptadecenoic	1.37 ± 0.01	3.08 ± 0.02	nd
$C_{18:0}$	Stearic	4.32 ± 0.03	2.52 ± 0.01	3.48 ± 0.03
$C_{18:1}$	Oleic	16.56 ± 0.15	19.31 ± 0.18	14.69 ± 0.13
$C_{18:2}$ (ω-6)	Linoleic (*cis*)	63.19 ± 0.60	7.02 ± 0.06	67.89 ± 0.65
$C_{18:3}$ (ω-3)	Linolenic	0.41 ± 0.00	1.56 ± 0.01	0.41 ± 0.00
$C_{20:0}$	Arachidic	0.42 ± 0.00	0.33 ± 0.00	nd
Saturated FAs		17.74	67.72	16.23
Unsaturated FAs		82.26	32.28	83.77
Monounsaturated FAs		18.66	23.70	15.47
Polyunsaturated FAs		63.60	8.58	68.30

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3). Standard deviation values below 0.01 were equal to ±0.00.

[b] nd: not detected.

For example, Rodrigues *et al.*, (2009) identified linoleic acid (72.42%), oleic acid (10.03%) and palmitic acid (9.38%) as the main FAs in the lipid fraction of fruit from Brazil; the SFAs were 12.87% of total FAs. Similar results were reported by Mokhtar *et al.*, (2018) for seed/peel waste powder, in which four FAs were quantified, linoleic (77.78%), oleic (11.32%), palmitic (7.39%), and stearic (3.51%), at a SFA:UFA ratio of 10.9:89.1. In the study by Ramadan and Mörsel, (2003) the contents of the main FAs of CG seed oil were: linoleic (76.1%), oleic (11.7%), palmitic (7.29%), and stearic (2.51%), with a SFA:UFA ratio of 12.8:87.2. The FA profile of the seed/peel extracted oil was also dominated by linoleic (77.1%), oleic (10.3%), palmitic (7.95%), and stearic (2.61%), at a SFA:UFA ratio of 12.8:88.2 (Ramadan, 2012). As it can be seen, the ratio of linoleic to oleic acid in these studies was about 7:1. While our results suggested a lower value, about 4:1; the relative share of UFA was slightly lower in this study, too (SFA:UFA ratios of 17.7:82.3 and 16.2:83.8, for seed/peel and seed oils). Nevertheless, the results from this study supported the evaluation of CG fruit oil as especially suitable for nutritional application, due to its high proportions of PUFAs.

The individual sterol composition of the oils is presented in Table 3. The results revealed that the total sterol content in the unsaponifiable fraction was comparable in seed and seed/peel oils (33.33-44.59%), but that it was considerably lower in the peel oil (2.14%). The total amount of unsaponifiables was in a reversed order, i.e. higher in the peel oil (61.33%) compared to seed and seed/peel oils (3.02-4.21%). With a share of 80.23% in the total sterol content, β-sitosterol was the dominating sterol in the oil of CG peels, while the other two oils contained campesterol, Δ^5-avenasterol and β-sitosterol as their main sterols (constituting 67.65% and 77.80% of the sterol fraction in seed and seed/peel oil, respectively). These results were very close to the findings by Ramadan, (2012), who also reported high levels of unsaponifiables in CG pomace oil (22.0 g/kg), and campesterol (4.70 g/kg), Δ^5-avenasterol (2.63 g/kg) and lanosterol (1.60 g/kg) as responsible for about 75% of total sterols.

The results from the analysis of the individual tocopherol composition of CG oil are presented in Table 4. Four tocopherols were identified in each of the oils, and the data revealed that γ-tocopherol was noticeably the dominating structure in the peel oil, while β-tocopherol, δ-tocopherol and γ-tocopherol had similar individual shares (31.40-34.15%) in both seed and seed/peel waste oils. As tocopherols are known to be potent antioxidants, it can be suggested that the high amount of tocopherols in the studied CG oils, and especially the high γ-tocopherol levels,

Table 3. Sterol composition of Cape gooseberry oil

Sterols (% of total)	**Seed/peel waste oil** [a]	**Peel oil**	**Seed oil**
Cholesterol	1.39 ± 0.01 [b]	2.52 ± 0.02 [c]	0.31 ± 0.00 [d]
Ergosterol	nd [e]	nd	1.62 ± 0.01
Campesterol	57.23 ± 0.56 [b]	5.56 ± 0.05 [c]	22.56 ± 0.21 [d]
Stigmasterol	6.39 ± 0.06 [b]	0.47 ± 0.00 [c]	9.03 ± 0.08 [d]
Δ^7-Campesterol	8.74 ± 0.08 [b]	6.45 ± 0.06 [c]	nd
β-Sitosterol	9.36 ± 0.09 [b]	80.23 ± 0.79 [c]	18.32 ± 0.17 [d]
Lanosterol	nd	nd	2.11 ± 0.01
Δ^5-Avenasterol	11.21 ± 0.10 [b]	1.52 ± 0.01 [c]	26.77 ± 0.25 [d]
$\Delta^{7,25}$-Stigmastadienol	0.89 ± 0.00 [b]	0.44 ± 0.00 [b]	nd
Δ^7-Stigmasterol	3.92 ± 0.03 [b]	2.08 ± 0.01 [c]	14.72 ± 0.14 [d]
Δ^7-Avenasterol	0.87 ± 0.00 [b]	0.73 ± 0.00 [b]	4.56 ± 0.04 [c]
Total sterols in the unsaponifiable fraction (%)	33.33	2.14	44.59
Unsaponifiables (% in the oil)	4.21	61.33	3.02

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3). Standard deviation values below 0.01 were equal to ±0.00.

[b-d] Values with different superscripts within a row differed significantly (by Tukey's test at 5% probability).

[e] nd: not detected.

Table 4. Tocopherol composition of Cape gooseberry oil

Tocopherols (% of total)	Seed/peel waste oil [a]	Peel oil	Seed oil
α-Tocopherol	1.72 ± 0.01 [b]	19.30 ± 0.18 [c]	1.14 ± 0.01 [b]
β-Tocopherol	33.04 ± 0.30 [b]	nd [d]	34.15 ± 0.33 [b]
γ-Tocopherol	31.46 ± 0.30 [b]	72.78 ± 0.70 [c]	31.40 ± 0.30 [b]
γ-Tocotrienol	nd	3.59 ± 0.03	nd
δ-Tocopherol	33.78 ± 0.31 [b]	4.33 ± 0.04 [c]	33.31 ± 0.32 [b]

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3).

[b-c] Values with different superscripts within a row differed significantly (by Tukey's test at 5% probability).

[d] nd: not detected.

would be a preventive factor against lipid oxidation processes during the storage of oil and oil-containing products.

These results were in compliance with the findings by Ramadan and Mörsel, (2003), who also identified γ- and α-tocopherols as the main constituents in the pulp/peel oil of CG fruit (45.5 mg/kg and 28.3 mg/kg, respectively), and β-tocopherol and γ-tocopherol in the seed oil. In another study (Ramadan, 2012), β-tocopherol comprised 47% of the tocopherol fraction in the CG seed/pulp oil (2.10 g/kg), γ-tocopherol was 26% (1.08 g/kg), δ-tocopherol 18.5% (0.85 g/kg), and α-tocopherol about 6% (0.34 g/kg). There were no sufficient data in the literature about the tocopherol content in CG fruit peel alone, so it was difficult to make a more detailed comparison of our results with previous findings.

3.2. Minerals, fiber, protein, and amino acids in CG waste

As already stated, the two by-products obtained from CG fruit, seed cakes and seed/peel waste, represent raw materials which are underutilized, but valuable in terms of both quantity and functionality. In this study, the peels were found to constitute 11.4% of fresh fruit weight, and the seeds 13.2%. For example, the combined seed/peel fraction made up nearly a quarter of the fruit weight. Our results were very close to the findings by Ramadan, (2011) for CG seed/peel pomace at 27.4% of fruit weight. On the other hand, CG waste fractions were previously indicated as potential sources of nutrients, e.g. vitamins, minerals, carbohydrates, protein, etc. (Ramadan *et al.*, 2008; Ramadan, 2011, 2012). On these grounds, the two waste products from fruit processing in this study were considered worthy of further analysis in order to determine the contents in some components with nutritional value, e.g. minerals, fiber, protein, and amino acids.

3.2.1. Mineral elements in seed cakes and seed/peel waste

The data presented in Table 5 revealed that CG seed cakes and seed/peel waste were a source of minerals, with some differences in both macro- and microelement concentrations between the two waste products. K and Mg dominated in the group of macroelements, and Zn, Cu and Fe in the group

Table 5. Mineral elements (mg/kg) in Cape gooseberry seed/peel waste and seed cakes

Fruit sample	Mineral elements (mg/kg)										
	K	Na	Ca	Mg	Fe	Mn	Cu	Zn	Pb	Cd	Cr
Seed/peel waste	4527.00±12.31[a]	112.63±1.09	<0.10 [b]	1750.00±8.22	42.85±0.39	17.77±0.09	10.71±0.08	34.65±0.12	<0.10	<0.01 [c]	<0.10
Seed cake	3911.00±11.78	124.44±1.06	<0.10	2095.00±8.67	52.36±0.48	24.44±0.11	114.62±0.81	130.60±0.39	<0.10	<0.01	0.75±0.02

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3).

[b] Not quantified.

[c] Not detected.

of microelements. These results supported the assumption that CG waste products have possible use as ingredients in functional foods or as additives in forage mixtures for animal nutrition, as they are carriers of macro- and microelements with nutritional value. There were some interesting variations in the distribution of minerals between the two waste products. For example, Cu and Zn were in considerably lower amounts in the seed/peel waste compared to the seed cakes, but the opposite situation was observed for K (Table 5). This variance could be attributed to the different mobility and accumulation of those minerals in the different fruit parts (pulp, peel, seeds). Therefore, the differences were conditioned by the nature of the two analyzed CG waste samples in the study, reflecting the presence of peels in the seed/peel waste (and partly juice, as the waste was not additionally rinsed). Such an assumption is in compliance with the observations by Morais *et al.*, (2017) for the distribution of K, Cu and Zn among the seeds, pulp and peels of fruits like papaya, passion fruit, watermelon, and melon. K had the highest contents in the peels and pulps compared to the seeds, while the microminerals tended to be higher in the seeds.

The results from the study were in full compliance with previous findings about high K contents in CG, and exceeded those found in many other fruits (Olivares-Tenorio *et al.*, 2016; Mayorga *et al.*, 2001; Rodrigues *et al.*, 2009; Ozturk *et al.*, 2017; Zhang *et al.*, 2013; Mokhtar *et al.*, 2018). This was a certain asset of the studied CG fruit wastes, as K represents an intracellular element involved in neural and muscle electrochemical processes and in the acid-base regulation in the body. The levels of Na and Mg were higher than the results achieved by Rodrigues *et al.*, (2009) (Na - 1.1 mg/100g DW, Mg - 34.7 mg/100g whole fruit), Ozturk *et al.*, (2017) (Mg - 102.5 mg/100 g), Eken *et al.*, (2014) (Mg - 145 mg/100g fruit) or Leterme *et al.*, (2006) (Mg - 19 mg/100 g, Na – 6 mg/100 g fruit pulp). Ca was practically absent, similar to the previously observed low levels of this macromineral in CG fruit (values between 7.0 and 43.65 mg/100 g in whole fruit or fruit pulp) (Leterme *et al.*, 2006; Rodrigues *et al.*, 2009; Puente *et al.*, 2011; Zhang *et al.*, 2013; Eken *et al.*, 2014; Olivares-Tenorio *et al.*, 2016; Ozturk *et al.*, 2017). In general, the contents of microelements registered in this study, Fe, Zn, Mn, and Cu, were higher than the data reported by most of the above mentioned authors (Rodrigues *et al.*, 2009; Puente *et al.*, 2011; Olivares-Tenorio *et al.*, 2016), although some variation also existed. The contents of Fe and Zn were lower than those reported by Eken *et al.*, (2014) for whole fruit (Fe - 36 mg/100 g fruit, Zn - 11.4 mg/100 g fruit). Our results varied from the data for CG seed/peel waste analyzed by Mokhtar *et al.*, (2018), regarding Fe (13.04 mg/100 g), Zn (0.88 mg/100 g), and Mn (0.67 mg/100 g). The heavy metals Pb, Cd and Cr were practically undetected in CG seed cakes and seed/peel waste, similar to the findings by Rodrigues *et al.*, (2009) and Eken *et al.*, (2014). All those numerical variations could be explained by the influence of fruit origin (production conditions) and sample preparation (the analyzed fruit fraction).

The results confirmed the studied CG waste products to be a potent source of macro- and microminerals for human and animal nutrition, which might be of practical importance in the development of functional foods and feeds.

3.2.2. Amino acids, protein and fiber in seed cakes

CG seed cakes had a protein content of 24.32 ± 0.22% DW, total nitrogen content of 3.89 ± 0.03%, DW and cellulose contents (crude fiber) of 42.94 ± 0.31% DW. Thus, CG seed cakes could be considered a relatively rich source of protein, similar to linseeds (20-28% DW) (Heuzé *et al.*, 2015b) and rapeseeds (17-24% DW) (Heuzé *et al.*, 2019), and richer than grape seeds (9-11%) (Heuzé and Tran, 2017), sunflower and safflower (about 17-17.5%) (Heuzé *et al.*, 2015a, 2016). In turn, the cellulose content was also significantly high, approximating that of seed cakes from safflower (30-40%), sunflower (27-31%) or grape seeds (over 33%) (Heuzé *et al.*, 2015a, 2016; Heuzé and Tran, 2017).

The amino acid composition of CG seed cake is presented in Table 6. Surprisingly, lysine (27.22 mg/g) was the second most abundant amino acid, next to aspartic acid (32.11 mg/g). Relatively high levels of alanine (17.96 mg/g), arginine (14.67 mg/g), histidine (12.32 mg/g), and threonine (12.30 mg/g) were also found. Our results suggested an amino acid profile of CG seed cakes which differed from the data by Mokhtar *et al.*, (2018), who found glutamic acid (18.09 g/100 g protein), arginine (11.57 g/100 g protein), aspartic acid (7.82 g/100 g protein), and leucine (5.87 g/100

Table 6. Amino acids in Cape gooseberry seed cakes

Amino acids	Content (mg/g)
Cysteine	1.55 ± 0.01[a]
Methionine	2.30 ± 0.02
Valine	8.73 ± 0.07
Isoleucine	11.43 ± 0.09
Leucine	2.13 ± 0.02
Lysine	27.22 ± 0.22
Histidine	12.23 ± 0.11
Threonine	12.30 ± 0.11
Tyrosine	9.26 ± 0.09
Phenylalanine	10.50 ± 0.08
Aspartic acid	32.11 ± 0.23
Serine	11.90 ± 0.09
Glutamic acid	7.65 ± 0.08
Glycine	2.46 ± 0.02
Arginine	14.67 ± 0.12
Alanine	17.96 ± 0.14
Proline	7.17 ± 0.05

[a] All data are presented as mean value ± standard deviation (from a three-fold repetition, n=3).

g protein) as the dominant amino acids in dehydrated CG waste powder. Further comparisons to previous data were hard to make, as there were no detailed records of CG amino acids or protein quality (Puente *et al.*, 2011). The dominant share of lysine in CG seed cake was an interesting finding, as its low levels (limiting amino acid) are an issue which is common to many oil seed cakes, such as sunflower, safflower and others. Regarding the potential use in livestock feed, it should be noted that the levels of the two other limiting amino acids (beside lysine) in pig and poultry nutrition, methionine and cysteine, were relatively low, suggesting that CG seed cakes must be carefully combined with other animal feed ingredients.

4. CONCLUSIONS

The results from the study demonstrated that the seeds and peels of CG fruit, a waste from juice production, could be a valuable source of functional nutrients for human and animal nutrition. The yield of edible oil from CG seed and seed/peel fractions was sufficiently high, over 21%, making oil extraction feasible. The composition of the analyzed oils was favorable, too, as they were rich in bioactive compounds (such as campesterol, Δ^5-avenasterol, β-sitosterol, β-, δ- and γ-tocopherols) and unsaturated FAs (over 82% of the oil, mainly linoleic and oleic). The further valorization of the obtained CG wastes (seed cakes, seed/peel pomace) revealed them to be fully fit for incorporation in food and feed products. The studied wastes contained high levels of important macro- and microminerals (K, Mg, Na, Zn, Cu, Fe), they were rich in protein (about 24% in seed cakes), cellulose (about 42%) and essential AAs (especially lysine, threonine and histidine).

This study provides new data on Cape gooseberry (*P. peruviana* L.) composition, from the perspective of the contribution of the different parts of the fruit and their potential uses. Thus, the outcomes from the study might be of practical relevance in the development of functional foods and feeds, as they add new details to the existing knowledge about CG fruit. They might also serve as grounds for the development of more efficient approaches to the circular processing of CG fruit worldwide.

ACKNOWLEDGMENTS

The authors acknowledge the support from the Science Fund of the University of Food Technologies, Plovdiv, Bulgaria, project No 04/18-N.

REFERENCES

AOAC. 2016. AOAC Official Method 976.06. Protein (crude) in animal feed and pet food. In AOAC Official Methods of Analysis, 20th ed., AOAC International, Rockville, MD.

Brendel O, Iannetta PPM, Stewart D. 2000. A rapid and simple method to isolate pure α-cellulose. *Phytochem. Anal.* **11**, 7–10. https://doi.org/10.1002/(SICI)1099-1565(200001/02)11:1<7::AID-PCA488>3.0.CO;2-U

Eken A, Ünlü-Endirlik B, Baldemir A, Ilgün S, Soykurt B, Erdem O, Akay G. 2016. Antioxidant capacity and metal content of *Physalis peruviana* L. fruits sold in markets. *J. Clin. Anal. Med.* **7**, 291–294. https://doi.org/10.4328/JCAM.2709

FAO/WHO Codex Alimentarius Commission. 1999. *Standard for Named Vegetable Oils, CXS 210-1999.* FAO/WHO Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Rome (revised, amended 2019). http://www.fao.org/fao-who-codexalimentarius/codex-texts/list-standards/en/

Heuzé V, Tran G, Chapoutot P, Renaudeau D, Bastianelli D, Lebas F. 2015a. Safflower (*Carthamus tinctorius*) seeds and oil meal. *Feedipedia, a Programme by INRA, CIRAD, AFZ and FAO*. http://www.feedipedia.org/node/49 (accessed 22 November, 2019).

Heuzé V, Tran G, Hassoun P, Renaudeau D, Lessire M, Lebas F. 2015b. Linseeds. *Feedipedia, a programme by INRA, CIRAD, AFZ and FAO*. https://www.feedipedia.org/node/36 (accessed 22 November, 2019).

Heuzé V, Tran G, Hassoun P, Lessire M, Lebas F. 2016. Sunflower meal. *Feedipedia, a Programme by INRA, CIRAD, AFZ and FAO*. http://www.feedipedia.org/node/732 (accessed 22 November, 2019).

Heuzé V, Tran G. 2017. Grape seeds and grape seed oil meal. *Feedipedia, a programme by INRA, CIRAD, AFZ and FAO*. https://feedipedia.org/node/692 (accessed 22 November, 2019).

Heuzé V, Tran G, Sauvant D, Lessire M, Lebas F. 2019. Rapeseeds. *Feedipedia, a programme by INRA, CIRAD, AFZ and FAO*. https://www.feedipedia.org/node/15617 (accessed 22 November, 2019).

International Organization for Standardization. 2000. *ISO 18609:2000. Animal and Vegetable Fat and Oils. Determination of Unsaponifiable Matter (Method Using Hexane Extraction)*. International Organization for Standardization. https://www.iso.org/standard/33517.html (accessed 15 November, 2019).

International Organization for Standardization. 2011. *ISO 12966-2:2011. Animal and Vegetable Fats and Oils. Gas Chromatography of Fatty Acid Methyl Esters Part 2: Preparation of Methyl Esters of Fatty Acids*. International Organization for Standardization. https://www.iso.org/standard/43172.html (accessed 15 November, 2019)

International Organization for Standardization. 2014a. *ISO 10540-1:2014. Animal and Vegetable Fats and Oils. Determination of Phosphorus Content – Part 1: Colorimetric Method*. International Organization for Standardization. https://www.iso.org/standard/36178.html (accessed 15 November, 2019).

International Organization for Standardization. 2014b. *ISO 12228-1:2014. Part 1: Animal and Vegetable Fats and Oils. Determination of Individual and Total Sterols Contents. Gas Chromatographic Method*. International Organization for Standardization. https://www.iso.org/standard/60248.html (accessed 15 November, 2019).

International Organization for Standardization. 2014c. *ISO 12966-1:2014. Animal and Vegetable Fats and Oils. Gas Chromatography of Fatty acid Methyl Esters. Part 1: Guidelines on Modern Gas Chromatography of Fatty Acid Methyl Esters*. International Organization for Standardization. https://www.iso.org/standard/52294.html (accessed 15 November, 2019).

International Organization for Standardization. 2014d. *ISO 659:2014. Oilseeds. Determination of Oil Content (Reference Method)*. International Organization for Standardization. https://www.iso.org/standard/43169.html (accessed 15 November, 2019).

International Organization for Standardization. 2016. *ISO 9936:2016. Animal and Vegetable Fats and Oils. Determination of Tocopherol and Tocotrienol Contents by High-Performance Liquid Chromatography*. International Organization for Standardization. https://www.iso.org/standard/69595.html (accessed 15 November, 2019).

Kalugina I, Telegenko L, Kalugina Y, Kyselov S. 2017. The nutritional value of desserts with the addition of Gooseberry family raw materials from the Northern Black Sea region. *Ukrainian Food J.* **6,** 459–469.

Leterme P, Buldgen A, Estrada F, Londoño AM. 2006. Mineral content of tropical fruits and unconventional foods of the Andes and the rain forest of Colombia. *Food Chem.* **95**, 644–652. https://doi.org/10.1016/j.foodchem.2005.02.003

Mokhtar SM, Swailam HM, Embaby HE-S. 2018. Physicochemical properties, nutritional value and techno-functional properties of goldenberry (*Physalis peruviana*) waste powder. *Food Chem.* **248**, 1–7. https://doi.org/10.1016/j.foodchem.2017.11.117

Morais DR, Rotta EM, Sargi SC, Bonafe EG, Suzuki RM, Souza NE, Matsushita M, Visentainer JV. 2017. Proximate composition, mineral contents and fatty acid composition of the different parts and dried peels of tropical fruits cultivated in

Brazil. *J. Braz. Chem. Soc*. **28**, 308–318. https://doi.org/10.5935/0103-5053.20160178

Olivares-Tenorio ML, Dekker M, Verkerk R, van Boekel MAJS. 2016. Health-promoting compounds in Cape gooseberry (*Physalis peruviana* L.): Review from a supply chain perspective. *Trends Food Sci. Technol.* **57** (A), 83–92. https://doi.org/10.1016/j.tifs.2016.09.009

Ozturk A, Özdemir Y, Albayrak B, Simşek M, Yildirim KC. 2017. Some nutrient characteristics of goldenberry (*Physalis peruviana* L.) cultivar candidate from Turkey. *Sci. Papers. Ser. B. Horticulture* **61**, 293–297.

Popov A, Ilinov P. 1986. *Chemistry of Lipids*. Nauka i Iskustvo, Sofia.

Puente L, Pinto-Munoz G, Castro E, Cortes M. 2011. *Physalis peruviana* Linnaeus, the multiple properties of a highly functional fruit: a review. *Food Res. Int.* **44,** 1733–1740. https://doi.org/10.1016/j.foodres.2010.09.034

Ramadan MF, Mörsel J-T. 2003. Oil goldenberry (*Physalis peruviana* L.). *J. Agric. Food Chem.* **51**, 969–974. https://doi.org/10.1021/jf020778z

Ramadan MF, Sitohy M, Moersel J-T. 2008. Solvent and enzyme-aided aqueous extraction of goldenberry (*Physalis peruviana* L.) pomace oil: impact of processing on composition and quality of oil and meal. *Eur. Food Res. Technol.* **226**, 1445–1458. https://doi.org/10.1007/s00217-007-0676-y

Ramadan MF. 2011. Bioactive phytochemicals, nutritional value, and functional properties of cape gooseberry (*Physalis peruviana*): an overview. *Food Res. Int.* **44,** 1830–1836. https://doi.org/10.1016/j.foodres.2010.12.042

Ramadan MF. 2012. *Physalis peruviana* pomace suppresses high-cholesterol diet-induced hypercholesterolemia in rats. *Grasas Aceites* **63,** 411–422. https://doi.org/10.3989/gya.047412

Rodrigues E, Rockenbach I, Cataneo C, Gonzaga L, Chaves E, Fett R. 2009. Minerals and essential fatty acids of the exotic fruit *Physalis peruviana* L. *Ciencia Tecnol. Alime.* **29,** 642–654. https://doi.org/10.1590/S0101-20612009000300029

Sharma N, Bano A, Dhaliwal H, Sharma V. 2015. Perspectives and possibilities of Indian species of genus *Physalis* (L.) – a comprehensive review. *Eur. J. Pharm. Med. Res.* **2**, 326–353.

Yıldız G, İzli N, Ünal H, Uylaşer V. 2015. Physical and chemical characteristics of goldenberry fruit (*Physalis peruviana* L.). *J. Food Sci. Technol.* **52,** 2320–2327. https://doi.org/10.1007/s13197-014-1280-3

Zhang Y-J, Deng G-F, Xu X-R, Wu S, Li S, Li H-B. 2013. Chemical components and bioactivities of Cape gooseberry (*Physalis peruviana*). *Int. J. Food Nutr. Saf.* **3**, 15–24.

GRASAS Y ACEITES 72 (2)
April-June 2021, e403
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1146192

Chemical compositon, antibacterial and antioxidant activities of *Cnidium silaifolium* ssp. *orientale* (Boiss.) Tutin essential oils

A.E. Karadağ[a,b,✉], B. Demirci[c], Ö. Çeçen[d], F. Tosun[a] and F. Demirci[c,e]

[a]School of Pharmacy, Department of Pharmacognosy, Istanbul Medipol University, 34810, Istanbul, Turkey

[b]Graduate School of Health Sciences, Department of Pharmacognosy, Anadolu University, Eskişehir, Turkey

[c]Faculty of Pharmacy, Department of Pharmacognosy, Anadolu University, 26470, Eskişehir, Turkey

[d]Department of Plant and Animal Production, Medical and Aromatic Plants Programme, Ermenek Vocational High School, Karamanoğlu Mehmetbey University, 70400, Ermenek, Karaman, Turkey.

[e]Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, N. Cyprus, Mersin 10, Turkey

[✉]Corresponding author: ayseesraguler@gmail.com

Submitted: 28 November 2019; Accepted: 07 April 2020; Published online: 03 June 2021

SUMMARY: The chemical compositions of the essential oils (EOs) obtained by hydrodistillation from different parts of *Cnidium silaifolium* ssp. *orientale* (Boiss.) Tutin were analyzed both by GC-FID and GC/MS, simultaneously. One hundred nine compounds representing 90.1% of the total volatiles in the EOs were identified with the main characteristic compounds α-pinene (50.3%) in the root, germacrene D (20.3%) in the fruit, and β-caryophyllene (18.7%) in the aerial parts of *C. silaifolium* ssp. *orientale.* The antimicrobial activity against human pathogenic Gram-negative and Gram-positive bacteria was evaluated by the *in vitro* microdilution method. Antibacterial succeptibility was observed from the root and aerial part EOs against *Staphylococcus aureus* (0.039 and 0.156 mg/mL, respectively); while the fruit EO was most effective against *Bacillus cereus* at 0.07 mg/mL. The antioxidant capacities of the EOs were also evaluated by *in vitro* DPPH• and ABTS•+ scavenging assays, where no significant activity was observed compared to ascorbic acid and Trolox.

KEYWORDS: *Antimicrobial; Antioxidant; Apiaceae; Cnidium silaifolium ssp. orientale*

RESUMEN: ***Composición química, actividades antibacterianas y antioxidantes de* Cnidium silaifolia *ssp.* orientale *(Boiss.) de aceites esenciales de tutin.*** Se analizó por GC-FID y GC-MS la composición química de los aceites esenciales (AE) obtenidos por hidrodestilación de diferentes partes de *Cnidium silaifolium* ssp. *orientale* (Boiss.) tutin. Ciento nueve compuestos, que representan el 90.1% del total de volátiles de los AE, se identificaron. Los compuestos característicos principales fueron α-pineno (50.3%) en la raíz, germacreno D (20.3%) en la fruta y β-cariofileno (18.7%) en las partes aéreas de *C. silaifolium* ssp. *orientale.* La actividad antimicrobiana contra bacterias Gram negativas y Gram positivas patógenas humanas se evaluó mediante el método de microdilución in vitro. La susceptibilidad antibacteriana contra *Staphylococcus aureus*se observó desde la raíz a la parte aérea AEs (0.039 y 0.156 mg/mL, respectivamente), mientras que la fruta EO fue más efectiva contra *Bacillus cereus* a 0.07 mg/mL. La capacidad antioxidante de los AE también se evaluó mediante ensayos de eliminación in vitro de DPPH• y ABTS•+, donde no se observó actividad significativa en comparación con el ácido ascórbico y Trolox.

PALABRAS CLAVE: *Antimicrobiano; Antioxidante; Apiacea; Cnidium silaifolium ssp. orientale*

Citation/Cómo citar este artículo: Karadağ AE, Demirci B, Çeçen Ö, Tosun F, Demirci F. 2021. Chemical compositon, antibacterial and antioxidant activities of *Cnidium silaifolium* ssp. *orientale* (Boiss.) Tutin essential oils. *Grasas Aceites* **72** (2), e403. https://doi.org/10.3989/gya.1146192

1. INTRODUCTION

Cnidium silaifolium ssp. *orientale* (Boiss.) Tutin of Apiaceae is the only representative of the *Cnidium* species in Turkey, and is known as "galyabişotu" (Yüzbaşıoğlu *et al*., 2018). Previous *Cnidium* studies reported acaricidal, antioxidant, antipruritic, anticancer, hepatoprotective, and anti-inflammatory activities (Oh *et al.,* 2002; Jeong *et al.,* 2009; Li *et al.,* 2015; Hong *et al.,* 2017; Lim *et al.,* 2018; Tran *et al.,* 2018; Kim *et al.,* 2018). However, there are only a few previous reports on the essential oil (EO) compositions,where aerial parts were investigated in two different studies (Kapetanos *et al.,* 2008; Polat *et al.,* 2011).

There are very few studies on *C. silaifolium* ssp. *orientale* and the EO compositions of its aerial parts were investigated previously in two different studies (Kapetanos *et al.,* 2008; Polat *et al.,* 2011). So far, the EO composition of *C. silaifolium* ssp. *orientale* fruit and root parts have not been characterized. Here, comparative EO compositions of the aerial parts, fruits, and roots of *C. silaifolium* ssp. *orientale* were reported using gas chromatography with flame ionization detector (GC-FID) and mass spectrometry (GC-MS) systems. Natural products are an important resource for antimicrobial agents, and the essential oils are useful for many applications due to their antimicrobial properties. Antimicrobial essential oils are used as aromas, cosmetics and pharmaceuticals (Arici *et al.,* 2005; Selim, 2011; Başer and Buchbauer 2016). Because of this, the antibacterial and antioxidant activities of the aforementioned EOs were determined by broth microdilution and DPPH - ABTS radical scavenging methods, respectively.

The aim of this present study was to evaluate the *in vitro* antimicrobial and antioxidant activities of the different parts of *C. silaifolium* ssp. *orientale* EOs. To the best of our knowledge, this is the first comparative study on the chemistry of the volatiles and biological activities of the EOs from different parts of *C. silaifolium* ssp. *orientale* from its natural habitat in Turkey. The EOs were extracted by hydrodistillation followed by chromatographic analyses, and *in vitro* biological evaluation using selected human pathogenic strains and DPPH and ABTS radicals as scavenger targets.

2. MATERIALS AND METHODS

2.1. Plant material

The aerial parts, fruits, and roots of *C. silaifolium* ssp. *orientale* were collected in 16 July 2018 in Ermenek, Balkusan Village. The plant was identified by Ömer Çeçen and the voucher specimen (Herbarium No: 28000) was deposited at the Herbarium of the Selcuk University (KNYA), Konya, Turkey.

2.2. Hydrodistillation

Air-dried aerial parts, fruits, and roots (100 g) were crushed and hydrodistilled by distilled water (200 mL) using a Clevenger apparatus (ILDAM LTD., Ankara, Turkey) for eight hours, individually. The obtained EOs were dried by anhydrous sodium sulfate (Sigma, Germany) and kept in suitable conditions at 4 °C until GC and GC/MS analyses as well as biological assays were performed.

2.3. Chromatographic analyses

GC/MS analyses of the essential oils were performed using an Agilent 5975 GC-MSD system, (SEM Ltd., Istanbul, Turkey) where an HP-Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness, Agilent, Walt & Jennings Scientific, Delaware, USA) was used with Helium as carrier gas with a 0.8 mL/min flow rate. The GC oven temperature was maintained at 60 °C for 10 min. The oven was set to 220 °C (4°C/min), and kept for 10 min. and then heated to 240 °C (1°C/min). The split ratio was set to 40:1. The injection temperature was 250 °C. The Mass Spectra (MS) were recorded at 70 eV, and the mass ranges were from *m/z* 35 to 450.

FID temperature was set to 300 °C for GC analyses using an Agilent 6890N system (SEM Ltd., Istanbul, Turkey). Simultaneous auto-injection was applied using the same conditions as described in the GC/MS part. Relative percentages (%) of the detected volatile compounds were determined. Identification of these compounds was carried out by comparing their linear retention indexes (LRI) to a series of C_9–C_{20} *n*-alkane standard solutions (Fluka, Buchs, Switzerland). Computer matching was carried out using commercial (Wiley GC/MS Library, MassFinder Software 4.0), and the in-house 'Başer Library of Essential Oil Constituents' library as well as the literature was performed (Demirci *et al.*, 2018).

Table 1. The Chemical composition of *Cnidium silaifolium* ssp. *orientale* essential oils

*RRI	Compounds	**CsH %	**CsF %	**CsR %	***IM
1032	α-Pinene	0.2	0.6	50.3	t_R, MS
1035	α-Thujene	-	tr	-	MS
1076	Camphene	-	-	0.6	t_R, MS
1093	Hexanal	-	-	0.2	t_R, MS
1118	β-Pinene	0.1	0.1	1.8	t_R, MS
1132	Sabinene	0.1	0.2	1.2	t_R, MS
1174	Myrcene	0.1	0.8	4.0	t_R, MS
1176	α-Phellandrene	-	-	0.5	t_R, MS
1183	*p*-Mentha-1,7(8)-diene (=*Pseudolimonene*)	-	-	0.1	MS
1197	Methyl hexanoate	-	-	0.1	t_R, MS
1203	Limonene	0.1	0.5	5.4	t_R, MS
1218	β-Phellandrene	-	0.1	3.6	t_R, MS
1246	(*Z*)-β-Ocimene	0.1	0.2	4.5	MS
1255	γ-Terpinene	-	0.1	1.0	t_R, MS
1266	(*E*)-β-Ocimene	0.1	0.2	0.8	MS
1280	*p*-Cymene	tr	0.1	-	t_R, MS
1290	Terpinolene	-	tr	-	t_R, MS
1294	1,2,4-Trimethyl benzene	-	-	0.2	MS
1296	Octanal	-	-	0.1	t_R, MS
1355	1,2,3-Trimethyl benzene	-	-	0.1	MS
1400	Nonanal	-	0.1	-	t_R, MS
1429	Perillene	-	-	0.1	t_R, MS
1452	α,*p*-Dimethylstyrene	-	-	0.2	MS
1452	1-Octen-3-ol	-	-	0.1	MS
1466	α-Cubebene	0.3	0.1	-	MS
1477	4,8-Epoxyterpinolene	-	-	0.4	MS
1479	δ-Elemene	-	0.1	-	MS
1492	Cyclosativene	0.6	-	-	MS
1497	α-Copaene	7.5	5.3	0.3	MS
1499	α-Campholene aldehyde	-	-	0.6	MS
1519	1,7-Diepi-α-Cedrene (=α-Funebrene)	0.4	0.3	-	MS
1520	3,5-Octadien-2-one	-	-	0.1	MS
1535	β-Bourbonene	0.4	0.2	-	MS
1549	β-Cubebene	1.0	2.0	-	MS
1553	Linalool	tr	tr	0.1	t_R, MS
1571	*trans*-*p*-Menth-2-en-1-ol	-	-	0.1	MS
1577	α-Cedrene	1.1	0.7	-	t_R, MS
1586	Pinocarvone	-	-	0.2	t_R, MS
1597	β-Copaene	0.4	-	-	MS
1600	β-Elemene	2.1	13.7	-	MS
1604	Thymol methyl ether (=*Methyl thymol*)	-	-	0.4	t_R, MS
1611	Terpinen-4-ol	2.0	-	-	t_R, MS
1612	β-Caryophyllene	18.7	11.3	0.1	t_R, MS

*RRI	Compounds	**CsH %	**CsF %	**CsR %	***IM
1614	Carvacrol methyl ether (=*Methyl carvacrol*)	-	-	0.3	t_R, MS
1614	Acora-2,4-diene	-	0.3	-	MS
1648	Myrtenal		-	0.2	MS
1650	γ-Elemene	tr	0.1	-	MS
1670	*trans*-Pinocarveol	-	-	0.7	t_R, MS
1668	(*Z*)-β-Farnesene	1.2	0.4	-	MS
1683	*trans*-Verbenol	-	-	1.2	t_R, MS
1687	α-Humulene	2.5	6.0		t_R, MS
1690	Cryptone	-	-	0.4	MS
1693	β-Acoradiene	1.0	0.6	-	MS
1700	*p*-Mentha-1,8-dien-4-ol (=*Limonen-4-ol*)	-	-	0.1	t_R, MS
1704	γ-Muurolene	-	1.2	-	MS
1704	γ-Curcumene	-	1.6	-	MS
1706	α-Terpineol	2.2	-	-	t_R, MS
1725	Verbenone	-	-	0.3	t_R, MS
1726	Germacrene D	9.2	20.3	-	t_R, MS
1742	β-Selinene	2.2	3.4	-	MS
1744	α-Selinene	1.3	1.3	-	MS
1751	Carvone	-	-	0.1	t_R, MS
1755	Bicyclogermacrene	1.4	0.6	-	MS
1773	δ-Cadinene	5.7	-	-	MS
1783	β-Sesquiphellandrene	-	4.4	-	MS
1786	*ar*-Curcumene	2.9	5.1	0.2	MS
1796	Selina-3,7(11)-diene	-	1.5	-	MS
1797	*p*-Methyl acetophenone	-	-	0.2	MS
1804	Myrtenol	-	-	0.3	MS
1827	(*E,E*)-2,4-Decadienal	-	-	0.1	MS
1845	*trans*-Carveol	0.3	-	0.5	t_R, MS
1849	Cuparene		0.2	-	MS
1854	Germacrene-B	0.5	2.0	-	MS
1864	*p*-Cymen-8-ol	0.2	-	1.4	MS
1868	(*E*)-Geranyl acetone	-	0.1	-	MS
1870	Hexanoic acid	-	-	0.2	t_R, MS
1878	2,5-Dimethoxy-*p*-cymene	-	-	0.1	MS
1900	*epi*-Cubebol	-	0.1	-	MS
1925	2,3,4-Trimethyl benzaldehyde	-	-	0.2	MS
1941	α-Calacorene	0.7	tr	-	MS
1945	1,5-Epoxy-salvial(4)14-ene	0.2	0.1	-	MS
1957	Cubebol	-	0.1	-	MS
1984	γ-Calacorene	-	0.1	-	MS
2001	Isocaryophyllene oxide	-	0.1	-	MS
2008	Caryophyllene oxide	3.4	1.3	-	t_R, MS
2019	2,3,6-Trimethylbenzaldehyde	3.9	-	1.6	t_R, MS
2037	Salvial-4(14)-en-1-one	1.3	0.1	-	MS

*RRI	Compounds	**CsH %	**CsF %	**CsR %	***IM
2050	(*E*)-Nerolidol	0.4	-	-	t_R, MS
2071	Humulene epoxide-II	0.6	0.1	-	MS
2084	Octanoic acid	-	-	tr	t_R, MS
2100	Heneicosane	-	0.1	-	t_R, MS
2109	*cis*-Methyl isoeugenol	-	-	0.2	MS
2131	Hexahydrofarnesyl acetone	0.1	-	-	MS
2144	Spathulenol	5.9	0.8	-	MS
2161	Muurola-4,10(14)-dien-1-ol	tr	0.1	-	MS
2192	Nonanoic acid	tr	0.1	-	t_R, MS
2200	3,4-Dimetil-5-pentyl-5H-furan-2-one	tr	tr		MS
2239	Carvacrol	-	-	0.1	t_R, MS
2242	Methyl palmitate	-	-	0.2	MS
2255	α-Cadinol	-	0.1	-	MS
2262	Ethyl palmitate	-	-	tr	MS
2273	Selin-11-en-4α-ol	-	0.4	-	MS
2278	Torilenol	0.1	0.1	-	MS
2324	Caryophylla-2(12),6(13)-dien-5α-ol (=*Caryophylladienol II*)	0.7	-	-	MS
2369	Eudesma-4(15),7-dien-4β-ol	-	0.2	-	MS
2392	Caryophylla-2(12),6-dien-5β-ol (=*Caryophyllenol II*)	1.0	-	-	MS
2456	Methyl oleate	-	-	0.1	t_R, MS
2509	Methyl linoleate	-	-	0.3	t_R, MS
2655	Benzyl benzoate	5.9	0.4	0.7	t_R, MS
	Monoterpene Hydrocarbons	0.8	2.9	73.7	
	Oxygenated Monoterpenes	4.7	tr	7.3	
	Sesquiterpene Hydrocarbons	61.1	82.8	0.6	
	Oxygenated Sesquiterpenes	13.6	3.6		
	Fatty acid+esters	tr	0.1	0.9	
	Others	9.9	0.7	4.4	
	Total	90.1	90.1	86.9	

*RRI Relative retention indices calculated against *n*-alkanes , % calculated from FID data; tr Trace (< 0.1 %)

**CsH: aerial parts, CsF: fruits. CsR: *C.* roots

***Method of Identification by *t*R: retention times of standards on the HP Innowax column [a]; MS: Mass spectra identified on the basis of computer matching with those of the Wiley and MassFinder libraries and comparison with literature data

2.4. Antimicrobial activity

The antimicrobial activity of the Eos was determined using the broth microdilution assay as described before (Karadag *et al.,* 2019). *Acinetobacter baumanii* ATCC 19606, *Salmonella typhi* ATCC 6539, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 6538, and *Listeria monocytogenes* ATCC 19115 strains were grown in Mueller Hinton Broth (MHB, Merck, Germany). All microorganisms were standardized to 1 × 10^8 CFU/mL using McFarland No: 0.5 in sterile saline (0.85%) using a tubidometer (Biolab, Turkey). Intially, stock solutions of each essential oil and standard antimicrobial agent were prepared in diluted DMSO, serial dilutions were prepared and each strain along with the diluted samples were added to the wells and then allowed to incubate at 37 °C for 24 hours (Karadağ *et al*., 2019).

Helicobacter pylori ATCC 43504 was inoculated for 24 hours in Brucella broth containing 5% (v/v)

horse blood Colombia agar (Oxoid, Germany) and containing 10% (v/v) fetal bovine serum (FBS, Sigma Aldrich, Germany) at 37 °C in an anaerobic incubation system (5% CO_2). After the incubation, 100 μL of 1:10 diluted and density adjusted pathogenic strain were put onto each microplate (Karadağ *et al.,* 2019).

Mycobacterium avium was inoculated in Middlebrook 7H11 agar (Sigma Aldrich) and incubated at 37 °C under aerobic conditions for 4-5 days. Subsequently cultures were vortexed, and after 30 min. diluted bacterial suspensions (10^6 CFU/mL) were added to each well and then allowed to incubate at 37 °C for 5 days. The minimum inhibitory concentrations (MIC) were determined by XTT staining and the results were calculated as a mean of three repetitions. The standard antimicrobial compounds were Chloramphenicol, as shown in Table 3. (Chung *et al.,* 1995; Sun *et al.,* 2007).

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging assay

The antioxidant capacity was determined in terms of hydrogen donating or radical scavenging ability using 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Sigma, Germany) for its capability to bleach the stable radical (Blois 1958). The reaction mix contained 100 μM DPPH• in methanol and EOs at 1 mg/mL concentration. After 30 min, absorbance was read at 517 nm by using a UV–Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 25±2 °C.

Ascorbic acid (Merck, USA) was used as the reference, methanol was used for negative control. IC_{50} values were determined from a calibration curve, where each experiment was performed in triplicate (Blois 1958; Okur *et al.,* 2018).

2.5.2. ABTS radical scavenging assay

The total antioxidant activity of the EOs was measured using the ABTS· assay (Re *et al.,* 1999). ABTS• was produced by reacting ABTS• (Sigma, Germany) with 2.45 mM potassium persulfate. The mixture was left at room temperature overnight. Then, the colored ABTS radical cation was diluted with ethanol. The absorbances were measured at 734 nm at room temperature. In the assay Trolox (Supelco, Italy) was used as a positive control, as well as the water-soluble α-tocopherol (Sigma-Aldrich, Germany) analogue and blank ethanol was used for negative control. The assays were performed in triplicate.

3. RESULTS AND DISCUSSION

Comparative EO compositions of the aerial parts, fruits, and roots of *C. silaifolium* ssp. *orientale* were were reported using gas chromatography with flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS) systems. The air-dried root, fruit, and aerial part materials were hydro distilled in a Clevenger-type apparatus for 8 hours to yield a light-yellow oil. The *C. silaifolium* ssp. *orientale* aerial part, fruit, and root oil yields were 0.9% (v/w), 1.2% (v/w), 0.7% (v/w), respectively which were consequently analyzed both by GC-FID and GC-MS simultaneously. One hundred-nine compound were identified in *C. silaifolium* ssp. *orientale* EOs obtained from different parts constituting approximately 90% of the total oil. The aerial part and fruit EOs were dominated by sesquiterpene hydrocarbons. Otherwise, the EO of the root consisted of monoterpene hydrocarbons, mainly. These compounds are listed in Table 1 with their relative percentages. The main components were found to be β-caryophyllene (18.7%), germacrene D (9.2%), α-copaene (7.5%), spathulenol (5.9%), benzyl benzoate (5.9%) for aerial part; α-pinene (50%), limonene (5.4%), (*Z*)-β-ocimene (4.5%) and myrcene (4%)

Table 2. Antioxidant activity of *C. silaifolium* ssp. *orientale* essential oils (1 mg/mL concentration)

	IC_{50} ±SD (mg/mL)			
	CsH	**CsF**	**CsR**	**References**
DPPH·	1.32 ± 0.05	1.28 ±0.17	1.45 ± 0.03	0.004± 0.001 (Ascorbic acid)
ABTS·	1.14± 0.06	0.91± 0.07	1.29± 0.07	0.015± 0.008(Trolox)

**CsH: aerial parts, CsF: fruits. CsR: *C.* roots

Table 3. Antimicrobial activity of *C. silaifolium* ssp. *silaifolium* essential oils (MICs in mg/mL)

Bacteria Sample	St	Sa	Lm	Ab	Hp	Bc	Ma
CsH	>10	**0.156**	0.625	>10	>10	0.625	>10
CsF	>10	>10	>10	>10	>10	**0.078**	>10
CsR	>10	**0.039**	0.625	>10	>10	1.25	>10
Chloramphenicol	0.062	0.007	0.001	0.125	0.007	0.031	-

(- control) DMSO. **CsH: aerial parts, CsF: fruits. CsR: *C.* roots *St: Salmonella typhii Sa: Staphyllococcus aureus Lm: Listeria monocytogenes Ab: Acinetobacter baumanii Hp: Helicobacter pylori Bc: Bacillus cereus Ma: Mycobacterium avium*

β-phellandrene (3.6%) for root; germacrene D (20.3%), β-elemene (13.7%), β-caryophyllene (11.3%) and α-humulene (6%) for fruit EO, respectively. In previous studies, the EOs of the aerial parts of *C. silaifolium* ssp. *orientale* from two different localities were analyzed (Kapetanos *et al.,*2008; Polat *et al.,*2011). One of the studies was *C. silaifolium* ssp. *orientale* from Central Balcan, which was investigated for its EO composition and α-pinene was found to be the main component in this study (Kapetanos *et al.,* 2008). In other respects, the EO composition of *C. silaifolium* ssp. *orientale* aerial parts from Turkey was analyzed and kessane was found to be the main component of the EO composition (Polat *et al.,* 2011). The first five main components were found to be completely different compared to these two previous studies. Also, in this present study, compared to previous studies, it was seen that the content in EO was investigated more as a percentage and that more compounds were detected than in other studies (Kapetanos *et al.,*2008; Polat *et al*., 2011). These differences can be considered to be due to the collection of plant materials from different locations and different seasons. It is possible to see from the results that location differences in plants can change the phytochemistry of plants and hence biological activities.

The results for DPPH and ABTS radical scavenging activities are shown in Table 2. According to the DPPH· testing system, free radical scavenging activity IC_{50} value of *C. silaifolium* ssp. *orientale* aerial part, fruit, and root EOs were determined as 1.32, 1.28, and 1.45 mg/mL, respectively. For the ascorbic acid results (0.004 mg/mL) the oils were less effective than those of the ascorbic acid standard. In addition, the ABTS radical scavenging activity was also found at moderate levels (1.14, 0.91, and 1.29 mg/mL) and the results were compared to the Trolox standard (0.015 mg/mL).

Selected Gram (-) and (+) bacteria are given in Table S3 and were subjected to *C. silaifolium* ssp. *orientale* EOs. Among the tested bacteria in this study, *S. aureus* was the most sensitive to the aerial part and root and *B. cereus* was the most sensitive to the fruit EOs. The growth of *S. aureus* was remarkably inhibited by the EO of *C. silaifolium* ssp. *orientale* aerial and root parts. These results show that aerial and root EOs of *C. silaifolium* ssp. *orientale* can be used as a natural antibacterial agent for the prevention of *S. aureus* infections. The results indicated that these volatile oils can be natural and potential antimicrobial agents for wound healing and throat infections.

The biological activities of EOs are often explained by synergistic effects caused by combinations of major components. In previous studies, it was found that a *Zantoxylum* species and *Phlomis cretia* EOs, the major components of EO similar to the root EO used in this study, had a moderate antimicrobial activity. as in this study (Tatsadjieu *et al.,*2003; Aligiannis *et al*., 2004). The essential oils of *C. officinale* leaves and rhizomes, another *Cnidium* species, were studied against some human pathogens and moderate activity was detected (Sim and Shin 2014). In particular, the high antimicrobial activity of *C. officinale* leaf essential oil against *B. cereus* is similar to the *C. silaifolium* ssp. *orientale* leaf essential oil used in this study.

In conclusion, the EOs of the different parts of *C. silaifolium* ssp. *orientale* have moderate antioxidant activity. In addition, aerial part and root EOs showed

significant inhibition against *S. aureus*, while *B. cereus* was susceptible to fruit EO. To the best of our knowledge, this is the first comparabe report on the volatiles and *in vitro* biological activities of *C. silaifolium* ssp. *orientale* aerial part, root, and fruit EOs.

ACKNOWLEDGMENTS

Part of this work was presented at the International Symposium on Essential Oil Research 2019, in Vienna, Austria.

REFERENCES

Aligiannis N, Kalpoutzakis E, Kyriakopoulou I, Mitaku S, Chinou IB. 2004. Essential oils of *Phlomis* species growing in Greece: Chemical composition and antimicrobial activity. *Flavour Fragr. J.* **19**, 320–324. https://doi.org/10.1002/ffj.1305

Arici M, Sagdic O, Gecgel U. 2005. Antibacterial effect of Turkish black cumin (*Nigella sativa* L.) oils. *Grasas Aceites* **56**, 259–262.

Başer KHC, Buchbauer G. 2016. *Handbook of Essential Oils Science, Technology and Applications.* 2nd edition. London, CRC Press, 121-130.

Demirci F, Karaca N, Tekin M, Demirci B. 2018. Anti-inflammatory and antibacterial evaluation of *Thymus sipyleus* Boiss. subsp. *sipyleus* var. *sipyleus* essential oil against rhinosinusitis pathogens. *Microb. Pathog.* **122**, 117–121. https://doi.org/10.1016/j.micpath.2018.06.025

Hong H, An JC, De La Cruz JF, Hwang SG. 2017. *Cnidium officinale* makino extract induces apoptosis through activation of caspase-3 and p53 in human liver cancer HepG2 cells. *Exp. Ther. Med.* **14**, 3191–3197. https://doi.org/10.3892/etm.2017.4916

Jeong JB, Ju SY, Park JH, Lee JR, Yun KW, Kwon ST, Lim JH, Chung GY, Jeong HJ. 2009. Antioxidant activity in essential oils of *Cnidium officinale* makino and *Ligusticum chuanxiong* hort and their inhibitory effects on DNA damage and apoptosis induced by ultraviolet B in mammalian cell. *Cancer Epidemiol.* **33**, 41–46. https://doi.org/10.1016/j.canep.2009.04.010

Kapetanos C, Karioti A, Bojović S, Marin P, Veljić M, Skaltsa H. 2008. Chemical and principal-component analyses of the essential oils of *Apioideae taxa* (Apiaceae) from Central Balkan. *Chem Biodivers.* **5**, 101–119. https://doi.org/10.1002/cbdv.200890000

Karadağ AE, Demirci B, Çaşkurlu A, Demirci F, Okur ME, Orak D, Sipahi H, Başer KHC. 2019. *In vitro* antibacterial, antioxidant, anti-inflammatory and analgesic evaluation of *Rosmarinus officinalis* L. flower extract fractions. *South African J. Bot.* **125**, 214–220. https://doi.org/10.1016/j.sajb.2019.07.039

Karadag AE, Demirci B, Cecen O, Tosun F. 2019. Chemical characterization of *Glaucosciadium cordifolium* (Boiss.) B.L. Burtt and P.H. Davis essential oils and their antimicrobial, and antioxidant activities. *Istanbul J. Pharm.* **49**, 77–80. https://doi.org/10.26650/IstanbulJPharm.2019.19013

Kim K-T, Kim M-H, Park J-H, Lee J-Y, Cho H-J, Yoon I-S, Kim D-D. 2018. Microemulsion-based hydrogels for enhancing epidermal/dermal deposition of topically administered 20(S)-protopanaxadiol: in vitro and in vivo evaluation studies. *J. Ginseng Res.* **42**, 512–523. https://doi.org/10.1016/j.jgr.2017.07.005

Li YM, Jia M, Li HQ, Zhang ND, Wen X, Rahman K, Zhang QY, Qin LP. 2015. *Cnidium monnieri*: A review of traditional uses, phytochemical and ethnopharmacological properties. *Am. J. Chin. Med.* **43**, 835–877. https://doi.org/10.1142/S0192415X15500500

Lim EG, Kim GT, Kim BM, Kim EJ, Kim SY, Kim YM. 2018. Ethanol extract from *Cnidium monnieri* (L.) Cusson induces cell cycle arrest and apoptosis via regulation of the p53-independent pathway in HepG2 and Hep3B hepatocellular carcinoma cells. *Mol. Med. Rep.* **17**, 2572–2580. https://doi.org/10.3892/mmr.2017.8183

Oh H, Kim JS, Song EK, Cho H, Kim DH, Park SE, Lee HS, Kim YC. 2002. Sesquiterpenes with hepatoprotective activity from *Cnidium monnieri* on tacrine-induced cytotoxicity in Hep G2 cells. *Planta Med.* **68**, 748–749. https://doi.org/10.1055/s-2002-33796

Polat T, Özer H, Cakir A, Kandemir A, Mete E, Özturk E, Yildiz G. 2011. Volatile Constituents of *Cnidium silaifolium* (Jacq.) Simonkai subsp. *orientale* (Boiss.) Tutin from Turkey. *J. Essent. Oil-Bearing Plants* **14**, 453–457. https://doi.org/10.1080/0972060X.2011.10643600

Selim SA. 2011. Chemical composition, antioxidant and antimicrobial activity of the essential oil and methanol extract of the egyptian lemongrass cymbopogon proximus stapf. *Grasas Aceites* **62**, 55–61.

Sim Y, Shin S. 2014. Antibacterial activities of the essential oil from the leaves and rhizomes of *Cnidium officinale* Makino. *J. Essent. Oil Res.* **26**, 452–457. https://doi.org/10.1080/10412905.2014.951456

Tatsadjieu LN, Essia Ngang JJ, Ngassoum MB, Etoa FX. 2003. Antibacterial and antifungal activity of *Xylopia aethiopica, Monodora myristica, Zanthoxylum xanthoxyloïdes* and *Zanthoxylum leprieurii* from Cameroon. *Fitoterapia* **74**, 469–472. https://doi.org/10.1016/S0367-326X(03)00067-4

Tran HNK, Cao TQ, Kim JA, Youn UJ, Kim S, Woo MH, Min BS. 2018. Anti-inflammatory activity of compounds from the rhizome of *Cnidium officinale*. *Arch. Pharm. Res*. **41**, 977–985. https://doi.org/10.1007/s12272-018-1048-9

Yüzbaşıoğlu S, Altınözlü H, Kandemir A, Özbek MU. 2018. Flora of Kemaliye (Erzincan) District. *Hacettepe J. Biol. Chem.* **4**, 533–557.

GRASAS Y ACEITES 72 (2)
April-June 2021, e404
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0217201

Effects of virgin olive oil phenolic compounds on health: solid evidence or just another fiasco?

J.M. Castellano[a] and J.S. Perona[a, ✉]

[a]Instituto de la Grasa-CSIC. Campus University Pablo de Olavide, Building 46. 41013 Seville (Spain).
✉Corresponding author: perona@ig.csic.es

Submitted: 14 February 2020; Accepted: 18 April 2020; Published online: 03 June 2021

SUMMARY: Current research suggests that virgin olive oil (VOO) phenolics are potent preventive and therapeutic agents against metabolic diseases associated with inflammation and oxidative stress. Evidence-based medicine requires these effects be proved in randomized controlled trials (RCT), which are then assessed in meta-analyses, to ensure that the alleged health benefits really proceed in humans. The available evidence is limited to the ability of VOO phenolic compounds to protect lipoproteins from oxidation and to reduce systolic pressure in hypertensive individuals. No RCT assessing the effects of VOO phenolics on diabetes and neurodegenerative diseases have been performed, and those focused on osteoarthritis and cancer provided very scarce information. Therefore, RCT in extensive and diverse population groups, with different disorders and phenolic doses adjusted to usual VOO consumptions are necessary to achieve high quality scientific evidence before nutritional recommendations can be given to the general public.

KEYWORDS*: Evidence; Health; Hydroxytyrosol; Oleocanthal; Phenolic Compounds; Virgin Olive Oil*

RESUMEN: ***Efectos de los compuestos fenólicos del aceite de oliva virgen en la salud: ¿evidencia sólida o simplemente otro fiasco?*** Las investigaciones actuales indican que los compuestos fenólicos del aceite de oliva virgen (AOV) son potentes agentes preventivos y terapéuticos contra las enfermedades metabólicas asociadas con la inflamación y el estrés oxidativo. La medicina basada en la evidencia requiere que estos efectos se prueben en ensayos aleatorizados controlados (RCT), que son después evaluados en meta-análisis, para garantizar que los supuestos beneficios para la salud realmente se registran en humanos. La evidencia disponible se limita a la capacidad de los compuestos fenólicos del AOV para proteger las lipoproteínas de la oxidación y reducir la presión sistólica en individuos hipertensos. No se han realizado RCT que evalúen el efecto de estos compuestos sobre la diabetes y las enfermedades neurodegenerativas, y los que se centraron en la osteoartritis y el cáncer han proporcionado información muy escasa. Por lo tanto, nuevos RCT, en grupos de población extensos y diversos, con diferentes patologías y con dosis de fenoles ajustadas a los consumos habituales de VOO, deben desarrollarse, para lograr evidencia científica de alta calidad antes de que se puedan dar recomendaciones nutricionales al público en general.

PALABRAS CLAVE*: Aceite de Oliva Virgen; Compuestos Fenólicos; Evidencia; Hidroxitirosol; Oleocantal; Salud*

Citation/Cómo citar este artículo: Castellano JM, Perona JS. 2021. Effects of virgin olive oil phenolic compounds on health: solid evidence or just another fiasco? *Grasas Aceites* **72** (2), e404. https://doi.org/10.3989/gya.0217201

1. INTRODUCTION

In 2014, Francesco Visioli published an editorial response to a review by Tang *et al*., (2014) on the effects on cardiovascular health of resveratrol, a natural phenolic compound found in red wine (Visioli, 2014). Resveratrol was alleged to interact with multiple targets in a variety of cardiovascular disease models to exert protective effects or induce a reduction in cardiovascular risks. However, Visioli's article was entitled "The resveratrol fiasco" and concluded that after more than 20 years of well-funded research, resveratrol had no proven human activity. He claimed that there was a lack of clinical trials and the majority of available evidence came from cell culture and animal models. He also suggested that the resveratrol fiasco is not the only one of that kind in pharma-nutrition research. Considering that the association of virgin olive oil (VOO) phenolic compounds and health was initiated in the late 90s (some of the early studies were carried out by Visioli himself (Visioli *et al*., 1998)) and that the bulk of experimental data were obtained from *in vitro* and animal models, are VOO phenolics another example of scientific fiasco? In order to answer this question, the present review summarizes the current knowledge on the effects of VOO phenolic compounds on human health, focusing on the data obtained from randomized clinical trials (RCT) and their meta-analyses and systematic reviews, which provide the highest level of scientific evidence.

2. VOO PHENOLIC COMPOUNDS

There is ample evidence indicating that VOO consumption provides benefits in key processes associated with the development of a number of diseases and pathophysiological conditions. These include atherosclerosis, diabetes mellitus, obesity, metabolic syndrome, cancer, arthritis and neurodegenerative diseases (Covas *et al*., 2015). Despite its high oleic acid content, VOO is more than just a monounsaturated fatty acid-rich fat. VOO contains minor compounds with potent pharmacological activity, which are classified into two large groups: those that form part of the unsaponifiable fraction and those with a phenolic nature. The former are lipophilic and may be extracted with organic solvents after saponification of the oil, while the latter are water soluble.

More than 30 phenolic compounds that can play a role in the health promoting qualities of VOO have been identified, among which there is considerable variation regarding their concentration (0.02 to 600 mg/kg) (Servili *et al.,* 2009). This variability depends on the type of phenolic compound, but also on many other factors such as the olive tree variety, geographical origin, cultivation techniques, ripening stage at the time of harvest, processing and storage. Among VOO phenolics, secoiridoids are present in the greatest amount, but the most interesting ones from the point of view of health are probably oleuropein aglycone and its metabolite hydroxytyrosol. However, the presence of oleocanthal should not be ignored, as this compound has received a great deal of attention since it was suggested that it might have anti-inflammatory activity (Beauchamp *et al.*, 2005).

Several experimental studies in *in vitro* systems and animal models have shown that the possible benefits of VOO phenolics are associated with their anti-inflammatory, antioxidant and vasodilatory activity, which in theory makes them key preventive or therapeutic agents for metabolic diseases related to oxidative stress and inflammation (Covas *et al*., 2006). However, most of these investigations used supraphysiological doses of phenolics (> 10 μM) (Catalán *et al.*, 2015) and, therefore, it is difficult to translate their results into physiological relevance for humans. VOO phenolics are bioavailable in humans, i.e. they are susceptible to being absorbed and to exert a bioactive effect on the organism (de la Torre, 2008). It has been proposed that in humans hydroxytyrosol is dose-dependently absorbed and excreted in urine after the intake from VOO (Rubió *et al*., 2012; Oliveras-López *et al.*, 2014). However, a more recent study in Sprague-Dawley rats suggested that different dosages of hydroxytyrosol do not provide a linear, dose-dependent plasma concentration or excretion in urine (Domínguez-Perles *et al*., 2017). Nevertheless, it has been estimated that the amount of phenolics ingested from VOO consumption does not exceed 9 mg/day in Mediterranean countries (Parkinson and Cicerale., 2016).

Since 2011, the European Food Safety Authority (EFSA) accepts a claim about the benefits of daily intake of VOO rich in phenolic compounds over the oxidation of low density lipoproteins (LDL), maintenance of normal (fasting) blood levels of triglycerides, HDL-cholesterol and blood glucose

(EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011). The acceptance of these claims by EFSA authorizes their inclusion on the labels of olive oil bottles. However, this health claim is focused on the protection provided by hydroxytyrosol and there are currently no accepted claims for the other benefits that have been attributed to VOO or its phenolic compounds.

Although a large number of *in vitro* and experimental animal studies are available, according to Evidence-Based Medicine (Woolf *et al*., 1990), the healthy properties of a food and/or its components must be proved in RCT, in order to ensure that the alleged health benefits really stand out. The highest degree of scientific evidence is obtained through meta-analyses and systematic reviews of these RCT. However, at the moment the number and variety of RCT carried out using VOO phenolic compounds is very limited and there is only one meta-analysis and less than five systematic reviews assessing the healthy effects of VOO phenolic compounds. With regard to RCT, there are currently about 50 published articles that can be classified as such in which the effect of VOO phenolic compounds on health has been evaluated. A high number of these studies were published by the group of Covas and their collaborators and the rest by three or four research teams, the absolute majority of which were Spanish or Italian.

3. VOO PHENOLIC COMPOUNDS AND CARDIOVASCULAR DISEASE

The role of VOO phenolics as cardiovascular protectors has been a matter of research for a long time. However, as very recently displayed by Visioli *et al*., (2020), most investigations used isolated compounds in pharmacological *in vitro* approaches, rather than more nutritional human trials. Still, enough data from human studies assessing surrogate markers of cardiovascular disease have been generated (Bogani *et al*., 2007; Visioli *et al*., 2005; Covas *et al*., 2006) to allow for the completion of a meta-analysis of RCT (Hohmann *et al.,* 2015) and two systematic reviews (Bahramsoltani *et al*., 2019; Schwingshackl *et al*., 2019).

Indeed, this meta-analysis was aimed at assessing the effects of high phenolic olive oil on risk factors for cardiovascular disease. It was published in 2015 and included the results of 8 human RCT (Hohmann *et al.,* 2015). The sample size of these trials was quite variable, ranging from 24 participants in the study published by Moreno-Luna *et al*., (2012), to 200 individuals in the one carried out by Covas *et al*., (2006). In addition, there was variability in the health status of the participants. In five of these studies, the subjects were healthy and in the remaining three ones, the recruited individuals already had a cardiovascular event. The doses used in these tests ranged from 0 to 19.5 mg of total phenolics. Furthermore, in all these trials what was compared was the intake of a high phenolic VOO with a refined olive oil. Therefore, only the total phenolic content was considered and the concentrations in different phenolic compounds were not distinguished.

The intake of high phenolic VOO only seemed to produce a modest but significant reduction in oxidized LDL-cholesterol ($Z=1.98$, $p=0.05$) and blood pressure ($Z=4.04$, $p<0.001$). For the rest of parameters related to cardiovascular disease (malondialdehyde, LDL-cholesterol, HDL-cholesterol, total cholesterol and triglycerides), no significant effects were found. Therefore, the conclusion of this meta-analysis was that a high phenolic VOO provides small beneficial effects on systolic pressure and plasma oxidative status. No association between the intake of phenolic compounds from VOO and cardiovascular risk could be obtained.

In addition to this meta-analysis, two systematic reviews were published, both of them in 2019. Bahramsoltani *et al*., (2019) published a comprehensive review about dietary phenolic compounds and atherosclerosis and, even more recently, Schwingshackl *et al*., (2019) carried out a systematic review and network meta-analysis to assess the impact of different types of olive oil on cardiovascular risk factors.

The review by Bahramsoltani *et al*., (2019) included only six RCT, while the aforementioned meta-analysis by Hohmann and colaborators included eight. Of these 6 trials, only one reached a score of 3 on the Jadad Scale (Jadad *et al.*, 1996) which measures the methodological quality of a clinical trial from 0 (very poor) to 5 (rigurous) points. The others stayed at 2 or below. What was observed in the best designed study (Covas *et al.,* 2006) was an effect on the postprandial isoprostane F2 levels in plasma, which is a biomarker of oxidative stress. In this double-bind trial, 40 mL of three olive oils

with different phenolic contents were administered in a single dose: low (2.7 mg/kg), medium (164 mg/kg) and high (366 mg/kg). The authors observed that the concentration in total phenolic compounds in LDL increased in the postprandial period in a direct relationship with the phenolic content of the oils ingested. Moreover, plasma concentrations of tyrosol, hydroxytyrosol, and 3-O-methyl-hydroxytyrosol correlated with changes in the total phenolic compound contents in LDL. The other RCT included in the review also confirmed these results, as well as reduced lipid peroxidation (measured as 8-iso-PGF2α) and inflammation (assessed as intercellular adhesion molecule-1, ICAM-1).

The systematic review by Schwingshackl *et al*., (2019) included thirteen RCTs with 611 participants (mainly healthy) and compared refined olive oil, commercial olive oil (blend of refined and virgin olive oils), low-phenolic extra VOO and high-phenolic VOO. No differences for total cholesterol, HDL-cholesterol, triglycerides, and diastolic blood pressure were observed. However, the phenolic content positively correlated with a slight reduction in LDL-cholesterol (mean difference -0.14 mmol/L, 95%-CI: -0.28, -0.01) and oxidized LDL-cholesterol (standardized mean difference: -0.68, 95%-CI: -1.31, -0.04). Both, high- and low-phenolic VOO reduced systolic blood pressure compared to refined olive oil (range of mean difference: -2.99 to -2.87 mmHg). The authors concluded that high phenolic VOO may improve some cardiovascular risk factors, although the implications for public health were limited due to the overall low or moderate level of evidence provided and also because the duration of these RCTs was too short (≤12 weeks) and no data could be found for relevant outcomes such as cardiovascular events.

From these meta-analysis and systematic reviews, it can be concluded that the protective effect of VOO phenolics is related to blood pressure and LDL oxidation only. Nonetheless, although the oxidative hypothesis of atherosclerosis has been accepted for decades, the true contribution of LDL oxidation to cardiovascular disease is still unclear (Arsenault *et al*., 2017; Visioli *et al*., 2020). While the hypothesis is supported by hundreds of *in vitro* and animal studies, it does not explain why human trials with some antioxidants, such as vitamin E, did not provide sufficient convincing evidence for cardiovascular prevention (Sesso *et al*., 2008; Guallar *et al*., 2013). Possibly, the story is not as simple as it was believed. For instance, it has been proposed that LDL can become atherogenic even before oxidation. Modification of these lipoproteins begins with a desialylation and it is followed by a cascade of other physical and chemical alterations that increase LDL atherogenicity, including particle size reduction, increase in its density and negative electrical charge and loss of lipids (Summerhill *et al*., 2019). Therefore, the effects of VOO phenolics on cardiovascular disease via LDL modification are, as of today, dubious and there is need of more RCT that directly address the claim.

4. VOO PHENOLIC COMPOUNDS AND TYPE 2 DIABETES MELLITUS/METABOLIC SYNDROME

Accumulated data obtained from experimental models indicate that VOO phenolics have the potential to normalize metabolic syndrome and its pathophysiological complications, including diabetes. Diabetes and metabolic syndrome are linked to each other through insulin resistance, and subjects diagnosed with metabolic syndrome have a high risk of developing T2DM (Shin *et al.,* 2013). However, there are currently no RCT aimed at specifically assessing the effect of VOO phenolics on Type 2 diabetes (T2DM) patients, although two systematic reviews on the effects of these compounds on metabolic syndrome have been published (Chiva-Blanch and Badimon, 2017; Saibandith, 2017).

The review by Chiva-Blanch and Badimon (2017) was focused on human intervention trials administering phenolic-rich foods to patients with metabolic syndrome. They included a single RCT, carried out by Venturini *et al*., (2015), which administered extra VOO (10 mL/day). That trial reported an increase in the total radical-trapping antioxidant parameter (TRAP)/uric acid ratio, with no apparent effects on other markers of oxidative stress. On the other hand, the authors did not find changes in the lipid profile, plasma glucose, insulin resistance or blood pressure.

The systematic review by Saibandith *et al*., (2017) did assess the effects of VOO phenolics on components of the metabolic syndrome (i.e. glucose levels, blood pressure, central obesity, triglycerides and HDL-cholesterol). They summarized the current knowledge obtained from 18 clinical trials that were

not specifically aimed at this syndrome. Saibandith and collaborators confirmed the effects of VOO phenolics on systolic blood pressure observed in previous systematic reviews and meta-analyses, but they did not draw significant outcomes on obesity, triglycerides or HDL. Still, some reductions in plasma glucose and in biomarkers associated with glucose homeostasis were reported, but only in three of those trials. Surprisingly, the authors did not include the RCT by Venturini *et al*., (2015) that had been published a couple of years before, and therefore, they did not analyzed any RCTs involving patients with metabolic syndrome. The systematic review could only conclude that there was good evidence showing that, when consumed at appropriate doses, VOO phenolics may reduce blood pressure in hypertensive subjects and improve plasma glucose in pre-diabetic individuals.

It is noteworthy that the RCT involving patients with metabolic syndrome and published in 2016 by D'Amore *et al.,* was not included in both systematic reviews stated above. In that trial, 12 subjects with metabolic syndrome and 12 healthy controls received a single dose of 50 mL VOO from two olive varieties: *coratina*, providing 491 ppm of phenolic compounds, and *peranzana*, which had 270 ppm of phenolics. The most interesting result found in this study was the modification of the transcriptome of peripheral blood mononuclear cells, switching them to a less deleterious inflammatory phenotype. However, no relevant changes in the components of the metabolic syndrome were observed.

5. VOO PHENOLIC COMPOUNDS AND ALZHEIMER'S DISEASE

AD is pathologically characterized by substantial neuronal and synaptic losses, and decreased cognitive abilities, which are associated with cerebral deposits of amyloid-beta-enriched plaques and neurofibrillary aggregates of the Tau protein, as well as with chronic inflammation and oxidative stress (Rosales-Corral *et al*., 2015) elicited by the pathological activation of glial cells (Scimemi *et al*., 2013). Brain has revealed nowadays as an insulin-sensitive organ, where the hormone regulates important physiological processes, such as nutrient homeostasis, reproduction, cognition, and memory, and also exerts neurotrophic, neuromodulatory, and neuroprotective effects (Blazquez *et al*., 2014). Disturbances in insulin signaling in the brain may contribute to the development of several clinical entities, including T2DM and Alzheimer's disease. The close association between cerebral insulin resistance and Alzheimer's Disease brought some authors to propose the name "type 3 diabetes" for this illness (de la Monte and Wands, 2008).

For the moment, there are no RCTs available in which olive oil phenolics have been tested on Alzheimer's disease or other neurodegenerative disorders. Therefore, no systematic reviews or meta-analysis of RCT have been performed so far. All available information refers to observational studies and those carried out in animal models, which, as mentioned above, provide a low level of scientific evidence.

Regarding the potential neuroprotective effects of olive phenolics, it has been reported that dietary supplementation with oleuropein aglycone reduced the amount of amyloid-beta oligomers in the brain of Alzheimer's model mice (Luccarini *et al*., 2015), at the same time that they significantle improved their cognitive functions, presenting greater learning and memory capacities (Pantano *et al*., 2017). Oleuropein also enhanced the endogenous antioxidant response in the CA1 hippocampal area of rats suffering from colchicine-induced cognitive dysfunction, improving the redox status of glutathione, and increasing the activity of antioxidant enzymes, such as superoxide dismutase or catalase (Pourkhodadad *et al*., 2016). Similarly, hydroxytyrosol has been related to the amelioration of insulin resistance in the brain of mice (Kulas *et al*., 2020). In APP/PS1 mice, rodent models of AD, hydroxytyrosol improved cognitive function, increased the expression of antioxidant enzymes and phase 2 response genes, and reduced inflammatory factors in the brain of these animals (Peng *et al*., 2016).

Oleocanthal has also been associated with a reduction in amyloid-beta oligomers, as well as an attenuation of astrocytes activation and a reduction in systemic inflammation in neurons and astrocytes cells lines (Batarseh *et al*., 2017). Likewise, oleocanthal has demonstrated that it may modulate the astrocyte activation in TgSwDI mice, a model of Alzheimer's disease (Qosa *et al*., 2015).

6. VOO PHENOLIC COMPOUNDS AND CANCER

Only one systematic review about the effects of VOO phenolic compounds on cancer has

been published, which implies that there are no meta-analyses available and, in consequence, the highest level of scientific evidence cannot be reached. In their review, Fabiani *et al*., (2016) included 16 animal studies and 5 RCTs. Most of the animal studies confirmed the ability of secoiridoid compounds to inhibit carcinogenesis at both initiation and promotion/progression phases. However, all human intervention trials included in this review only investigated the effects of VOO phenolics on DNA damage and did not evaluate their effect on the incidence or development of any kind of cancer. These trials only reported determinations related to the oxidation of nucleic acids, in particular the concentrations of 8-oxo-7,8-dihydro-2'-deoxyguanosine, which is a marker of DNA oxidation. Between 10 and 182 subjects participated in these RCT, and the phenolic doses from VOO ranged from 10 to 592 mg/kg. Three of the five human trials showed that VOO phenolics reduced the 8-oxo-7,8-dihydro-2'-deoxyguanosine levels in urine, mitochondrial DNA of mononuclear cells and lymphocyte DNA. The other two trials failed to find a protective effect on DNA oxidation. Four of these studies were conducted by the same research group, and had similar experimental designs (Weinbrenner *et al.*, 2004; Hillestrøm *et al*., 2006; Machowetz *et al*., 2007; Romeu *et al*., 2016). Therefore, there is very little evidence relating the intake of VOO phenolic compounds with cancer. In fact, the authors suggested that further investigations are necessary to clarify the real chemopreventive potential of these compounds and that intervention studies on populations at high cancer risk are needed.

7. VOO PHENOLIC COMPOUNDS AND ARTHRITIS

There is currently only one RCT evaluating the effect of VOO phenolic compounds on arthritis (Takeda *et al.*, 2013). This trial was aimed at determining whether hydroxytyrosol intake could reduce knee pain in individuals with gonarthrosis (n=25). An extract of *Olea europaea* containing approximately 22% of hydroxytyrosol, at a dose of 11 mg/day for 4 weeks, was administrated to the participants. The conclusion of the trial was that the subjects informed a reduction in knee pain, as compared to the administration of a placebo.

8. CONCLUSIONS

The benefits of VOO phenolic compounds on health have been extensively investigated, and recent studies support the belief that these components may play a key role in the amelioration of pathophysiological conditions. In particular, studies on *in vitro* systems and animal models have shown that oleuropein derivatives, hydroxytyrosol and oleocanthal exert potent pharmacological activities on markers of cancer, atherosclerosis and metabolic diseases. More specifically, on those associated with inflammatory processes and oxidative stress. However, meta-analyses of RCT, from which the highest level of scientific evidence should be obtained, do not support those effects on humans, except for some markers of cardiovascular risk. In fact, the strongest piece of evidence available has been found for the ability of VOO phenolic compounds to protect lipoproteins from oxidation and to reduce systolic blood pressure in hypertensive individuals (Table 1).

Unfortunately, there is not enough high-level evidence at the moment to confirm that the intake of phenolic compounds isolated or as components of the VOO can be healthy (Table 2). No RCT have been carried out to assess the direct effect of VOO phenolics on diabetes or neurodegenerative diseases and very scarce information can be collected from the ones that have focused on osteoarthritis and cancer. Therefore, it is still necessary to develop double-bind RCT in extensive and diverse population groups, with different disorders and with doses of phenols adjusted to usual VOO consumptions, in order to provide a greater degree of scientific evidence before nutritional recommendations may be given to the general population.

ACKNOWLEDGMENTS

This review was presented as an oral communication to the VI Conference of the Spanish Society of Horticultural Sciences "Phenols in olives and olive oil: origin and evolution", which was held in Madrid on the 26-27th June, 2019.

Table 1. Availability of studies and degree of evidence for the effect of virgin olive oil phenolics intake on different disorders according to evidence-based medicine.

Disorder	Meta-Analyses	Systematic reviews	RCT	In vivo (animals)	In vitro (cells)	Degree of evidence
Cardiovascular disease	Yes (1)	Yes (2)	Yes (13)	Yes	Yes	Medium (High por BP and ox LDL)
Cancer	No	Yes (1)	Yes (5)	Yes	Yes	Low
Alzheimer's disease	No	No	No	Yes	Yes	Very low
Arthritis	No	No	Yes (1)	Yes	Yes	Low
Diabetes mellitus	No	No	No	Yes	Yes	Low
Metabolic syndrome	No	Yes (2)	Yes (2)	Yes	Yes	Low (High for BP)

[a]Numbers in parentheses refer to the published number of studies of that kind. RCT, randomized controlled trial; BP, blood pressure; LDL, low-density lipoprotein.

Table 2. Summary of the studies cited in the text.

Disorder	Study design	n	Intervention	Dose (day)/time	Time	Main markers	References
CVD	RCT	22	2 olive oils (VOO vs ROO)	40 mL	7 weeks	TXB2, isoprostanes	Visioli *et al*., 2005
	RCT	200	3 olive oils (different PC)	25 mL	3 weeks	TG, HDL, oxLDL	Covas *et al*., 2006
	RCT	12	EVOO, OO and CO	50 mL	Postprandial	TXB2, LTB4	Bogani *et al*., 2007
	RCT	24	2 olive oils (different PC)	30 mg	8 weeks	BP, CRP, ox-LDL	Moreno-Luna *et al*., 2012
	Systematic review	6 RCT	Olive oils (different PC)	Various	Single-8 weeks	Lipids, oxidation, inflammation	Bahramsoltani *et al*., 2019
	Systematic review	13 RCT	Olive oils (different PC)	Various	3-12 weeks	TC, LDL, HDL, TG, ox-LDL, BP	Schwingshackl *et al*., 2019
	Meta-analysis	8 RCT	Olive oils (different PC)	Various	3-12 weeks	TC, LDL, HDL, TG, ox-LDL, BP	Hohmann *et al*., 2015
Cancer	RCT	12	3 olive oils (different PC)	25 mL	4 days	8-OHdG	Weinbrenner *et al*., 2004
	RCT	28	3 olive oils (different PC)	25 mL	3 weeks	Etheno–DNA adducts	Hillestrøm *et al*., 2006
	RCT	58	3 olive oils (different PC)	25 mL	3 weeks	8-Oxo-guanine	Machowetz *et al*., 2007
	RCT	33	2 olive oils (different PC)	25 mL	3 weeks	8-OHdG	Romeu *et al*., 2008
	Systematic review	5 RCT	2-3 olive oils (different PC)	25 mL. 50 g	4 days – 8 weeks	8-OHdG	Fabiani *et al*., 2016
Alzheimer's	Astrocyte cell line	N/A	Oleocanthal	5 µM	3-7 days	GLT1, GLUT1, IL-6	Batarseh *et al*., 2017
	Murine model (Tg-CRND8)	6/group	Oleuropein aglycone	50 mg/kg of diet	8 weeks	Aß42, pE3-Aß aggregation	Luccarini *et al*., 2015
	Murine model (TgSwDI)	6/group	Oleocanthal	6 mg/kg injection	4 weeks	Aß, IL-1ß	Qosa *et al*., 2015

Disorder	Study design	n	Intervention	Dose (day)/time	Time	Main markers	References
	Rat model	7/group	Oleuropein	10-20 mg/kg diet	10 days	SOD, Catalase, NO, MDA	Pourkhodadad *et al*., 2016
	Murine model (APP/PS1)	9/group	Hydroxytyrosol	5 mg/kg gavage	6 months	Cognitive, SOD, inflammation	Peng *et al*., 2016
	Murine model (Tg-CRND8)	6/group	Oleuropein aglycone	12.5 mg/kg of diet	8 weeks	Aß42 , pE3-Aß aggregation	Pantano *et al*., 2017
Arthritis	RCT	25	Hydroxytyrosol	11 mg/day	4 weeks	Knee pain	Takeda *et al*., 2013
MetS	RCT	102	Usual diet, EVOO, fish oil	10 mL/day	12 weeks	TRAP, uric acid	Venturini *et al*., 2015
	RCT	24	EVOO two varieties	50 mL	Postprandial	PBMC transcriptome	D'Amore *et al*., 2016
	Systematic review	1 RCT[b]	Usual diet, EVOO, fish oil	10 mL/day	12 weeks	TRAP, uric acid	Chiva-Blanch and Badimon, 2017
	Systematic review	18 RCT[c]	EVOO, olive leaf extract	Various	1 week -1 year	BP, HbA1C, Glucose insulin, WC	Saibandith, 2017

[a]8-OHdG, 8-hydroxy-deoxyguanosine; BP, blood pressure; CO, corn oil; CRP, C-reactive protein; EVOO, extra-virgin olive oil; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LTB4, leukotriene B4; OO, olive oil; ox-LDL, oxidized LDL; PC, phenolic content; RCT, randomized controlled trial; ROO, refined olive oil; TG, triglycerides; TXB2, thromboxane B2;

[a]Aß, amiloid beta; EVOO, extra-virgin olive oil; GLT1, glutamine transporter 1; GLUT1, glucose transporter 1; HbA1C, glycosylated hemoglobin; IL-1ß, interleukin 1 beta; IL-6, interleukin-6; MDA, malodialdehyde; MetS, metabolic syndrome; N/A, not applicable; NO, nitric oxide; PBMC, Peripheral blood mononuclear cellRCT, randomized controlled trial; SOD, superoxide dismutase; TRAP, peroxyl radical-trapping antioxidant potential; WC, waist circumference. [b]Review focused on phenolics from different sources. 1 RCT from VOO. [C] Review focused on MetS components, no RCT with MetS diagnosed subject

REFERENCES

Arsenault BJ, Bourgeois R, Mathieu P. 2017. Do oxidized lipoproteins cause atherosclerotic cardiovascular diseases? *Can. J. Cardiol.* **33** (12), 1513-1516.

Bahramsoltani R, Ebrahimi F, Farzaei MH, Baratpourmoghaddam A, Ahmadi P, Rostamiasrabadi P, Rasouli Amirabadi AH, Rahimi R. 2019. Dietary polyphenols for atherosclerosis: A comprehensive review and future perspectives. *Crit. Rev. Food Sci. Nutr*. **59** (1), 114-132. https://doi.org/10.1080/10408398.2017.1360244

Batarseh YS, Mohamed LA, Al Rihani SB, Mousa YM, Siddique AB, El Sayed KA, Kaddoumi A. 2017. Oleocanthal ameliorates amyloid-β oligomers' toxicity on astrocytes and neuronal cells: *In vitro* studies. *Neuroscience* **352**, 204-215. https://doi.org/10.1016/j.neuroscience.2017.03.059

Beauchamp GK, Keast RS, Morel D, Lin J, Pika J, Han Q, Lee CH, Smith AB, Breslin PA. 2005. Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. *Nature* **437**, 45-6.

Blázquez E, Velázquez E, Hurtado-Carneiro V, Ruiz-Albusac JM. 2014. Insulin in the brain: its pathophysiological implications for states related with central insulin resistance, type 2 diabetes and Alzheimer's disease. *Frontiers Endocrinol*. **5,** 161. https://doi.org/10.3389/fendo.2014.00161

Bogani P, Galli C, Villa M, Visioli F. 2007. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* **190** (1), 181-6.

Catalán Ú, López de Las Hazas MC, Rubió L, Fernández-Castillejo S, Pedret A, de la Torre R, Motilva MJ, Solà R. 2015. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. *Mol. Nutr. Food Res.* **59**, 2523–2536. https://doi.org/10.1002/mnfr.201500361

Chiva-Blanch G, Badimon L. 2017. Effects of Polyphenol Intake on Metabolic Syndrome:

Current Evidences from Human Trials. *Oxid. Med. Cell Longev*. **2017**, 5812401. https://doi.org/10.1155/2017/5812401

Covas M-I, de la Torre R, Fitó M. 2015. Virgin olive oil: a key food for cardiovascular risk protection. *Br. J. Nutr.* **113**, S19 – S28.

Covas MI, Nyyssonen K, Poulsen HE, Kaikkonen J, Zunft HJ, Kiesewetter H, Gaddi A, de la Torre R, Mursu J, Baumler H, Nascetti S, Salonen JT, Fito M, Virtanen J, Marrugat J, 2006. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. *Ann. Intern. Med*. **145** (5), 333–341.

D'Amore S, Vacca M, Cariello M, Graziano G, D'Orazio A, Salvia R, Sasso RC, Sabbà C, Palasciano G, Moschetta A. 2016. Genes and miRNA expression signatures in peripheral blood mononuclear cells in healthy subjects and patients with metabolic syndrome after acute intake of extra virgin olive oil. *Biochim. Biophys. Acta* **1861** (11), 1671-1680. https://doi.org/10.1016/j.bbalip.2016.07.003

Domínguez-Perles R, Auñón D, Ferreres F, Gil-Izquierdo A. 2017. Gender differences in plasma and urine metabolites from Sprague-Dawley rats after oral administration of normal and high doses of hydroxytyrosol, hydroxytyrosol acetate, and DOPAC. *Eur. J. Nutr*. 56 (1), 215-224.

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). 2011. Scientific Opinion on the substantiation of health claims related to olive oil and maintenance of normal blood LDL-cholesterol concentrations (ID1316, 1332), maintenance of normal (fasting) blood concentrations of triglycerides (ID 1316, 1332), maintenance of normal blood HDL-cholesterol concentrations (ID 1316, 1332) and maintenance of normal blood glucose concentrations (ID 4244) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J*. **9**, 2044.

Fabiani R. 2016. Anti-cancer properties of olive oil secoiridoid phenols: a systematic review of in vivo studies. *Food Funct.* **7** (10), 4145-4159.

Fitó M, Covas MI, Lamuela-Raventós RM, Vila J, Torrents L, de la Torre C, Marrugat J. 2000. Protective effect of olive oil and its phenolic compounds against low density lipoprotein oxidation. *Lipids* **35** (6),633-8.

Guallar E, Stranges S, Mulrow C, Appel LJ, Miller ER 3rd. 2013. Enough is enough: Stop wasting money on vitamin and mineral supplements. *Ann. Intern. Med.* **159** (12), 850-851.

Hillestrøm PR, Covas MI, Poulsen HE. 2006. Effect of dietary virgin olive oil on urinary excretion of etheno-DNA adducts. *Free Radic. Biol. Med*. **41** (7),1133-1138.

Hohmann CD, Cramer H, Michalsen A, Kessler C, Steckhan N, Choi K, Dobos G. 2015. Effects of high phenolic olive oil on cardiovascular risk factors: A systematic review and meta-analysis. *Phytomedicine* **22** (6), 631-40. https://doi.org/10.1016/j.phymed.2015.03.019

Jadad AR, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. 1996. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin. Trials* **17** (1), 1-12.

Kulas JA, Weigel TK, Ferris HA. 2020. Insulin resistance and impaired lipid metabolism as a potential link between diabetes and Alzheimer's disease. *Drug Dev. Res*. **81** (2), 194-205. https://doi.org/10.1002/ddr.21643

Leszek J, Trypka E, Tarasov VV, Ashraf GM, Aliev G. 2017. Type 3 Diabetes Mellitus: A Novel Implication of Alzheimers Disease. *Curr. Top. Med. Chem.* **17** (12), 1331-1335. https://doi.org/10.2174/1568026617666170103163403

Luccarini I, Grossi C, Rigacci S, Coppi E, Pugliese AM, Pantano D, la Marca G, Ed Dami T, Berti A, Stefani M, Casamenti F. 2015. Oleuropein aglycone protects against pyroglutamylated-3 amyloid-ß toxicity: biochemical, epigenetic and functional correlates. *Neurobiol. Aging*. **36** (2), 648-63.

Machowetz A, Poulsen HE, Gruendel S, Weimann A, Fitó M, Marrugat J, de la Torre R, Salonen JT, Nyyssönen K, Mursu J, Nascetti S, Gaddi A, Kiesewetter H, Bäumler H, Selmi H, Kaikkonen J, Zunft HJ, Covas MI, Koebnick C. 2007. Effect of olive oils on biomarkers of oxidative DNA stress in Northern and Southern Europeans. *FASEB J.* **21** (1), 45-52.

Monte SM de la, Wands JR. 2008. Alzheimer's Disease Is Type 3 Diabetes—Evidence Reviewed. *J. Diabetes Sci. Technol*. 2, 1101-1113.

Moreno-Luna R, Muñoz-Hernandez R, Miranda ML, Costa AF, Jimenez-Jimenez L, Vallejo-Vaz AJ, Muriana FJ, Villar J, Stiefel P. 2012. Olive oil polyphenols decrease blood pressure and

improve endothelial function in young women with mild hypertension. *Am. J. Hypertens*. **25** (12), 1299–1304.

Oliveras-López M-J, Berná G, Jurado-Ruiz E, López-García de la Serrana H, Martín F. 2014. Consumption of extra-virgin olive oil rich in phenolic compounds has beneficial antioxidant effects in healthy human adults. *J. Func. Foods* **10**, 475-484.

Pantano D, Luccarini I, Nardiello P, Servili M, Stefani M, Casamenti F. 2017. Oleuropein aglycone and polyphenols from olive mill waste water ameliorate cognitive deficits and neuropathology. *Br. J. Clin. Pharmacol.* **83** (1), 54-62. https://doi.org/10.1111/bcp.12993

Parkinson L, Cicerale S. 2016. The Health Benefiting Mechanisms of Virgin Olive Oil Phenolic Compounds. *Molecules* **16**, E1734. https://doi.org/10.3390/molecules21121734

Peng Y, Hou C, Yang Z, Li C, Jia L, Liu J, Tang Y, Shi L, Li Y, Long J, Liu J. 2016. Hydroxytyrosol mildly improve cognitive function independent of APP processing in APP/PS1 mice. *Mol. Nutr. Food Res.* **60** (11), 2331-2342. https://doi.org/10.1002/mnfr.201600332

Pourkhodadad S, Alirezaei M, Moghaddasi M, Ahmadvand H, Karami M, Delfan B, Khanipour Z. 2016. Neuroprotective effects of oleuropein against cognitive dysfunction induced by colchicine in hippocampal CA1 area in rats. *J. Physiol Sci.* **66** (5), 397-405. https://doi.org/10.1007/s12576-016-0437-4

Puerta R de la, Ruiz Gutierrez V, Hoult JR. 1999. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* 57 (4), 445-449.

Qosa H, Batarseh YS, Mohyeldin MM, El Sayed KA, Keller JN, Kaddoumi A. 2015. Oleocanthal enhances amyloid-β clearance from the brains of TgSwDI mice and in vitro across a human blood-brain barrier model. *ACS Chem. Neurosci*. **6** (11), 1849-59. https://doi.org/10.1021/acschemneuro.5b00190

Romeu M, Rubió L, Sánchez-Martos V, Castañer O, de la Torre R, Valls RM, Ras R, Pedret A, Catalán Ú, López de las Hazas M del C, Motilva MJ, Fitó M, Solà R, Giralt M. 2016. Virgin Olive Oil Enriched with Its Own Phenols or Complemented with Thyme Phenols Improves DNA Protection against Oxidation and Antioxidant Enzyme Activity in Hyperlipidemic Subjects. *J. Agric. Food Chem.* **64** (9), 1879-88. https://doi.org/10.1021/acs.jafc.5b04915

Rosales-Corral S, Tan DX, Manchester L, Reiter RJ. 2015. Diabetes and Alzheimer disease, two overlapping pathologies with the same background: oxidative stress. *Oxid. Med. Cell Longev*. **2015**, 985845.

Rubió L, Valls RM, Macià A, Pedret A, Giralt M, Romero MP, de la Torre R, Covas MI, Solà R, Motilva MJ. 2012. Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites. *Food Chem*. **135** (4), 2922-9. https://doi.org/10.1016/j.foodchem.2012.07.085

Saibandith B, Spencer JPE, Rowland IR, Commane DM. 2017. Olive Polyphenols and the Metabolic Syndrome. *Molecules* **29**, 22 (7).

Schwingshackl L, Krause M, Schmucker C, Hoffmann G, Rücker G, Meerpohl JJ. 2019. Impact of different types of olive oil on cardiovascular risk factors: A systematic review and network meta-analysis. *Nutr. Metab. Cardiovasc. Dis*. **29** (10), 1030-1039.

Scimemi A, Meabon JS, Woltjer RL, Sullivan JM, Diamond JS, Cook DG. 2013. Amyloid-β_{1-42} slows clearance of synaptically released glutamate by mislocalizing astrocytic GLT-1. *J. Neurosci.* **33** (12), 5312-8. https://doi.org/10.1523/JNEUROSCI.5274-12.2013

Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R, Montedoro GF. 2009. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacol.* **17,** 76–84. https://doi.org/10.1007/s10787-008-8014-y

Sesso HD, Buring JE, Christen WG, Kurth T, Belanger C, MacFadyen J, Bubes V, Manson JE, Glynn RJ, Gaziano JM. 2008. Vitamins E and C in the prevention of cardiovascular disease in men: The Physicians' Health Study II randomized controlled trial. *JAMA* **300** (18), 2123-2133. https://doi.org/10.1001/jama.2008.600

Shin JA, Lee JH, Lim SY, Ha HS, Kwon HS, Park YM, Lee WC, Kang MI, Yim HW, Yoon KH, Son HY. 2013. Metabolic syndrome as a predictor of type 2 diabetes, and its clinical interpretations and usefulness. *J. Diabetes Investig*. **4** (4), 334-

43. https://doi.org/10.1111/jdi.12075

Summerhill VI, Grechko AV, Yet SF, Sobenin IA, Orekhov AN. 2019. The atherogenic role of circulating modified lipids in atherosclerosis. *Int. J. Mol. Sci*. **20**, 20 (14), E3561. https://doi.org/10.3390/ijms20143561

Takeda R, Koike T, Taniguchi I, Tanaka K. 2013. Double-blind placebo-controlled trial of hydroxytyrosol of *Olea europaea* on pain in gonarthrosis. *Phytomedicine* **20** (10), 861-864. https://doi.org/10.1016/j.phymed.2013.03.021

Tang PC, Ng YF, Ho S, Gyda M, Chan SW. 2014. Resveratrol and cardiovascular health-promising therapeutic or hopeless illusion? *Pharmacol. Res*. **90,** 88-115. https://doi.org/10.1016/j.phrs.2014.08.001

Torre R de la. 2008. Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacol*. 16 (5), 245-247. https://doi.org/10.1007/s10787-008-8029-4

Venturini D, Simão AN, Urbano MR, Dichi I. 2015. Effects of extra virgin olive oil and fish oil on lipid profile and oxidative stress in patients with metabolic syndrome. *Nutrition* **31** (6), 834-40. https://doi.org/10.1016/j.nut.2014.12.016

Visioli F, Bellomo G, Galli C. 1998. Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun*. **247,** 60-64.

Visioli F, Caruso D, Grande S, Bosisio R, Villa M, Galli G, Sirtori C, Galli C. 2005. Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. *Eur. J. Nutr.* **44** (2), 121-7.

Visioli F. 2014. The resveratrol fiasco. *Pharmacol. Res*. **90,** 87. https://doi.org/10.1016/j.phrs.2014.08.003

Visioli F, Davalos A, López de Las Hazas MC, Crespo MC, Tomé-Carneiro J. 2020. An overview of the pharmacology of olive oil and its active ingredients. *Br. J. Pharmacol*. **177** (6), 1316-1330. https://doi.org/10.1111/bph.14782

Weinbrenner T, Fitó M, de la Torre R, Saez GT, Rijken P, Tormos C, Coolen S, Albaladejo MF, Abanades S, Schroder H, Marrugat J, Covas MI. 2004. Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men. *J. Nutr.* **134** (9), 2314-2321.

Woolf SH, Battista RN, Anderson GM, Logan AG, Wang E. 1990. Assessing the clinical effectiveness of preventive maneuvers: analytic principles and systematic methods in reviewing evidence and developing clinical practice recommendations. A report by the Canadian Task Force on the Periodic Health Examination. *J. Clin. Epidemiol.* **43,** 891-905.

GRASAS Y ACEITES 72 (2)
April-June 2021, e405
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0220201

Application of mixed starter culture for table olive production

Z.Ş. Erdemir Tıraş[a] and H. Kalkan Yıldırım[b,] ✉

[a]Olive Research Institute, 35100, Izmir, Turkey

[b]Ege University, Engineering Faculty, Department of Food Engineering, 35100, Izmir, Turkey
✉Corresponding author: hatice.kalkan.yildirim@ege.edu.tr

Submitted: 24 February 2020; Accepted: 19 April 2020; Published online: 07 June 2021

SUMMARY: The fermentation of olives is usually carried out spontaneously by natural microbiota. Spontaneous fermentation has some disadvantages, such as the formation of defects in the end product due to the activities of undesirable microorganisms. The use of starter cultures could be a promising option to provide a more controlled fermentation environment and to reduce the risk of spoilage. Mixed starter culture use (generally selected *Lactobacillus* strains with or without yeasts) could reduce pH in a shorter time, producing a higher amount of lactic acid and enhancing microbial safety compared to fermentation with starter cultures containing single species or natural fermentation. Their use could also enhance the organoleptical properties of table olives. Particularly the use of yeast (such as strains of *W. anomolus*, *S. cerevisiae)* in the fermentation of olives, in combination or sequentially with lactic acid bacteria could result in an increase in volatile compounds and a more aromatic final product.

KEYWORDS*: Controlled fermentation; Mixed starter cultures; Table olive*

RESUMEN: ***Aplicación de un cultivo iniciador mixto para la producción de aceituna de mesa.*** La fermentación de la aceituna generalmente se lleva a cabo espontáneamente por la microbiota natural. Sin embargo, la fermentación espontánea tiene algunas desventajas, como la formación de defectos en el producto final debido a las actividades de microorganismos indeseables. El uso de cultivos iniciadores podría ofrecerse como una opción importante para proporcionar un entorno de fermentación más controlado y reducir el riesgo de deterioro. El uso de cultivos mixtos iniciadores (cepas generalmente seleccionadas de *Lactobacillu*s con/sin levaduras) podría reducir el pH en un tiempo más corto, produciendo una mayor cantidad de ácido láctico y mejorando la seguridad microbiana, en comparación con la fermentación con cultivos iniciadores que contienen especies individuales o fermentación natural. Su uso también podría mejorar las propiedades organolépticas de las aceitunas de mesa. En particular, el uso de la levadura (como las cepas de *W. anomolus*, *S. cerevisiae*) en la fermentación de aceitunas, en combinación o secuencialmente con bacterias de ácido láctico podría dar lugar a un aumento de los compuestos volátiles y a la obtención de un producto final más aromático.

PALABRAS CLAVE: *Aceituna de mesa; Cultivos iniciadores mixtos; Fermentación controlada*

Citation/Cómo citar este artículo: Erdemir Tıraş ZŞ, Kalkan Yıldırım H. 2021. Application of mixed starter culture for table olive production. *Grasas Aceites* **72** (2), e405. https://doi.org/10.3989/gya.0220201

1. INTRODUCTION

Table olives are regarded as one of the oldest and most popular fermented foods in the world. Due to their rich nutritional components such as monounsaturated fatty acids, antioxidants (e.g α-tocopherol) and bioactive compounds (e.g. phenolic substances), table olives are a significant part of the diet and promise health benefits for consumers (Aktan *et al*., 1999; Sakouhi *et al.*, 2008; Malheiro *et al*., 2012).

According to the Trade Standard Applying to Table Olives of International Olive Oil Council, table olives are defined as "the sound fruit of varieties of the cultivated olive trees (*Olea europea* L.), which are chosen for their production of olives when their volume, shape, flesh to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing; treated to remove their bitterness and preserved by natural fermentation; or by heat treatment, with or without the addition of preservatives; packed with or without covering liquid." (IOOC, 2004).

Olive fruits are not suitable food products for direct consumption due to phenolic compounds, particularly oleuropein, which make the fruit taste bitter. For this reason, olives should be processed first in order to hydrolyze oleuropein and remove this bitter taste (Değirmencioğlu, 2016). The most well-known processing methods used in the world are Spanish style, Californian style and natural processing. In Spanish style, olives are immersed in an alkali (NaOH) solution to remove bitterness by chemical hydrolysis of oleuropein (Figure 1) and subjected to fermentation in brine (Johnson *et al*., 2018). Californian style includes a lye treatment, and the solution is ventilated with the aim of obtaining a dark color through oxidation. In this technique, there is no fermentation step and the olives are preserved by sterilization (Charoenprasert *et al*., 2014). Natural processed olives are not treated with alkali and are directly fermented into brine (Johnson *et al*., 2018). Greek style is also a natural processing technique with air in the fermentation step for color improvement (Boskou, 2006). In natural processing, the removal of bitterness is carried out by the diffusion of oleuropein from fruit to brine and the occurrence of non-bitter compounds through enzymatic hydrolysis (Figure 1), namely with the beta-glucosidase and esterase activity found in olives, as well as enzymatic activities of natural microbiota (Ozdemir *et al*., 2014).

The fermentation of table olives is generally an artisanal process, but the inconsistent end product and spoilage risks exist under these uncontrolled conditions. Starter cultures could be an option for a more controlled fermentation process and a high-quality product (Bonatsou *et al*., 2017). The use of mixed culture in olive production is relatively new and could contribute further to the beneficial effects expected from the use of starter culture. The aim of this review is to present an overview to the studies in which combined cultures were used in table olive production.

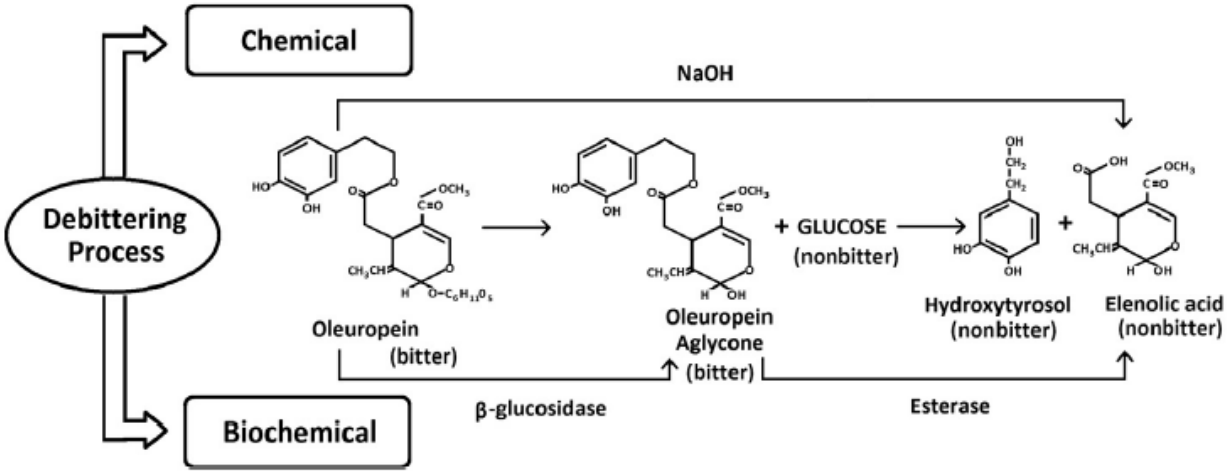


Figure 1. Chemical and biochemical hydrolysis of oleuropein (Boskou *et al.*, 2015)

2. SPONTANEOUS FERMENTATION OF TABLE OLIVES

The use of starter cultures is not a common practice in table olive fermentation. The endogenous microbiota of the olive carries out the fermentation process (Heperkan, 2013). The olive microbiota is variable depending upon the processing methods and olive cultivar. In addition, other variables, such as temperature, salt, pH or geographical zone may affect the microbiota in the olives (Corsetti *et al*., 2012). Lactic acid bacteria (LAB) and yeasts are the key actors to performing this process (Heperkan, 2013; Tufariello *et al*., 2016; Bonatsou *et al*., 2017).

The fermentation stages of table olives could be divided into three time periods. In the first days of fermentation, the pH level is high, with the value range of 6 – 11, and *Enterobacteriaceae* could be the most predominant microbial group. At the same time, some Gram-positive bacteria such as *Leuconostoc*, *Pediococcus* or *Bacillus* spp. are generally present. This step is crucial for ensuring the reduction in pH level, because if not, the fermentation quality could be affected negatively due to the growth of undesired bacteria which could cause adverse organoleptic properties (Bevilacqua *et al*., 2015).

After this phase, a reduction in the pH level and "primary fermentation" begin. LAB ferment the substrates and produce lactic acid; thus pH reduction and acidification are provided. *Lactobacillus* species play a major role due to their homofermentative metabolism and high acid production capacity. As the pH reduces to 5, *L. plantarum* and *L. pentosus* predominate. In this way, they also provide microbiological stability by eliminating undesired bacteria through the production of some antimicrobial compounds such as lactic acid, hydrogen peroxide and bacteriocins. The number of Gram-negative bacteria is reduced due to their sensitivity to such an acidic environment. In the last step, which is called as "secondary fermentation", *L. plantarum* becomes dominant and reduces the pH level below 5 (Hurtado *et al*., 2012, Bevilacqua *et al*., 2015). Yeast species, especially some strains of *Candida*, *Pichia* and *Saccharomyces* could also contribute to the flavor formation in table olives by producing some components like glycerol, ethanol, esters, organic acids and aldehydes. Additionally, esterase and lipase activities of the yeast species could increase the free fatty acid content in olives, which in turn could provide the formation of important components for aroma development, such as propanol and 2-butanol. The contribution of yeasts to the fermentation process could also be the degradation of phenolics with their beta-glucosidase activity (Arroyo-López *et al*., 2008; Bevilacqua *et al*., 2013; Bonatsou *et al*., 2015).

Spontaneous fermentation has many drawbacks since it is uncontrollable and unpredicted outcomes can occur. Spoilage microorganisms could grow and cause a final product with undesired quality (Tufariello *et al*., 2016). For example, in the first stage of the fermentation, if adequate acid levels cannot be reached, *Enterobacteriaceae* (can grow pH 4.4-9.0) and some other Gram-negative bacteria could grow in high numbers. They could exhaust sugar and produce CO_2, causing the formation of gas pockets, which end up softening the olives. Moreover, if pH levels remain higher than necessary (i.e. 4.2), *Clostridium butyricum* could bring about butyric and putric fermentation which cause the product to have an unpleasant smell and can crack the fruits (Lanza, 2013). The softening of olive tissue could also stem from the activity of pectolytic yeasts and moulds, which are able to degrade pectic compounds (Arroyo-Lopez, 2012b). When lactic acid fermentation is complete, the pH level should be lower than 4 to ensure microbiological safety if the product will not be pasteurized. Unless acidity and salt contents (should be over 8%) are sufficient, zapatera spoilage could occur due to the actions of *Clostridium* and *Propionibacterium*. This is characterized by the production of malodorous organic acids which make the product inconsumable (García *et al*., 2004). Alcohol fermentation by yeasts and the resultant carbon dioxide, ethanol and organic acids can also cause sensory abnormalities such as the formation of a winey-vinegar taste in olives (Lanza, 2013). The use of starter culture is proposed in order to minimize these problems, which could occur during spontaneous fermentation.

3. STARTER CULTURE USE IN TABLE OLIVE PRODUCTION

Starter cultures can be defined as preparations that include microorganisms with desirable metabolic characteristics for the fermentation environment (Heperkan, 2013). The aims that could be achieved with the use of starter cultures can be listed as follows (Bonatsou *et al*., 2017):

- obtaining a more controlled fermentation environment
- increasing the initial number of desired microorganisms
- accelerating the de-bittering process
- enhancing the organoleptical properties of the final product
- reducing the risk of spoilage and pathogen growth
- preserving/improving the healthy and nutritional characteristics of the product
- obtaining a final product with extended shelf-life
- providing the food with a functional property, such as probiotic characteristics

Microorganism should have some desired characteristics in order to be selected as starter culture in table olive fermentations. They should degrade phenolic substances to some extent with their beta-glucosidase and esterase activity, grow in high numbers (10^6-10^7 cfu/ml) and predominate in the fermentation environment. They should not be sensitive to high salt concentrations (8-10%), acidity and phenolics and produce lactic acid in high amounts. They also should have low nutrient requirement and grow at low temperatures. If they are used in commercial application, they should survive in frozen forms (Bevilacqua *et al*., 2015).

Although Spanish style table olives are normally fermented between 60-120 days, the fermentation of directly brined olives takes much longer, 8-12 months in general, when it is carried out spontaneously. Such a long fermentation period retards the introduction of the product into the market and this is undesirable for manufacturers (Tufariello *et al*., 2016). Due to their technological superiority, starter cultures could easily colonize into the fermentation medium by eliminating indigenous microbiota, lower the pH at desired levels in a shorter time and shorten the time that is required for de-bittering (Corsetti *et al*., 2012).

The use of a starter culture could also be regarded as an alternative to NaOH in olive de-bittering (Tufariello *et al*., 2016). In Spanish style olive processing, when NaOH is used to remove oleuropein from olives, a considerable amount of phenols is removed, which means a high level of nutritional loss. Additionally, large amounts of water are wasted, which is hazardous for the environment. The use of starter cultures which exert beta-glucosidase activity to hydrolyze oleuropein could diminish the need for alkali treatment and provide an advantage from nutritional and environmental perspectives (Chranioti *et al*., 2018).

The most commonly used starter cultures for table olive production are lactic acid bacteria (Table 1) (Campus *et al*., 2018).

Yeasts could have dual effects on table olive processing. Firstly, they could spoil the product. Zaragoza *et al*., (2017) stated that the use of pectolytic *S. cerevisiae* UCDFST 09-448 caused softening and spoilage to Sicilian style green olives. However, the strains of some yeast species could affect olive processing positively and could be chosen as starters (Table 2). *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, *C. diddensiae*, *Pichia galeiformis*, *P. membranifaciens* and *Kluyveromyces lactis* are the most emphasized yeasts in this respect (Arroyo-Lopez *et al*., 2012a). In a recent study, Ciafardini *et al.,* (2019) revealed the good performance of *C. diddensiae*, *C. adriatica* and *W. anomalus* in the fermentation of Taggiasca black olives, particularly when the olives are brined with citric acid and 12% salt concentration. Schaide *et al*.,

Table 1. The most common used LAB starters in the fermentation of olives (adapted from: Campus *et al*., 2018)

Most frequently used	Less frequently used	Rarely used
Lactobacillus plantarum	*Lactobacillus paraplantarum*	*Enterococcus casseliflavus*
Lactobacillus pentosus	*Lactobacillus casei*	
	Lactobacillus brevis	
	Lactobacillus paracasei	
	Leuconostoc mesenteroides	
	Pediococcus acidilactici *Leuconostoc cremoris*	

Table 2. The negative and positive impacts of yeasts on the fermentation of olives (adapted from: Arroyo-López *et al*., 2012b)

Negative effects	Positive effects
Off odors/flavors	The formation of volatile compounds that are important for flavor enhancement (with lipase and esterase activity)
CO_2 production and so blister formation on olive	Biodegredation of phenolic substances with their beta-glucosidase activity
Clouding of brines	Toxic protein production that can be effective in inhibiting fungi and harmful yeasts
Fruit softening due to enzymes (proteases, xylanases and pectinases)	Promoting the growth of LAB by synthesizing substrates

(2019) used *S. cerevisiae* in combination with olive leaf extract in the Spanish style fermentation of olives of the Carrasqueña cultivar. The authors indicated that the use of the yeast strain could have contributed to the sensorial quality of the final product. Some yeast species are also reported to possess probiotic characteristics (Tufariello *et al*., 2016).

Along with the safety and quality of the products, consumers also care about their usual, intrinsic and traditional sensory characteristics. Preserving the desired quality properties of olives by using commercial starters is not easy. Hence, selecting microorganisms from their own fermentation environment and using the ones with technological properties as starters could be a better approach. These selected autochthonous starters could be more successful at driving fermentation in comparison to the commercial starters because they could easily adapt to the environment and dominate (Campus *et al*., 2018). To isolate and characterize LAB and yeasts, culture-dependent methods are applied and culture-independent methods can be used for identification (Heperkan, 2013). Culture-dependent methods are the conventional microbiological methods where molecular techniques are applied; however, the genetic material is directly extracted from the food matrix while applying the culture-independent methods. These methods should be applied attentively in order to select the most proper strains as starters in the production of table olives (Botta *et al*., 2012).

Papadelli *et al*., (2015) used autochthonous *Leuconostoc mesenteroides* subsp. *mesenteroides* Lm139 and *Lactobacillus pentosus* DSM 16366 separately as starters in the natural fermentation of black olives of the Kalamon cultivar. They reported a faster acidification, successful lactic acid fermentation, inhibition of *Enterobacteriaceae* in a shorter time and a more controlled fermentation environment in conditions with a lower level of salt, through the use of starters in comparison to spontaneous fermentation. Panagou *et al*., (2008) evaluated a commercial *L. pentosus* starter and a *L. plantarum* strain isolated from cassava for the fermentation of the Conservolea black olives. The starter inoculation accelerated the fermentation process, provided a rapid pH reduction and decreased the number of Gram-negative bacteria, which lowered the risk of spoilage. The authors observed that *L. pentosus* performed better than *L. plantarum*, probably due to the different origin of the latter. It was proposed by authors that the use of starter culture could contribute to quality and produce a safe product with desirable organoleptic properties. A similar study was carried out more recently by using autochthonous *L. pentosus* B281 in Greek style processing of the same olive cultivar. The use of the starter increased the physiochemical and sensory quality of the final product (Grounta *et al*., 2016). Marsilio *et al*., (2005) used a *L. plantarum* strain which is able to degrade oleuropein and observed that the de-bittering time was reduced; the acidification and good flavor in the fermentation of the Ascolana tenera cultivar green olives were increased. The authors indicated that the panellists had a less bitter and more aromatic taste with the starter.

The use of probiotic strains could also make table olives have a functional property in addition to its nutritional value (Bonatsou *et al*., 2017). Through the use of a human origin probiotic strain, *L. paracasei* IMPC2.1, De Bellis *et al*., (2010) paved the way for producing a new functional food. Besides being a starter culture, the inoculated strain is colonized on the surface and survived in Bella di Cerignola green table olives. Some strains of *L. pentosus*, *L. plantarum* and *L. paracasei* subsp. *paracasei* were

isolated from table olives and reported to show probiotic activity along with the starter culture properties (Argyri *et al*., 2013).

4. MIXED STARTER CULTURE USE IN TABLE OLIVE PRODUCTION

The use of starter cultures with desired characteristics is advantageous in order to obtain products with consistent and enhanced qualities. The mixed started culture is used more commonly in the dairy, meat or cereal industries; however, it is less widely used for the production of table olives (Di Cagno *et al*., 2008). A mixed starter culture may comprise the same microbial group, such as bacteria or a combination of different groups, such as bacteria and yeasts. In fact, most of the spontaneous food fermentations are based on these kinds of combinations and create a product with desirable properties (Hesseltine, 1992; Adebo *et al*., 2018).

The use of mixed culture starters could have positive effects compared to the use of a single strain. For instance, different metabolic pathways could be used by strains due to the synergistic interactions and multiple transformations of substrates. Microorganisms could also adapt better to the fermentation environment by means of these enhanced metabolic processes (Adebo *et al*., 2018). Such a complex microflora could make the microorganisms more versatile and more robust. There are two main reasons for this situation. Firstly, the microorganisms can interact with each other via a mechanism called quorum sensing (QS). The molecular signals and metabolites could be transferred between the members of microbiota through the QS. Secondly, the metabolic activities that need to be carried out are shared between microorganisms and then merged, which provides a more productive and overall yield (Smid *et al*., 2013).

The products could lose their unique organoleptic properties and become plain when fermented by a single strain starter. The reason for this could be a decreased microbial flora in the fermentation environment. Mixed cultures could provide improved organoleptic characteristics due to rich biodiversity. They could also contribute to the acidification rate, reduce fermentation time and improve functionality and nutritional quality (Table 3) (Adebo *et al*., 2018). In olive fermentation, more than one starter culture can be used either sequentially or simultaneously in a combined form.

As LAB, particularly *Lactobacillus* are the most used starter cultures, there are studies in which the starter culture combinations are formed with the selected *Lactobacillus* strains. Ruiz-Barba *et al.,* (2012) used two selected *L. pentosus* starter strains in the Spanish style fermentation of olives. As compared to the uninoculated samples, the paired starter combination served to reduce the pH level quickly and obtain a higher amount of lactic acid at the end of the fermentation. Starters adapted to the fermentation environment by overwhelming the natural flora. The authors reported that in this regard, the combined starters were more efficient than a single strain starter which they used previously for fermentation. Some of their abilities such as bacteriocin production and survival at high pH levels, which is particularly important for the Spanish style processing, were important for managing the fermentation. Perpetuini *et al*., (2018) combined two *L. pentosus* strains (C8 and C11) and used this for the Greek style processing of Itrana cultivar olives. LAB growth and the pH reduction was quicker in inoculated samples compared to uninoculated ones. A complete disappearance of the oleuropein concentration was observed only in inoculated olives after 30 days of fermentation, which shows the high ability of the combined starter in the de-bittering of olives. In a similar study, an undefined mixture of *L. pentosus* strains decreased the processing time of Tonda di Cagliari cultivar olives by 3 months compared to spontaneous fermentation and suppressed the growth of spoilage bacteria (Campus *et al*., 2017). Chranioti *et al*., (2018) compared the effects of a commercial starter and a mix of *L. plantarum* strains originating from olives in the fermentation of Conservolea olives, which are processed with a natural fermentation or in accordance with the Spanish style. Regardless of the production type, the number of lactic acid bacteria was higher in the fermentation with mixed starters compared to spontaneous fermentation and the commercial starter. The mixed culture sped up and controlled the fermentation and contributed to obtaining a safe end product with desirable organoleptic characteristics. It was observed that phenolic compounds in the olive flesh diminished during processing, especially with the alkali treatment. The mixed starter use without alkali was advantageous at this point because of the smallest decrease in the phenolic components

and the highest antioxidant capacity which were obtained in this way. Bitterness was also reduced in the sensory analysis with the mixed starter. Similarly, Campus *et al.,* (2015) fermented Tonda di Cagliari cultivar olives with autochthonous *L. plantarum* and a combination of *L. pentosus* strains. Along with the good microbiological quality, the hydroxytyrosol was more abundant in inoculated olives when compared to spontaneous fermentation due to the enhanced enzymatic activity executed by the starters. It is a desired feature because hydroxytyrosol is one of the most significant bioactive compounds with antioxidant activity in olives. The antioxidant capacity of the mixed culture fermented samples was also higher than that of single-strain samples during processing. In addition, a more firm and elastic olive

Table 3. Improved features of table olives fermented with mixed culture starter cultures

Olive cultivar	Process conditions	Starter culture combinations	Enhanced beneficial effect	Reference
Nocellara del Belice	Spanish style	*L. pentosus* OM13-*L. coryniformis* OM68	Improved sensory properties	Aponte *et al*., (2012)
Tonda di Cagliari	Directly brined	Undefined mixed culture of *L. pentosus*	Better adaptation to the fermentation environment	Comunian *et al.,* (2017)
Bella di Cerignola	Spanish style	Probiotic *L. plantarum* c10- *L. plantarum* c16-*L. plantarum* c19 with 0.5% glucose	pH reduction to a safe level (4.3-4.5), control of yeast growth, probiotic characteristic	Perricone *et al*., (2010)
Gemlik	Gemlik method with low (7%) salt concentration	*L. brevis*-*Leuconostoc cremoris*; *L. brevis*-*L. paramesenteroides*; *L. brevis*- *Leuconostoc cremoris*- *L. paramesenteroides*	Higher acidity, control of yeast growth (when *L. cremoris* is present in the mixture)	Kumral *et al*., (2009)
Kalamata Chaldikis	Alkali treatment, fermentation in low salt brine (2.3% NaCl, 32.3 Mm Ca-acetate, 33.9 Mm Ca-lactate and 4% NaCl)	*L. plantarum* Lp 15-Lp 20-Lp 28-Lp 40-Lp 48	Reduction of the fermentation time, decrease in the risk of *Enterobacteriaceae* spoilage, increase in hydroxytyrosol and tyrosol formation, inactivation of *E. coli* O157 EDL-932 and *L. monocytogenes* ScottA more than 6 logs within ≤ 24 hour	Tataridou *et al*., (2015)
Nocellara Etnea	Sicilian style without alkali	*L. plantarum* UT2.1-*L. paracasei* N24- *L. pentosus* TH969; *L. paracasei* N24- *L. pentosus* TH969; *L. plantarum* UT2.1- *L. pentosus* TH969; *L. plantarum* UT2.1- *L. paracasei* N24	Faster acidification, reaching a high biodiversity that positively correlates with ester compounds which give fruity and floral aromas; preventing *Enterobacteriaceae* growth at the end of fermentation	Randozzo *et al*., (2017), Randazzo *et al*., (2018)
Nocellara Etnea	Directly brined	*L. plantarum* UT 2.1-probiotic L.paracasei N24	Accelerating fermentation process, higher reduction of pH, inhibiting *Enterobacteriaceae* growth, potential probiotic characteristics due to high survival rate of the probiotic strain	Pino *et al*., (2018)
Kalamata Conservolea	Greek style	*Leuconostoc mesenteroides* K T5-1- *S. cerevisiae* KI 30-16; *L. plantarum* A 135–5-*Debaryomyces hansenii* A 15-44	Increasing the amount of hydroxytyrosol and tyrosol with sequential inoculation (first yeast, then LAB), enhanced antioxidant content Most aromatic and acceptable Kalamata olives with co-inoculation of LAB and yeast	Chytiri *et al*., (2019)
Bella di Cerignola	Greek style	Commercial *L. plantarum*-*W. anomalus* DiSSPA73 (SY); commercial *L. plantarum*-*W. anomalus* DiSSPA73-*L. plantarum* DiSSPA1A7-*L. pentosus* DiSSPA7(SYL)	Sweeter taste perception and the highest sensory appreciation for SYL; increase in some phenolic and volatile compounds for SY and SYL	De Angelis *et al*., (2015)

structure was determined for the combined starter use. The authors concluded that the use of mixed autochthonous *L. pentosus* strains could be a cheaper alternative to the commercial starter cultures for industrial use.

While forming the starter culture mixtures, the use of yeast/LAB communities has drawn attention in studies. As stated above, the use of yeast as an adjunct culture could contribute to the survival of LAB and supress the growth of spoilage bacteria and wild yeasts (Hurtado *et al*., 2012). Tsapatsaris *et al.,* (2004) inoculated *Debaryomyces hansenii* to the brine 24 and 48 hours before *L. plantarum,* and in the latter case the growth rate of *L. plantarum* reached its maximum level. This could be associated with the formation of substances which are essential for the growth of *L. plantarum*, such as vitamins. In a similar study, *Saccharomyces cerevisiae* enhanced the growth of *L. pentosus* in the fermentation of green olives (Segovia-Bravo *et al*., 2007). However, Pistarino *et al.,* (2013) did not observe a statistical difference when they used *L. plantarum* and *S. cerevisiae* together for the fermentation of the Taggiasca black olives. Hurtado *et al*., (2010) studied the use of *C. diddensiae* C6B19, *L. plantarum* V10A2 and *L. pentosus* FXMA1, either alone or in combination with *L. pentosus* 5E3A18 in natural Arbequina table olive fermentation. Microbial quality was enhanced with mixed inoculations when compared to single strain use. This effect was more notable when the yeast strain and *L. pentosus* 5E3A18 were used together. The presence of the yeast strain, alone or in combination with LAB, created a remarkable decrease in the *Enterobacteriacea* population. The authors advised that *C. diddensiae* C6B19 could be a promising adjuvant starter which could be effective against unwanted wild yeast and pathogen bacteria. De Castro *et al*., (2002) tried both the simultaneous and sequential use of *Enterococcus casseliflavus* and *L. pentosus* in green olive fermentation and got better results in terms of LAB growth when *L. pentosus* was inoculated 24 hours later than the yeast. De Angelis *et al*., (2015) observed a rapid and consistent fermentation process with a combined inoculation of some *Lactobacillus* strains and *Wickeramomyces anomalus* during the fermentation of the Bella di Cerignola olives.

Yeasts can produce glycoproteins or other toxic proteins which could reduce the need for chemical preservatives, and thus less salty and natural final products could be obtained with the use of yeasts in starter culture mixtures (Arroyo-López, 2012a). Psani *et al.,* (2006) determined the killer activity of some strains of *Debaryomyces hansenii* and *Torulaspora delbrueckii* isolated from fermented black olives against some pathogen bacteria/wild yeasts. They revealed their potential use as adjunct cultures for enhancing the quality of the product.

The organoleptic characteristics of table olives are created with the joint contribution of LAB and yeasts in fermentation. Through the production of ethanol, glycerol, organic acids, esters, aldehydes and free fatty acids, desired aroma and flavor are formed in the olives (Sabatini *et al*., 2008; Tufariello *et al.*, 2016; Campus *et al*., 2018). One of the main purposes of creating a mixed starter combination is increasing the organoleptical properties and especially aroma (Benitez-Cabello *et al*., 2019). Tufariello *et al*., (2015) used two Italian (Cellina di Nardo and Leccino) and two Greek olive cultivars (Kalamata and Conservolea) and inoculated them with one yeast starter and 63 days later one LAB starter. *S. cerevisiae*/*L. plantarum* for Leccino, *P. anomala*/*L. plantarum* for Cellina di Nardò, *S. cerevisiae* and *L. mesenteroides* for Kalamàta, *D. hansenii* and *L. plantarum* for Conservolea were used. As for the inoculated samples, total organic acid (especially lactic and acetic acids) levels were higher or comparable to those of natural fermentations. In the first month of the fermentation aldehydes (herbaceous flavor) were determined markedly, however terpens and higher alcohols became more prominent in the second month. The authors attributed this situation to yeast activity. In the last part of the fermentation esters (fruity notes) were abundant possibly because of enzymes by LAB. The authors emphasized that this sequential inoculation technology enhanced the organoleptic properties of olives and decreased the fermentation time by 9 months. Pino *et al*., (2019) used *L. plantarum* F3.3 and *L. paracasei* N24, a potentially probiotic strain after 60 days in the fermentation of Sicilian table olives. They observed a significant increase in volatile compounds, especially for floral and fruity notes with the inoculation of the probiotic strain. Benitez-Cabello *et al*., (2019) evaluated the volatile compound profile of Spanish style Manzanilla olives. Two *L. pentosus*, one *L. plantarum* and a yeast strain, *W. anomalus,* were used either separately

or all together. The most significant result of the study was enhanced volatile compound formation when the yeast existed. Therefore, the authors suggested that creating an inoculum that included yeasts could be promising in order to obtain a more aromatic end product. Grounta *et al*., (2016) obtained a less acidic taste for Conservolea black olives with co-inoculation of *L. pentosus* B281 and *P. membranifaciens* M3A, which was more appreciated in the sensory analysis.

Probiotic strain use could enhance the functionality of table olives and produce a final product with positive health benefits. The colonization capacity and formation of biofilms by LAB on the olive surface are significant characteristics in this regard (Argyri *et al*., 2014). The probiotics used survived during the mixed culture fermentation of Giarraffa and Grossa di Spagna table olives with the selected *Lactobacillus* strains (Randazzo *et al*., 2014). Blana *et al*., (2014) combined potential probiotic *L. pentosus* B281 and *L. plantarum* B282 for the production of Halkidiki cultivar table olives according to the Spanish style. The olives were colonized by *L. pentosus* B281 rather than *L. plantarum* B282. *L. plantarum* B282 could not colonize the olive surface when 10% salt concentration was used for brining although it survived in a concentration over 80% when the NaCl level was 8%. Similarly, Pino *et al*., (2019) reported a better survival of a potentially probiotic strain (*L. paracasei* N24) at 5% NaCl concentration in comparison to 8% NaCl when it was sequentially inoculated with a beta-glucosidase positive LAB (*L. plantarum* F3.3). The authors suggested that this could be a beneficial approach to obtaining functional and less salty olives.

When used together, LAB and yeasts can also form stable biofilms on the olives' surface. As the biofilms are ingested by consumers, the technological characteristics of the starters and synergy among them gain more importance (Grounta *et al*., 2014). The co-presence of yeasts and LAB in the biofilm shows that the mixed use of yeasts and LAB could be a good approach, especially when it comes to carrying probiotics to table olives. The mixed biofilm formation could increase their survival rate when they pass through the gastrointestinal tract (Arroyo-López *et al*., 2012b). Grounta *et al.,* (2014) reported that *L. pentosus* B281 and *P. membranifaciens* M3A could colonize in Halkidiki table olives processed according to the Californian style in high numbers in most of the different conditions of brining. Authors also indicated that *P. membranifaciens* increased the growth rate and colonization capacity of *L. pentosus* B281. In a similar study, although *L. pentosus* was quite successful in forming biofilm on the surface of Conservolea black olives, *P. membranifaciens* M3A could not be found in biofilms at the end of fermentation. However, it was reported that the yeast strain provided a milder taste to the product, which could be appreciated by consumers who do not prefer acidic flavors (Grounta *et al*., 2016).

5. CONCLUSION AND FUTURE PROSPECTS

The table olive is a valuable fermented food product due to its nutritional properties, and starter culture technology is a significant biotechnological approach for its processing. With the use of starters, a low-cost, shorter and more controlled fermentation process and a final product with increased shelf-life and enhanced organoleptic properties could be obtained (Campus *et al*., 2018; Bonatsou *et al*., 2017).

Food fermentations are generally carried out by a mixed microflora and they have a better ecological success in comparison to single strain fermentations (Sieuwerts *et al*., 2008). By simulating this, mixed starter cultures, sequentially or in combination, could be used and this approach could produce a higher quality, safer product. The results of the research have indicated that mixed culture fermentation has some important advantages in comparison to the single strain and natural fermentations in table olive production. In order to better understand the interactions between the microflora and the progress of the fermentation, and to determine its effects on the final product, more studies should be done in this field.

It has been emphasized that selecting functional starter cultures from their natural environment and using them is a better approach in comparison to using starters originating from different sources. Thus, metabolic activities in the fermentation environment could be enhanced (Chranioti *et al*., 2018). Therefore, the selection of new strains from olives/fermentation environments and the creation of suitable combinations between microorganisms in order to use them in table olive production would be more prominent in the future. Furthermore, in this way, in accordance with consumer demand, instead of producing a plain, uniform product, traditional

flavors could be preserved and transferred to the final product. Healthy, functional table olives would also be developed by the use of starter cultures with probiotic or other features, such as vitamin production (Bevilacqua *et al*., 2015).

The progress in genetic and genomic tools would allow for revealing functional and new mechanisms in order to comprehend the interactions in mixed cultures and their behaviors. New cultivation methods and innovative techniques, such as immobilization could be exploited to develop and disseminate the use of mixed culture starters (Smid *et al*., 2013). These approaches could be implemented in table olive production by moving this process to a further point.

REFERENCES

Adebo OA, Njobeh PB, Adeboye AS, Adebiyi JA, Sobowale SS, Ogundele OM, Kayitesi E. 2018. Advances in fermentation technology for novel food products, in Panda SK and Shetty PH (Ed.) *Innovations in Technologies for Fermented Food and Beverage Industries,* Springer, Cham, pp. 71-87. https://doi.org/10.1007/978-3-319-74820-7_4

Aktan N, Kalkan H. 1999. *Sofralık Zeytin Teknolojisi.* Ege Üniversitesi Basimevi, Bornova, Izmir, Turkey.

Angelis M de, Campanella D, Cosmai L, Summo C, Rizzello CG, Caponio F. 2015. Microbiota and metabolome of un-started and started Greek-type fermentation of Bella di Cerignola table olives. *Food Microbiol*. **52**, 18-30. https://doi.org/10.1016/j.fm.2015.06.002

Aponte M, Blaiotta G, La Croce F, Mazzaglia A, Farina V, Settanni L, Moschetti G. 2012. Use of selected autochthonous lactic acid bacteria for Spanish-style table olive fermentation. *Food Microbiol*. **30**, 8-16. https://doi.org/10.1016/j.fm.2011.10.005

Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou EZ, Tassou CC. 2013. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiol*. **33**, 282-291. https://doi.org/10.1016/j.fm.2012.10.005

Argyri AA, Nisiotou AA, Mallouchos A, Panagou EZ, Tassou CC. 2014. Performance of two potential probiotic *Lactobacillus* strains from the olive microbiota as starters in the fermentation of heat shocked green olives. *Int. J. Food Microbiol*. **171**, 68-76. https://doi.org/10.1016/j.ijfoodmicro.2013.11.003

Arroyo-López FN, Querol A, Bautista-Gallego J, Garrido-Fernández A. 2008. Role of yeasts in table olive production. *Int. J. Food Microbiol*. **128**, 189-196. https://doi.org/10.1016/j.ijfoodmicro.2008.08.018

Arroyo-López F, Romero-Gil V, Bautista-Gallego J, Rodríguez-Gómez F, Jiménez-Díaz R, García-García P, Querol A, Garrido-Fernández A. 2012a. Potential benefits of the application of yeast starters in table olive processing. *Front. Microbiol*. **3**, 1-4. https://doi.org/10.3389/fmicb.2012.00161

Arroyo-López F, Romero-Gil V, Bautista-Gallego J, Rodríguez-Gómez F, Jiménez-Díaz R, García-García P, Querol A, Garrido-Fernández A. 2012b. Yeasts in table olive processing: desirable or spoilage microorganisms? *Int. J. Food Microbiol*. **160**, 42-49. https://doi.org/10.1016/j.ijfoodmicro.2012.08.003

Bellis P de, Valerio F, Sisto A, Lonigro SL, Lavermicocca P. 2010. Probiotic table olives: microbial populations adhering on olive surface in fermentation sets inoculated with the probiotic strain *Lactobacillus paracasei* IMPC2.1 in an industrial plant. *Int. J. Food Microbiol*. **140**, 6-13. https://doi.org/10.1016/j.ijfoodmicro.2010.02.024

Benítez-Cabello A, Rodríguez-Gómez F, Morales M, Garrido-Fernández A, Jiménez-Díaz R, Arroyo-López F. 2019. Lactic Acid Bacteria and Yeast Inocula Modulate the Volatile Profile of Spanish-Style Green Table Olive Fermentations. *Foods* **8**, 280. https://doi.org/10.3390/foods8080280

Bevilacqua A, Beneduce L, Sinigaglia M, Corbo MR. 2013. Selection of yeasts as starter cultures for table olives. *J. Food Sci*. **78**, 742-751. https://doi.org/10.1111/1750-3841.12117

Bevilacqua A, De Stefano F, Augello S, Pignatiello S, Sinigaglia M, Corbo M. 2015. Biotechnological innovations for table olives. *Int. J. Food Sci. Nutr*. **66**, 127-131. https://doi.org/10.3109/09637486.2014.959901

Blana VA, Grounta A, Tassou CC, Nychas GJE, Panagou, EZ. 2014. Inoculated fermentation of green olives with potential probiotic *Lactobacillus pentosus* and *Lactobacillus plantarum* starter cultures isolated from industrially fermented

olives. *Food Microbiol*. **38**, 208-218. https://doi.org/10.1016/j.fm.2013.09.007

Bonatsou S, Benítez A, Rodríguez-Gómez F, Panagou EZ, Arroyo-López, FN. 2015. Selection of yeasts with multifunctional features for application as starters in natural black table olive processing. *Food Microbiol.* **46**, 66-73. https://doi.org/10.1016/j.fm.2014.07.011

Bonatsou S, Tassou CC, Panagou EZ, Nychas GJE. 2017. Table olive fermentation using starter cultures with multifunctional potential. *Microorganisms* **5**, 30. https://doi.org/10.3390/microorganisms5020030

Boskou G, Salta FN, Chrysostomou S, Mylona A, Chiou A, Andrikopoulos, NK. 2006. Antioxidant capacity and phenolic profile of table olives from the Greek market. *Food Chem*. **94**, 558-564. https://doi.org/10.1016/j.foodchem.2004.12.005

Boskou D, Camposeo S, Clodoveo ML. 2015. Table olives as sources of bioactive compounds, in Boskou D (Ed.) *Olive and Olive Oil Bioactive Constituents*, AOCS Press, Urbana, pp. 217-259. https://doi.org/10.1016/B978-1-63067-041-2.50014-8

Botta C, Cocolin L. 2012. Microbial dynamics and biodiversity in table olive fermentation: culture-dependent and-independent approaches. *Front. Microbiol*. **3**, 245. https://doi.org/10.3389/fmicb.2012.00245

Cagno R di, Surico RF, Siragusa S, De Angelis M, Paradiso A, Minervini F, De Gara L, Gobbetti M. 2008. Selection and use of autochthonous mixed starter for lactic acid fermentation of carrots, French beans or marrows. *Int. J. Food Microbiol.* **127,** 220-228. https://doi.org/10.1016/j.ijfoodmicro.2008.07.010

Campus M, Sedda P, Cauli E, Piras F, Comunian R, Paba A, Daga E, Schirru S, Angioni A, Zurru R, Bandino G. 2015. Evaluation of a single strain starter culture, a selected inoculum enrichment, and natural microflora in the processing of Tonda di Cagliari natural table olives: Impact on chemical, microbiological, sensory and texture quality. *LWT-Food Sci. Technol*. **64**, 671-677. https://doi.org/10.1016/j.lwt.2015.06.019

Campus M, Cauli E, Scano E, Piras F, Comunian R, Paba A, Daga E, Di Salvo R, Sedda P, Angioni A, Zurru R. 2017. Towards controlled fermentation of table olives: lab starter driven process in an automatic pilot processing plant. *Food Bioproc. Tech*. **10**, 1063-1073. https://doi.org/10.1007/s11947-017-1882-7

Campus M, Değirmencioğlu N, Comunian R. 2018. Technologies and trends to improve table olive quality and safety. *Front. Microbiol*. **9**, 617. https://doi.org/10.3389/fmicb.2018.00617

Castro A de, Montaño A, Casado FJ, Sánchez AH, Rejano L. 2002. Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter cultures for Spanish-style green olive fermentation. *Food Microbiol*. **19**, 637-644. https://doi.org/10.1006/fmic.2002.0466

Charoenprasert S, Mitchell A. 2014. Influence of California-style black ripe olive processing on the formation of acrylamide. *J. Agric. Food Chem.* **62**, 8716-8721. https://doi.org/10.1021/jf5022829

Chranioti C, Kotzekidou P, Gerasopoulos D. 2018. Effect of starter cultures on fermentation of naturally and alkali-treated cv. Conservolea green olives. *LWT-Food Sci. Technol.* **89**, 403-408. https://doi.org/10.1016/j.lwt.2017.11.007

Chytiri A, Tasioula-Margari M, Bleve G, Kontogianni VG, Kallimanis A, Kontominas MG. 2019. Effect of different inoculation strategies of selected yeast and LAB cultures on Conservolea and Kalamàta table olives considering phenol content, texture, and sensory attributes. *J. Sci. Food Agric*. **100**, 926-935. https://doi.org/10.1002/jsfa.10019

Ciafardini G, Zullo BA. 2019. Use of selected yeast starter cultures in industrial-scale processing of brined Taggiasca black table olives. *Food Microbiol*. **84**, 103-250. https://doi.org/10.1016/j.fm.2019.103250

Comunian R, Ferrocino I, Paba A, Daga E, Campus M, Di Salvo R, Cauli E, Piras F, Zurru R, Cocolin L. 2017. Evolution of microbiota during spontaneous and inoculated Tonda di Cagliari table olives fermentation and impact on sensory characteristics. *LWT Food Sci. Technol.* **84**, 64-72. https://doi.org/10.1016/j.lwt.2017.05.039

Corsetti A, Perpetuini G, Schirone M, Tofalo R, Suzzi G. 2012. Application of starter cultures to table olive fermentation: an overview on the experimental studies. *Front. Microbiol*. **3**, 248. https://doi.org/10.3389/fmicb.2012.00248

Değirmencioğlu, N. 2016. Modern techniques in the production of table olives, in Boskou D and Clodoveo MK (Ed.) *Products from Olive Tree*, IntechOpen, pp. 215. https://doi.org/10.5772/64988

García PG, Barranco CR, Durán-Quintana MC, Fernández AG. 2004. Biogenic amine formation and "zapatera" spoilage of fermented green olives: effect of storage temperature and debittering process. *J. Food Prot*. **67**, 117-123. https://doi.org/10.4315/0362-028X-67.1.117

Grounta A, Panagou EZ. 2014. Mono and dual species biofilm formation between *Lactobacillus pentosus* and *Pichia membranifaciens* on the surface of black olives under different sterile brine conditions. *Ann. Microbiol*. **64**, 1757-1767. https://doi.org/10.1007/s13213-014-0820-4

Grounta A, Doulgeraki AI, Nychas GJE, Panagou EZ. 2016. Biofilm formation on Conservolea natural black olives during single and combined inoculation with a functional *Lactobacillus pentosus* starter culture. *Food Microbiol*. **56**, 35-44. https://doi.org/10.1016/j.fm.2015.12.002

Heperkan D. 2013. Microbiota of table olive fermentations and criteria of selection for their use as starters. *Front. Microbiol*. **4**, 143. https://doi.org/10.3389/fmicb.2013.00143

Hesseltine, CW. 1992. Mixed Culture Fermentations. Applications of Biotechnology in Traditional Fermented Foods. *Applications of Biotechnology in Traditional Fermented Foods,* National Academies Press, Washington, Available from: https://www.ncbi.nlm.nih.gov/books/NBK234678/

Hurtado A, Reguant C, Bordons A, Rozès N. 2010. Evaluation of a single and combined inoculation of a *Lactobacillus pentosus* starter for processing cv. Arbequina natural green olives. *Food Microbiol*. **27,** 731-740. https://doi.org/10.1016/j.fm.2010.03.006

Hurtado A, Reguant C, Bordons A, Rozès N. 2012. Lactic acid bacteria from fermented table olives. *Food Microbiol*. **31**, 1-8. https://doi.org/10.1016/j.fm.2012.01.006

IOOC 2004. Trade Standard Applying to Table Olives. International Olive Oil Council COI/T20/Doc No 1. Madrid: IOOC.

Kumral A, Basoglu F, Sahin I. 2009. Effect of the use of different lactic starters on the microbiological and physicochemical characteristics of naturally black table olives of Gemlik cultivar. *J. Food Process. Pres*. **33**, 651-664. https://doi.org/10.1111/j.1745-4549.2008.00303.x

Johnson RL, Mitchell AE. 2018. Reducing Phenolics Related to Bitterness in Table Olives. *J. Food Qual*. 1-12. https://doi.org/10.1155/2018/3193185

Lanza B. 2013. Abnormal fermentations in table-olive processing: microbial origin and sensory evaluation. *Front. Microbiol*. **4**, 1-7. https://doi.org/10.3389/fmicb.2013.00091

Malheiro R, Casal S, Sousa A, De Pinho PG, Peres AM, Dias LG, Bento A, Pereira J. 2012. Effect of cultivar on sensory characteristics, chemical composition, and nutritional value of stoned green table olives. *Food Bioproc. Tech*. **5**, 1733-1742. https://doi.org/10.1007/s11947-011-0567-x

Marsilio V, Seghetti L, Iannucci E, Russi F, Lanza B, Felicioni M. 2005. Use of a lactic acid bacteria starter culture during green olive (*Olea europaea* L cv *Ascolana tenera*) processing. *J. Sci. Food Agr*. **85**, 1084-1090. https://doi.org/10.1002/jsfa.2066

Ozdemir Y, Guven E, Ozturk A. 2014. Understanding the characteristics of oleuropein for table olive processing. *J. Food Process Technol*. **5**, 1000328. https://doi.org/10.4172/2157-7110.1000328

Panagou EZ, Schillinger U, Franz CM, Nychas GJE. 2008. Microbiological and biochemical profile of cv. Conservolea naturally black olives during controlled fermentation with selected strains of lactic acid bacteria. *Food Microbiol*. **25**, 348-358. https://doi.org/10.1016/j.fm.2007.10.005

Papadelli M, Zoumpopoulou G, Georgalaki M, Anastasiou R, Manolopoulou E, Lytra I, Papadimitriou K, Tsakalidou E. 2015. Evaluation of Two Lactic Acid Bacteria Starter Cultures for the Fermentation of Natural Black Table Olives (*Olea europaea* L cv. *Kalamon*). *Pol. J. Microbiol.* **64**, 265-271. https://doi.org/10.5604/01.3001.0009.2121

Perpetuini G, Caruso G, Urbani S, Schirone M, Esposto S, Ciarrocchi A, Prete R, Garcia-Gonzalez N, Battistelli N, Gucci R, Servili M, Tofalo R, Corsetti A. 2018. Changes in polyphenolic concentrations of table olives (cv. Itrana) produced under different irrigation regimes during spontaneous or inoculated fermentation. *Front. Microbiol.* 9, 1287. https://doi.org/10.3389/fmicb.2018.01287

Perricone M, Bevilacqua A, Corbo MR, Sinigaglia M. 2010. Use of *Lactobacillus plantarum* and glucose to control the fermentation of "Bella di Cerignola" table olives, a traditional variety of Apulian region (southern Italy). *J. Food Sci*. **75**, 430-436. https://doi.org/10.1111/j.1750-3841.2010.01742.x

Pino A, De Angelis M, Todaro A, Van Hoorde K, Randazzo CL, Caggia C. 2018. Fermentation of Nocellara Etnea table olives by functional starter cultures at different low salt concentrations. *Front. Microbiol.* **9,** 1125. https://doi.org/10.3389/fmicb.2018.01125

Pino A, Vaccalluzzo A, Solieri L, Romeo F, Todaro A, Caggia C, Arroyo-López F, Bautista-Gallego J, Randazzo C. 2019. Effect of Sequential Inoculum of Beta-Glucosidase Positive and Probiotic Strains on Brine Fermentation to Obtain Low Salt Sicilian Table Olives. *Front. Microbiol*. **10**, 174. https://doi.org/10.3389/fmicb.2019.00174

Pistarino E, Aliakbarian B, Casazza AA, Paini M, Cosulich ME, Perego P. 2013. Combined effect of starter culture and temperature on phenolic compounds during fermentation of Taggiasca black olives. *Food Chem*. **138**, 2043-2049. https://doi.org/10.1016/j.foodchem.2012.11.021

Psani M, Kotzekidou P. 2006. Technological characteristics of yeast strains and their potential as starter adjuncts in Greek-style black olive fermentation. *World J. Microb. Biot.* **22**, 1329-1336. https://doi.org/10.1007/s11274-006-9180-y

Randazzo CL, Todaro A, Pino A, Pitino I, Corona O, Mazzaglia A, Caggia C. 2014. Giarraffa and Grossa di Spagna naturally fermented table olives: effect of starter and probiotic cultures on chemical, microbiological and sensory traits. *Food Res. Int*. **62**, 1154-1164. https://doi.org/10.1016/j.foodres.2014.05.056

Randazzo CL, Todaro A, Pino A, Pitino I, Corona O, Caggia C. 2017. Microbiota and metabolome during controlled and spontaneous fermentation of Nocellara Etnea table olives. *Food Microbiol.* **65**, 136-148. https://doi.org/10.1016/j.fm.2017.01.022

Randazzo CL, Russo N, Pino A, Mazzaglia A, Ferrante M, Conti GO, Caggia C. 2018. Effects of selected bacterial cultures on safety and sensory traits of Nocellara Etnea olives produced at large factory scale. *Food Chem. Toxicol*. **115**, 491-498. https://doi.org/10.1016/j.fct.2018.03.045

Ruiz-Barba JL, Jiménez-Díaz R. 2012. A novel *Lactobacillus pentosus*-paired starter culture for Spanish-style green olive fermentation. *Food Microbiol*. **30**, 253-259. https://doi.org/10.1016/j.fm.2011.11.004

Sabatini N, Mucciarella MR, Marsilio V. 2008. Volatile compounds in uninoculated and inoculated table olives with *Lactobacillus plantarum* (*Olea europaea* L., cv. *Moresca* and *Kalamata*). *LWT- Food Sci. Technol*. **41**, 2017-2022. https://doi.org/10.1016/j.lwt.2007.12.002

Sakouhi F, Harrabi S, Absalon C, Sbei K, Boukhchina S, Kallel H. 2008. α-Tocopherol and fatty acids contents of some Tunisian table olives (*Olea europea* L.): Changes in their composition during ripening and processing. *Food Chem*. **108**, 833-839. https://doi.org/10.1016/j.foodchem.2007.11.043

Schaide T, Cabrera-Bañegil M, Pérez-Nevado F, Esperilla A, Martín-Vertedor D. 2019. Effect of olive leaf extract combined with *Saccharomyces cerevisiae* in the fermentation process of table olives. *J. Food Sci. Technol*. **56**, 3001-3013. https://doi.org/10.1007/s13197-019-03782-x

Segovia-Bravo KA, López FA, García PG, Quintana MD, Fernández AG. 2007. Treatment of green table olive solutions with ozone. Effect on their polyphenol content and on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth. *Int. J. Food Microbiol*. **114**, 60-68. https://doi.org/10.1016/j.ijfoodmicro.2006.09.032

Sieuwerts S, De Bok FA, Hugenholtz J, van Hylckama-Vlieg JE. 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl. Environ. Microbiol.* **74**, 4997-5007. https://doi.org/10.1128/AEM.00113-08

Smid EJ, Lacroix C. 2013. Microbe–microbe interactions in mixed culture food fermentations. *Curr. Opin. Biotech*. **24**, 148-154. https://doi.org/10.1016/j.copbio.2012.11.007

Tataridou M, Kotzekidou P. 2015. Fermentation of table olives by oleuropeinolytic starter culture in reduced salt brines and inactivation of *Escherichia coli* O157: H7 and *Listeria monocytogenes*. *Int. J. Food Microbiol*. **208**, 122-130. https://doi.org/10.1016/j.ijfoodmicro.2015.06.001

Tsapatsaris S, Kotzekidou P. 2004. Application of central composite design and response surface methodology to the fermentation of olive juice by *Lactobacillus plantarum* and *Debaryomyces hansenii*. *Int. J. Food Microbiol*. **95**, 157-168. https://doi.org/10.1016/j.ijfoodmicro.2004.02.011

Tufariello M, Durante M, Ramires F, Grieco F, Tommasi L, Perbellini E, Falco V, Tasioula-Margari M, Logrieco A, Mita G, Bleve

G. 2015. New process for production of fermented black table olives using selected autochthonous microbial resources. *Front. Microbiol.* **6**, 1-15. https://doi.org/10.3389/fmicb.2015.01007

Tufariello M, Mita G, Bleve G. 2016. Biotechnology can improve a traditional product as table olives, in Boskou D and Clodoveo MK (Ed.) *Products from Olive Tree*, IntechOpen, pp. 235-260. https://doi.org/10.5772/64687

Zaragoza J, Bendiks Z, Tyler C, Kable M, Williams T, Luchkovska Y, Chow E, Boundy-Mills K, Marco M. 2017. Effects of exogenous yeast and bacteria on the microbial population dynamics and outcomes of olive fermentations. *mSphere*. **2**, 00315-316. https://doi.org/10.1128/mSphere.00315-16

GRASAS Y ACEITES 72 (2)
April-June 2021, e406
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1142192

Effects of different baking techniques on the quality of walnut and its oil

B.K. Niu[a], T.M. Olajide[a], H.A. Liu[a], H. Pasdar[a] and X.C. Weng[a,✉]

[a]School of Life Sciences, Shanghai University 333, Nanchen Road, Shanghai, 200444, China

✉Corresponding author: wxch@staff.shu.edu.cn

Submitted: 22 November 2019; Accepted: 29 April 2020; Published online: 07 June 2021

SUMMARY: The baking conditions of walnut kernels were optimized based on different cultivars and baking methods. The influence of the different baking techniques on the chemical properties of walnut oils was determined. The results showed that acid value, peroxide value and induction period (IP) all significantly increased in the baked samples compared to the unbaked ones. The highest increase in IP was from 6 to 17 h indicating that baking can improve the oxidative stability of walnut oils and prolong their shelf-life. Several aroma components increased after baking. However, among the different baking conditions, the strongest aroma in walnut oil was observed after baking was done for 20 min with sucrose (107%) at 153 °C. Nevertheless, baking had little effect on the fatty acid composition of walnuts.

KEYWORDS*: Aroma components; Baking technique; Chemical composition; Oil quality; Walnut oil*

RESUMEN: ***Efecto de diferentes técnicas de horneado en la calidad de las nueces y su aceite.*** Las condiciones de horneado de los granos de nueces se optimizaron en base a diferentes cultivares y métodos de horneado. Se determinó la influencia de las diferentes técnicas de horneado sobre las propiedades químicas de los aceites de nuez. Los resultados mostraron que el índice de acidez, el índice de peróxido y el período de inducción (PI) aumentaron significativamente en las muestras horneadas en comparación con las no horneadas. El mayor aumento en el PI fue de 6 a 17 h, lo que indica que el horneado puede mejorar la estabilidad oxidativa de los aceites de nuez y prolongar su vida útil. Varios componentes del aroma aumentaron después del horneado, sin embargo, entre las diferentes condiciones de horneado, se observó el aroma más fuerte en el aceite de nuez después del horneado durante 20 minutos con sacarosa (107%) a 153 °C. Sin embargo, el horneado tuvo poco efecto sobre la composición de ácidos grasos de las nueces.

PALABRAS CLAVE*: Aceite de nuez; Calidad de aceites; Componentes aromáticos; Composición química; Técnica de horneado*

Citation/Cómo citar este artículo: Niu BK, Olajide TM, Liu HA, Pasdar H, Weng XC. 2021. Effects of different baking techniques on the quality of walnut and its oil. *Grasas Aceites* **72** (2), e406. https://doi.org/10.3989/gya.1142192

1. INTRODUCTION

Walnuts (*Juglans regia* L.) are mainly grown in East Asia, Southeastern Europe and North America, and are among the oldest tree nuts as well as the most economically important nut in the world (Gunn *et al*., 2010). There are approximately 20 species of *Juglans regia* L distributed throughout the world. China is the largest producer of walnuts in the world. In Yangbi, Yunnan Province, the authors saw a walnut tree dated over 1160 years old, which still bears fruit and is acclaimed to be the oldest walnut tree in China. Yangbi is also known as "the town of walnuts". Three main fatty acids in walnuts, oleic (18:1 ω-9), linoleic (18:2 ω-6) and linolenic (18:3 ω-3) acids account for about 90% of total walnut oil, and its ratio of ω-3 to ω-6 unsaturated fatty acids is more reasonable and can significantly lower blood lipids and prevent cardiovascular and cerebrovascular diseases (Abbey *et al*., 1994). Polyphenols, flavonoids and vitamin E in walnuts have good antioxidant and anti-aging effects (Zhao *et al*., 2014) which can protect functional fatty acids from autoxidation.

Baking is usually carried out to remove the pellicles of kernels, inactivate enzymes, destroy microorganisms and reduce water activity. Moreover, it can also be used to improve the color, crispy texture and flavor of products (Burdack-Freitag and Schieberle, 2010). The thermal treatment applied during baking leads to physical changes such as dehydration (Amaral *et al*., 2006), color modifications (Alamprese *et al*., 2009), biochemical changes including lipid structure modification and improvement in alluring flavor formation through Maillard reaction (Martins *et al*., 2000). The Maillard reaction is a series of complex reactions between the carbonyl group of reducing sugars and amino groups of free amino acids, peptides and proteins (Hodge *et al*., 1953). So far, reaction models between free amino acids and carbonyl compounds have been studied extensively and suggested that different reaction mechanisms existed in regards to different reactants, e.g., free amino acids or peptides. Investigations using pure peptides in Maillard reaction models demonstrated that peptides could participate in the Maillard reaction through many pathways such as bond cleavage, cyclization, and glycation (Garbe *et al*., 2004).

Vaidya *et al*., (2013) showed that the baking of walnut kernels increased their oil's oxidative stability during storage in the dark. It has significant effects on the nutrients and quality of lipids in walnut oil. Oven-baking is one of the best methods for processing walnuts because it preserves the quality of its lipids and some nutrients. Zhang *et al*., (2012) observed that increasing the temperature during the baking of walnuts from 60 to 90 °C in a gradient style, shortens drying time and improves oxidative stability compared to other baking condition and the products were of good quality. Santos *et al*., (2017) showed that all walnut flours contained about 42% protein and a significant amount of dietary fiber (17%), and were not affected by the baking process. Ziaolhagh *et al*., (2017) optimized the temperature and time of the baking of walnut kernels used for producing walnut cream, as they aimed to extend the shelf-life of walnut cream by slowing its oxidation reactions and reducing oil separation. Finally, the temperature of 116 °C for 12 min was chosen as the optimized baking conditions for producing walnut cream.

Herein, the baking technique was explored to optimize the parameters for the production of walnut oil with very desirable aroma, as studies in this research area are very limited.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Three walnut cultivars were donated by Yunnan Jiye Biological Co., Ltd. (Yunnan, China), and included "Yangbi walnut, Chuxiong walnut, (two districts in Yunnan)" and "Iron (i.e. very hard shell) walnut". The samples were stored at 4 °C before baking to maintain freshness.

All solvents used were of AR grade: Anhydrous ether, petroleum ether, ethanol (95%), glacial acetic acid, chloroform, anhydrous methanol, potassium hydroxide, sodium hydroxide, anhydrous sodium carbonate, and potassium iodide were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium thiosulfate, dichloromethane, n-hexane and sodium methanol were from Macklin (Shanghai, China).

2.2. Baking procedure

Three kinds of baking techniques were employed: baking without additives, baking with sucrose, and

baking with sucrose and salt. Walnuts of all three categories were baked in a lab-scale electric oven with variability in temperature of ± 0.5 °C (SM-522, Xinmai Machinery Co., Ltd., Jiangsu, China). First, the walnuts were manually cracked and shelled with a nutcracker to afford edible kernels before baking. Then the walnut kernels were rinsed with tap water for 1 minute, allowed to dry and 100 ± 0.05 g were transferred into solutions of different concentrations of sucrose and/or salt for one hour. The walnut kernels were purposely soaked in these solutions in order to enhance their taste, flavor and appearance and also to study the effect of the additives on oxidative stability of the resulting walnut oil after baking. Nevertheless, soaking time should not be too long, otherwise walnut kernels may become soft, which would affect their taste. Next, the kernels were placed in the electric oven for baking at different times and temperatures, and turned over every 5 min. The baked walnut kernels were kept at room temperature to cool down.

2.3. Optimization of baking parameters

Baking without additives considered two factors: baking time and baking temperature. Walnut kernels were baked in the oven at seven levels of temperatures between 120 and 180 °C at 10 ± 0.5 °C increments for 5-25 min. The baked walnut kernels were then cooled at room temperature for an hour prior to sensory evaluation.

Baking with sucrose considered three factors: baking time, baking temperature and the amounts of sucrose added. An orthogonal experiment with 3 factors and 3 levels was designed to optimize baking conditions (Table 2). For Yangbi and Chuxiong walnuts, nine baking experiments were carried out at baking time (Yangbi: 10, 15, and 20 min; Chuxiong: 15, 20 and 25 min), baking temperature 140, 150, 160 °C and sucrose amount (sucrose: walnut weight) 90, 100 and 110% based on the single-factor test. For Iron walnuts, however, baking time was 15, 20, and 25 min, baking temperature 150, 160, 170 °C and sucrose amount was same as Yangbi and Chuxiong walnuts (sucrose: walnut weight) 90, 100, 110%. The average response value K (K_{1j}, K_{2j}, K_{3j}) of the factors at each level was calculated separately, and the extreme analysis R_j value of the effect of factor level on baking was also determined, indicating the order of influence i.e. the larger the R_j value, the greater the influence of the factor on the sensory evaluation score (Zhao *et al.*, 2014).

Baking with sucrose and salt involved four factors: baking time, baking temperature, the amounts of sucrose and salt added, and an orthogonal experiment with 4 factors and 3 levels was designed to optimize baking conditions (Table 3). Similar conditions and orthogonal analysis similar to the baking with sucrose experiment were carried out in this section including an additional factor, the salt amount (salt: walnut weight), at 10, 15, and 20% on the basis of single-factor test for the three walnut types.

2.4. Sensory evaluation

The sensory evaluation of the baked walnut kernels was carried out an hour after baking. 15 trained sensory assessors from the Food Science Department of Shanghai University, Shanghai, China, were selected and trained as panelists according to the standardized procedure (ISO/DIS 13299). The scorecard was developed according to a 10-point category scale (extremely dislike = 0; extremely like = 10), and each assessor was asked to assess the walnut kernels for overall quality based on shape, color, odor, and flavor. Sensory assessors were also asked to comment on what they liked or disliked about the samples.

2.5. Extraction of walnut oil

The extraction of walnut oil was performed according to the Soxhlet extraction method. Baked walnut kernels were finely ground for 1 min into paste by a pulverizer (FW100 model, Tianjin, China) and Soxhlet-extracted with petroleum ether (P.E) for 6 h. The temperature was controlled so that the extracting solution could reflux once every 6 to 8 min. Then, the solvent was removed by using a vacuum rotary evaporator (RE-52AA, Shanghai Yuhua Instrument Equipment Co., Ltd. Shanghai, China). The extracted oil was kept in sealed glass bottles at 4 °C until analysis.

2.6. Physicochemical properties of walnut oil

Kernel yield was measured according to the Chinese National Standard for quality testing GB/T 5499-2008.

The protein content in walnuts was determined according to the Kjeldahl method. The walnut kernels were ground into powder and digested in a digestion

apparatus. Protein content was determined using a SKD-2000 automatic Kjeldahl nitrogen analyzer (Shanghai Peiou Analytical Instrument Co., Ltd.).

Oxidative stability. The peroxide value (PV) was determined according to the Chinese National Standard method GB 5009.227-2016. PV is commonly used to determine oxidative rancidity in oils and fats. The Acid value (AV) is another useful oil quality parameter, which involves the quantification of the amount of free fatty acids (Olajide *et al.,* 2018), and in this study was done based on the Chinese National Standard method, GB 5009.229-2016. The oxidative stability index (OSI), also known as induction period (IP), was evaluated by applying an accelerated automated test using the 743 Rancimat (Metrohm Co., Herisau, Switzerland) method according to a previous method with slight modifications (Olajide *et al.,* 2020). Different extracted oil samples (5 ± 0.05 g) were weighed into test tubes and connected to the Rancimat. Air at 20 L/h flow rate was bubbled through the oil heated at 100 ± 0.2 °C.

2.7. Fatty acid composition

The fatty acids methyl esters (FAMEs) of walnut oil were prepared according to the AOCS method Ce 1b-89 (AOCS, 1993).

All GC-MS analyses were performed on a Shimadzu GC2010A (Kyoto, Japan) gas chromatography instrument coupled with a GCMS-QP2010 quadrupole mass spectrometer (Shimadzu). The gas chromatographic system was equipped with an HP-Innowax PEG capillary column, 30 m length and 0.25 mm i.d., and the chromatographic conditions were the same as Verardo *et al*., (2013).

Individual FAMEs were identified by comparison to the NIST98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral library as well as to the Wiley 6.0 (Wiley, New York, NY, USA) mass spectral library. A relative amount of each fatty acid methyl esters was expressed by using the peak area normalization method.

2.8. Determination of volatile flavor components

Simultaneous distillation–solvent extraction (SDE) was carried out in a micro-version apparatus. Dichloromethane was used as solvent. For each extraction, 10 ± 0.05 g walnut oil and 250 ml distilled water were added to a 500-ml flask, and 30 ml dichloromethane were added into another 50-ml flask. Steam distillation lasted two hours while the solvent extraction was continued for a further 15 min. The extract was placed in the refrigerator at -18 °C for 12 h. Dry anhydrous sodium sulfate was used to dehydrate the extract, which was then finally concentrated to 2 ml with a nitrogen purge. The concentrate was stored at -4 °C for GC-MS analysis.

All SDE extracts were analyzed using a Shimadzu GC2010A (Kyoto, Japan) gas chromatography instrument coupled with a GCMS-QP2010 quadrupole mass spectrometer (Shimadzu). A 30 m×0.25 mm CP-Wax52CB quartz capillary column with 0.25-μm film thickness was used to resolve the volatiles. The method of GC-MS referred to the method of Wang *et al*., (2015).

The mass spectral identifications of the volatiles were carried out by comparison to the NIST98 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral library as well as to the Wiley 6.0 (Wiley, New York, NY, USA) mass spectral library.

2.9. Statistical analysis

All assays were carried out in duplicate or triplicate and values are expressed as mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) and Duncan's multiple range test ($P < 0.05$) using SPSS software (PASW Statistics 18, SPSS Inc, Chicago, IL, USA) and MS Office Excel 2010 (Microsoft Co., Redmond, WA, USA).

3. RESULTS AND DISCUSSION

3.1. Optimization of baking parameters

3.1.1. Baking without additives

Table 1 shows the sensory evaluation scores of the three kinds of walnuts at different baking temperatures and time combinations. When the temperature was lower than 140 °C, the aroma of baked walnuts became stronger and the score was higher with the extension of baking time because Maillard reaction increased. When baking temperature was higher than 150 °C, and the baking time longer than 20 min, a burnt taste became prominent. For example, when baking was done at 180 °C for 25 min, a strong

TABLE 1. Sensory evaluation scores for the three kinds of walnuts baked at different time and temperature without additives.

Cultivar	Time (min)	Temperature (ºC)						
		120	130	140	150	160	170	180
Yangbi walnut	5	3.0±0.1	4.1±0.2	5.2±0.4	6.4±0.4	6.0±0.3	5.6±0.2	5.3±0.2
	10	3.8±0.3	5.0±0.4	6.6±0.3	7.0±0.1	6.4±0.2	5.5±0.2	5.0±0.1
	15	4.1±0.4	5.6±0.2	6.4±0.2	7.5±0.3	5.8±0.4	6.3±0.3	0
	20	5.3±0.2	6.2±0.3	5.1±0.3	0	0	0	0
	25	4.7±0.3	4.9±0.2	5.7±0.4	0	0	0	0
Chuxiong walnut	5	2.3±0.2	3.9±0.2	5.4±0.4	6.0±0.2	6.4±0.1	5.4±0.2	4.9±0.2
	10	3.7±0.3	5.3±0.3	6.3±0.4	6.7±0.3	6.0±0.2	5.1±0.1	4.5±0.2
	15	4.5±0.1	5.7±0.2	6.7±0.1	6.6±0.3	5.5±0.4	5.9±0.1	0
	20	4.8±0.3	6.0±0.3	7.3±0.3	0	0	0	0
	25	4.1±0.3	4.2±0.4	6.0±0.3	0	0	0	0
Iron walnut	5	3.1±0.3	4.2±0.2	5.3±0.1	6.3±0.1	6.4±0.2	5.4±0.3	4.9±0.2
	10	3.8±0.2	5.3±0.3	6.4±0.2	6.5±0.3	6.8±0.2	5.3±0.2	4.7±0.3
	15	4.4±0.3	5.7±0.2	5.7±0.2	6.8±0.2	7.1±0.3	5.9±0.2	5.5±0.2
	20	4.7±0.4	5.7±0.2	6.2±0.3	6.5±0.3	6.5±0.4	0	0
	25	3.9±0.3	4.2±0.1	5.8±0.2	5.9±0.1	0	0	0

Values are expressed as mean ± standard deviation of duplicate determinations.

n =15 responses for sensory evaluation.

The scorecard was developed with a 10-point category scale (dislike= 0; extremely like = 10).

unpleasant taste was produced, thereby zeroing the sensory evaluation score. Finally, according to the average highest score for the sensory evaluation, the best non-additive baking method for the three kinds of walnuts was obtained. The time and temperature of Yangbi walnuts baked without additives were 150 °C, for 15 min; Chuxiong walnuts baked without additives were 150 °C, for 20 min; and Iron walnuts were 160 °C, for 15 min. As can be seen from the data, the baking of Iron walnuts required more time and a higher temperature because they were harder and so required a higher temperature to bake than the other two cultivars.

3.1.2. Baking with sucrose

The sucrose-added baking experiment was carried out under the same conditions as above. Firstly, a single-factor experiment design was carried out to determine the range of the amount of sucrose. The sucrose amounts tested (sucrose: walnut weight) were 70, 80, 90, 100, 110 and 120%. The results of subsequent sensory evaluation, show that the increase in sucrose amount corresponded to higher sensory scores for the three walnuts (Figure 1). When the sucrose amount exceeded 90%, the sensory score did not change much. Therefore, 90, 100 and 110% sucrose amounts were selected as the three levels for the orthogonal experiment. The L_9 (3^3) orthogonal experiment was carried out after determining the range of sucrose amount.

Three factors and three levels of orthogonal experiments were carried out on three kinds of walnuts. The results are shown in Table 2. For the Yangbi walnut, the maximum sensory score was 8.1. However, the best score could not be selected based on the outcomes in Table 2 alone, so a further orthogonal analysis was done. Thus, the K and R values were calculated and listed in Table 2. As seen, the influence to the sensory score was in the order:

A (time) > B (temperature) > C (sucrose amount) according to the R values. The baking time was found to be the most important determinant factor for the sensory evaluation score. In other words, the maximum sensory score for the baking technique with sucrose was obtained when temperature, time and sucrose amount added to Yangbi walnut were 150 °C, 15 min and 110%, respectively. Using the same analysis method, the optimal baking conditions for Chuxiong walnuts and Iron walnuts were as follows: the baking temperature, baking time and sucrose amount added for Chuxiong walnuts were 150 °C, 20 min and 110%, and 160 °C, 25 min and 100%, respectively for Iron walnuts.

3.1.3. Baking with sucrose and salt

Similar optimal baking conditions to baking with sucrose were employed here. Firstly, a single factor experiment of salt addition was carried out to determine the range of salt amount, 5, 10, 15, 20, and 25% of salt amounts (salt: walnut weight) were selected. After the baking test, the walnuts were subjected to sensory evaluation. The results are shown in Figure 2, and from there it can be seen that the sensory score showed a positive correlation trend within 5%~15% of the salt amount. When the salt amount was higher than 15%, the sensory evaluation score began to decrease slightly. Therefore, 10, 15 and 20% salt amounts were

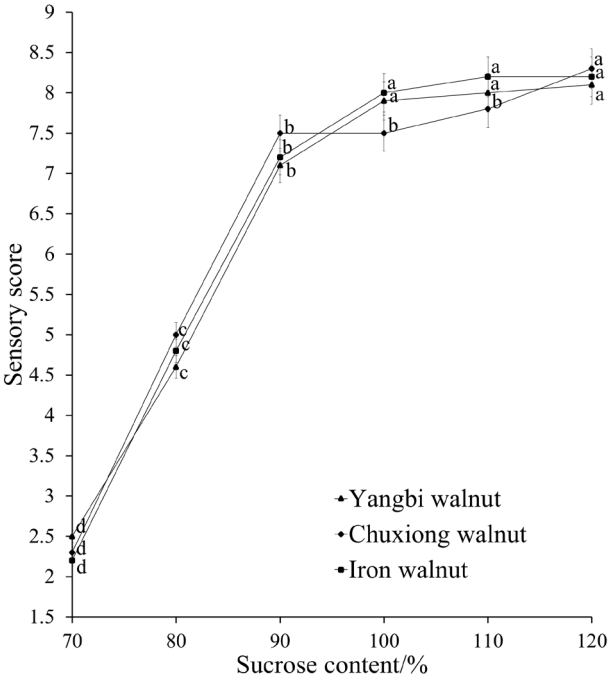


FIGURE 1. Single-factor experiments on different amounts of sucrose added during walnut baking. Each value is expressed as Mean ± SD (n = 3). Different letters are significantly different at $P < 0.05$ according to Duncan's test.

TABLE 2. Orthogonal experiment design and analysis for baking with sucrose.

Cultivar	Level	Orthogonal test factors			Analysis				
		A (time/ min)	B (temperature/°C)	C (sucrose amount/%)	Code	A (min)	B (°C)	C (%)	Sensory evaluation
Yangbi walnut	1	10	140	90	1	1	1	1	5.6±0.3^{c}
	2	15	150	100	2	1	2	2	7.2±0.3^{b}
	3	20	160	110	3	1	3	3	7±0.1^{b}
					4	2	1	2	8.1±0.3^{a}
					5	2	2	3	7.8±0.2^{a}
					6	2	3	1	6.7±0.2^{b}
					7	3	1	3	7.1±0.3^{b}
					8	3	2	1	7±0.4^{b}
					9	3	3	2	4.4±0.4^{d}
					K_{1j}	6.6	6.9	6.4	
					K_{2j}	7.5	7.3	6.6	
					K_{3j}	6.2	6.0	7.3	
					R_j	1.4	1.3	0.8	

Cultivar	Level	Orthogonal test factors			Code	Analysis			
		A (time/ min)	B (temperature/°C)	C (sucrose amount/%)		A (min)	B (°C)	C (%)	Sensory evaluation
Chu-xiong walnut	1	15	140	90	1	1	1	1	6.2±0.2^{f}
	2	20	150	100	2	1	2	2	7.8±0.2^{b}
	3	25	160	110	3	1	3	3	7.3±0.3^{cd}
					4	2	1	2	7.9±0.2^{b}
					5	2	2	3	8.5±0.3^{a}
					6	2	3	1	6.9±0.1^{de}
					7	3	1	3	6.8±0.2^{e}
					8	3	2	1	7.6±0.3^{bc}
					9	3	3	2	4.7±0.2^{g}
					K_{1j}	7.1	7.0	6.9	
					K_{2j}	7.8	8.0	6.8	
					K_{3j}	6.4	6.3	7.5	
					R_j	1.4	1.7	0.7	
Iron walnut	1	15	150	90	1	1	1	1	6.2±0.2^{e}
	2	20	160	100	2	1	2	2	7.9±0.2^{b}
	3	25	170	110	3	1	3	3	4.7±0.3^{f}
					4	2	1	2	6.9±0.3^{d}
					5	2	2	3	6.8±0.2^{d}
					6	2	3	1	7.6±0.1^{bc}
					7	3	1	3	8.5±0.3^{a}
					8	3	2	1	7.9±0.2^{b}
					9	3	3	2	7.3±0.3^{c}
					K_{1j}	6.3	7.2	7.2	
					K_{2j}	7.1	7.5	7.4	
					K_{3j}	7.9	6.5	6.7	
					R_j	1.6	1.0	0.7	

Values are expressed as mean ± standard deviation of triplicate determinations.
Means in the same row with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.
n =15 responses for sensory evaluation.
The scorecard was developed with 10-point category scale (dislike = 0; extremely like = 10)
K_{1j}, K_{2j}, and K_{3j} are the average values of sensory scores corresponding to the factors in different levels.
R_j refers to the result of extreme analysis.

selected as the three levels for the orthogonal experiment. The L_9 (3^4) orthogonal experiment was carried out after determining the range of salt addition.

Table 3 shows that the time, temperature, amounts of sucrose and salt added had the same effect on the baking of the three walnuts types: D (salt amount) > A (time) > B (temperature) > C (sucrose amount).

TABLE 3. Orthogonal experiment design and analysis for baking with sucrose and salt.

Cultivar	Level	Orthogonal test factors				Code	Analysis				
		A (time/ min)	B (temperature/°C)	C (sucrose amount /%)	D (salt amount/ %)		A (min)	B (°C)	C (%)	D (%)	Sensory evaluation
Yangbi walnut	1	10	140	90	10	1	1	1	1	1	6.2±0.2^{e}
	2	15	150	100	15	2	1	2	2	2	7.8±0.1^{b}
	3	20	160	110	20	3	1	3	3	3	7.3±0.3^{cd}
						4	2	1	2	3	8.6±0.4^{a}
						5	2	2	3	1	6.9±0.2^{d}
						6	2	3	1	2	7.2±0.2^{d}
						7	3	1	3	2	7.6±0.2^{bc}
						8	3	2	1	3	5.9±0.2^{e}
						9	3	3	2	1	4.7±0.2^{f}
						K_{1j}	7.1	7.5	6.4	5.9	
						K_{2j}	7.6	6.9	7.0	7.5	
						K_{3j}	6.1	6.4	7.3	7.3	
						R_j	1.5	1.1	0.8	1.6	
Chuxiong walnut	1	15	140	90	10	1	1	1	1	1	5.9±0.1^{e}
	2	20	150	100	15	2	1	2	2	2	6.8±0.3^{d}
	3	25	160	110	20	3	1	3	3	3	7.1±0.4^{d}
						4	2	1	2	3	8.5±0.2^{a}
						5	2	2	3	1	6.2±0.1^{e}
						6	2	3	1	2	7.9±0.2^{b}
						7	3	1	3	2	7.5±0.2^{c}
						8	3	2	1	3	5.2±0.1^{f}
						9	3	3	2	1	6.1±0.2^{e}
						K_{1j}	6.6	7.3	6.3	6.1	
						K_{2j}	7.5	6.1	7.1	7.4	
						K_{3j}	6.3	7.0	6.9	6.9	
						R_j	1.3	1.2	0.8	1.3	
Iron walnut	1	20	150	90	10	1	1	1	1	1	5.5±0.3^{e}
	2	25	160	100	15	2	1	2	2	2	8.6±0.3^{a}
	3	30	170	110	20	3	1	3	3	3	6.1±0.2^{d}
						4	2	1	2	3	5.4±0.4^{e}
						5	2	2	3	1	7.8±0.3^{b}
						6	2	3	1	2	8.5±0.3^{a}
						7	3	1	3	2	7.2±0.1^{c}
						8	3	2	1	3	4.8±0.3^{f}
						9	3	3	2	1	5.0±0.3^{ef}
						K_{1j}	6.7	6.0	6.3	6.1	
						K_{2j}	7.2	7.1	6.3	8.1	
						K_{3j}	5.7	6.5	7.0	5.4	
						R_j	1.6	1.0	0.8	2.7	

Values are means ± standard deviations of triplicate determinations.
Means in the same row with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.
n =15 responses for sensory evaluation.
The scorecard was developed with a 10-point category scale (disliked = 0; extremely liked = 10)
K_{1j}, K_{2j}, and K_{3j} are the average values of sensory scores corresponding to the factors in different levels.
R_j refers to the result of extreme analysis.

Nevertheless, the effect of salt addition on the sensory score of baked walnuts was most significant, i.e., a very high salt amount caused an obvious salty taste in the walnuts. Although the amounts of sucrose added had the smallest effect on baking, there is no denying its influence on the taste of the baked walnuts. It can be concluded that the optimum baking conditions of the three walnut types with sucrose and salt were as follows: the baking temperature of the Yangbi walnut was 150 °C, baking time 15 min, sucrose amount 110% and the salt amount 15%; Chuxiong walnut is 150 °C, baking time 20 min, sucrose amount 110% and salt amount 15%; Iron walnut was 160 °C, baking time 25 min, sucrose amount 100% and the salt amount 15%.

3.2. Physicochemical properties

Firstly, the physical characteristics of the three walnuts were compared based on their kernel productivity, fat content and protein content. The calculated data showed that the difference between the kernel yields of Yangbi and Chuxiong walnuts was not significant, 54.6 and 52.1%, respectively. However, the kernel productivity of Iron walnuts was significantly lower than that of Yangbi and Chuxiong, at only 30.4%. Their oil contents were slightly similar: the Chuxiong walnut kernel was 64.7%, which was higher than those of the other two walnuts; Iron walnut was 60.7%, and that of the Yangbi was 62.2%. According to Hao *et al*., (2002), the oil content of walnut kernels should be in the range 63-69%, and the protein content from 16 to 23%. Contrary to the oil content, the protein content of Iron walnut kernels was the highest among the three walnut kernels, which was 14.9%. The protein content of Chuxiong walnut kernels was slightly lower than that of Yangbi, at 11.0% and 13.4%, respectively. The content of protein of the walnut kernels studied herein was lower than those grown in the USA, at 17.6%, as reported by Sze-Tao *et al*., (2000) The variations in kernel productivity, oil and protein contents may be related to the different regions in which walnut trees were planted (Mao *et al*., 2014).

Oxidative stability. Walnut oil was extracted from walnuts processed according to different baking methods. The acid value (AV) and peroxide value (PV) of walnut oil were then evaluated

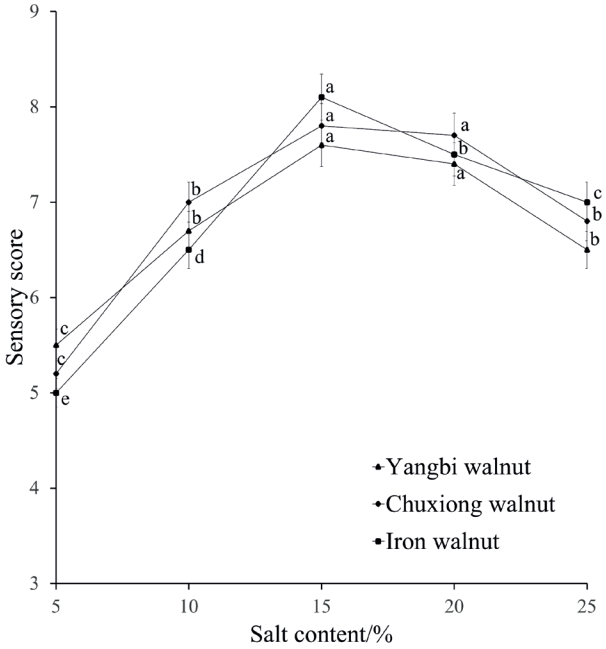


FIGURE 2. Single-factor experiments on different amounts of salt added during walnut baking. Each value is expressed as Mean ± SD (n = 3). Different letters are significantly different at $P < 0.05$ according to Duncan's test.

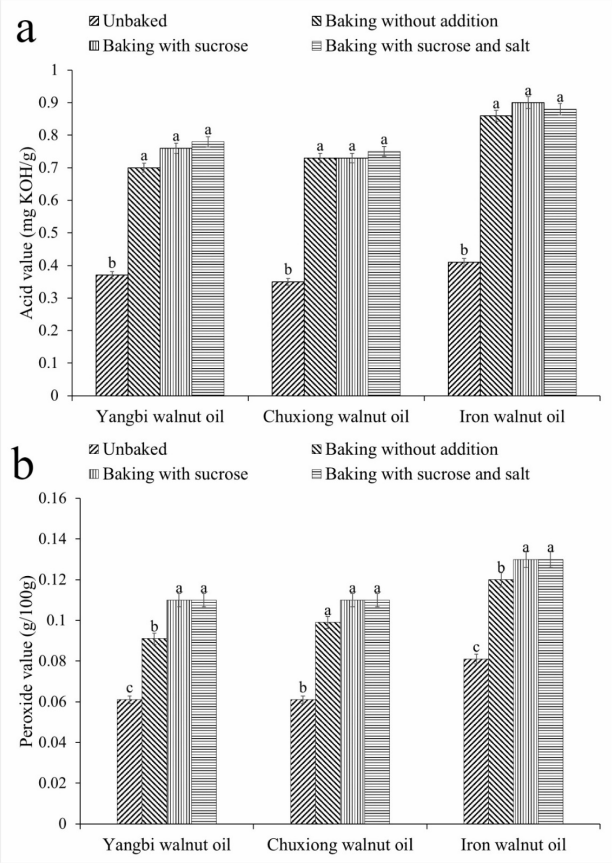


FIGURE 3. Effects of different baking techniques on the AV (a) and PV (b) of walnut oil. Each value is expressed as Mean ± SD (n = 3). Different letters are significantly different at $P < 0.05$ according to Duncan's test.

and compared. Under the action of lipase, some triglyceride molecules are hydrolyzed to release free fatty acids, which can influence the stability of oil (Fu *et al*., 2016). The AV can be used to express the degree of oil hydrolytic rancidity (Wang *et al*., 2015). The change in AV is shown in Figure 3(a). The AV of the three walnut oils were low prior to baking, but afterwards, the values increased significantly, and the three different baking processes had little to no positive effect on the AV of the walnut oils. It can be concluded that baking was the main influential factor in the change in AV, and the effect of adding sucrose or salt on the AV of walnut oil was small. This result is in agreement with the report of Vaidya *et al*., (2013). Branch *et al*., (1987) reported that the AV of oils extracted from baked peanuts were higher than those of unbaked peanuts.

The changes in PV in walnut oil is shown in Figure 3(b). Similar to the changes in AV, the PV of oils from the three walnut types were also greatly affected by baking. It significantly increased the PV of the walnut oils, especially those obtained by added sucrose, and sucrose and salt, which were slightly higher than without additives. Therefore, as with AV, the main influential factor of the PV change in walnut oil was also baking, and the effect of adding sucrose or salt on the PV of walnut oil was small. This result is in agreement with the report of Vaidya *et al*., (2013) who showed that the PV of baked walnut oil was significantly higher; however, the rate of increase in baked walnut oil was lower than that of the unbaked one during storage.

The oxidative stability indexes of the different walnut oils processed with different techniques were measured by the Rancimat test and expressed as induction period (IP) in Figure 4. It is obvious that the change in IPs of Yangbi and Chuxiong walnut oils was similar, at 6 and 7 h when unbaked to 9 and 10 h after baking, respectively. The IP of Iron walnuts was significantly prolonged from 6 to 14 h after baking, which means that its oil oxidative stability greatly improved. In addition, some Maillard reaction products produced after baking, such as 4-hydroxy-2,5-dimethylfuran-3-one and 2,3-dihydro-3,5-dihydroxy-6-methyl-4-pyranone, have antioxidant properties and can effectively improve the oxidative stability of walnut oil. As shown in Figure 4 their antioxidant

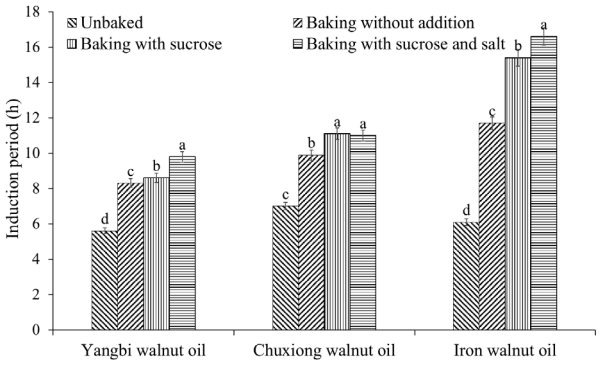


FIGURE 4. IPs of walnut oils extracted by different baking techniques. Each value is expressed as Mean ± SD (n = 3). Different letters are significantly different at $P < 0.05$ according to Duncan's test.

capacities are similar to that of ascorbic acid (Aoyama *et al*., 1982). Their relative content was higher when baking was done with added substances than without, but the stability indexes obtained by baking with sucrose and sucrose with salt were stronger.

Therefore, it can be concluded that the three baking techniques can decrease the oxidative degradation of walnut oils and prolong their shelf-life. This result is in agreement with the report of Vaidya *et al*., (2013) who showed that the baking of walnut kernels can be considered as an appropriate method for extending the oxidative stability of oil during storage.

3.3. Fatty acid composition

Both unbaked and baked walnut oils contained linoleic (57.43 and 58.4%, respectively) in the highest proportion, followed by oleic (27.1 and 25.6%, respectively), and linolenic acids (7.4 and 7.2%, respectively), the total contents in palmitic and stearic acids was < 7% in both oils. The amount of linoleic acid contained in the walnut oil from the cultivar grown in China was basically the same as those reported for walnuts cultivated in Portugal (57.5–62.5%), New Zealand (57.0–62.5%), and Serbia (57.2–65.1%) (Amaral *et al*., 2006). From the data we can draw the conclusion that there was no significant difference in the fatty acid composition between unbaked and baked walnut oils. Other studies also indicated that baking did not have an effect on the fatty acid composition of rice germ, safflower, or canola oils (Rabrenovic *et al*., 2011).

3.4. Volatile flavor components

Table 4 shows some aroma components as identified by the GC-MS analysis of walnut oil obtained with different baking techniques. The relative content of each aroma component was calculated by normalizing the peak area of the gas chromatograph. The relative content in alcohol in the unbaked walnut oil was the highest, higher than that in the baked ones, and the content in 1-penten-3-alcohol was the highest. After baking, the ketones, acids and aldehydes in the walnut oil increased the most, while the alcohols decreased the most. The main aroma components in baked walnut oil were Maillard reaction products. The pyrazine compounds contained in baked walnut oil are the key aroma components of the baking aroma produced. Furthermore, the alcohols, hydrocarbons and other substances in the oil from baked walnuts without additives were more abundant than those in the oil from baked walnuts with sucrose, while the ketones, esters and aldehydes in the oil from baked walnuts

TABLE 4. Aroma components of walnut oil obtained by different baking techniques.

Compound	Molecular formula	Molecular weight	Relative content/%			Aroma descriptions
			Sample 1	Sample 2	Sample 3	
Cyclobutanol	C_4H_8O	72	5.4±0.1	-	-	
2-Methyl butyraldehyde	$C_5H_{10}O$	86	-	0.9±0.1	3.6±0.3	Caramel, nutty
gamma-Butyrolactone	$C_4H_6O_2$	86	-	1.8±0.2	2.4±0.1	
1-Penten-3-ol	$C_5H_{10}O$	86	42.7±0.6	13.3±0.3	-	Almond, chocolaty
2-Methyl pyrazine	$C_5H_6N_2$	94	-	0.5±0.1	0.4±0.1	Rich baked nuts
Hexanal	$C_6H_{12}O$	100	1.6±0.2^c	3.8±0.3^b	5.2±0.3^a	Fat, nutty
2-Furaldehyde	C_4H_3OCHO	96	-	1.5±0.1	8.3±0.4	Caramel, baked food
Cyclohexanone	$C_6H_{10}O$	98	0.8±0.2^c	2.2±0.3^b	3.1±0.2^a	
2,3-Pentanedione	$C_5H_8O_2$	100	-	1.3±0.3	0.8±0.1	Cheese, grease aroma
2,3-Dimethyl pyrazine	$C_6H_8N_2$	108	0.3±0.1^b	3.9±0.4^a	3.7±0.2^a	Nutty, baked, chocolate
2,5-Dimethyl pyrazine	$C_6H_8N_2$	108	-	3.2±0.1	2.1±0.3	Potato flavor
Benzaldehyde	C_7H_6O	106	-	0.8±0.1	1.3±0.2	Nutty, fruity
2,4-Heptadienal	$C_7H_{10}O$	110	2.9±0.3	-	-	Fatty, nutty aroma
n-Hexanoic acid	$C_6H_{12}O_2$	116	10.6±0.3^c	17.2±0.6^a	16.1±0.4^b	
2-Ethyl-6-methyl pyrazine	$C_7H_{10}N_2$	122	-	2.1±0.4	2.7±0.6	Nuts, baking, and sweet aroma
5-Hydroxymethylfurfural	$C_6H_6O_3$	126	12.5±0.8^c	26.3±0.6^a	21.1±0.5^b	
4-Hydroxy-2,5-dimethyl furan-3-one	$C_6H_8O_3$	128	0.6±0.1^c	3.2±0.1^b	5.4±0.1^a	
1-octen-3-ol	$C_8H_{16}O$	128	1.5±0.2^a	0.7±0.1^b	0.3±0.1^c	Sweet, mushroom, greasy
2,3-Dihydro-3,5-dihydroxy-6-methyl-4-pyranone	$C_6H_8O_4$	144	1.7±0.1^c	4.3±0.4^b	7.6±0.5^a	

"-" means that the compound was not detected because its content was too low.
Values are means ± standard deviations of triplicate determinations.
Means in the same row with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.
Sample 1: unbaked walnut oil; Sample 2: oil of baked walnut without additive; Sample 3: oil of baked walnut with sucrose.

with sucrose were more abundant than those in the oil from baked walnuts without additives. It should be noted that the baking with sucrose and salt technique was not considered here, because the addition of salt has no effect on the change in the volatile substances content. Through a comprehensive comparison of the aroma components of walnut oil obtained with different baking methods, it was found that the unbaked walnut oil had the aroma of fresh nuts, and those of baked walnut oils had a characteristic baked nut smell. In addition, the caramel flavor of the oil of baked walnuts with sucrose was stronger than the oil of the baked walnut without additives.

CONCLUSIONS

This study optimized three different baking techniques through sensory evaluation. By analyzing AV, PV and IP, it was concluded that the oil quality of the studied walnuts was significantly influenced by the baking method, especially given that the IP of the walnut oil increased from 6 to 17 h after baking, which indicated that the oxidative stability improved and the oil can be preserved for a longer time. The relative content in pyrazines with a baked nutty flavor increased after baking. Therefore, compared to the walnut oil obtained without baking, oils with special aroma can be obtained by the baking techniques studied herein. In addition, some aldehydes were produced through a caramelization reaction during baking with sucrose, and walnut oil had the strongest aroma after baking for 20 min with sucrose at 153 °C. Finally, the small differences in fatty acid composition observed before and after baking guarantee healthy amounts which are beneficial to the human body.

ACKNOWLEDGEMENT

The authors thank the Yunnan Jiye Biology Co., Ltd. for the financial support and for providing various walnut samples for this research.

REFERENCES

Abbey M, Noakes M, Belling GB, Nestel PJ. 1994. Partial replacement of saturated fatty acids with almonds or walnuts lowers total plasma cholesterol and low-density-lipoprotein cholesterol. *Am. J. Clin. Nutr.* **59**, 995-999. https://doi.org/10.1093/ajcn/59.5.995

Alamprese C, Ratti S, Rossi M. 2009. Effects of roasting conditions on hazelnut characteristics in a two-step process. *J. Food Eng.* **95**, 272-279. https://doi.org/10.1016/j.jfoodeng. 2009.05.001

Amaral JS, Susana C, Seabra RM, Oliveira BPP. 2006. Effects of roasting on hazelnut lipids. *J. Agric. Food Chem.* **54**, 1315-1321. https://doi.org/10.1021/jf052287v

AOCS. 2007. *Official methods and recommended practices of the American Oil Chemists' Society.* 5th edition, Urbana, IL, USA.

Aoyama T, Nakakita Y, Nakagawa M, Sakai H. 1982. Screening for antioxidants of microbial [filamentous fungi] origin. *Agric. Biol. Chem.* https://doi.org/10.1080/00021369.1982. 10865439

Branch AL, Worthington RE, Roth IL, Chinnan MS, Nakayama TOM. 1987. Effect of Hot Water Immersion on Storage Stability of Non-Refrigerated Peanuts 1. *Peanut Sci.* **14**, 26-30. https://doi.org/10.3146/i0095-3679-14-1-7

Burdack-Freitag A, Schieberle P. 2010. Changes in the Key Odorants of Italian Hazelnuts (*Coryllus avellana* L. Var. *Tonda Romana*) Induced by Roasting. *J. Agric. Food Chem.* **58,** 6351-6359. https://doi.org/10.1021/jf100692k

Fu M, Qu Q, Yang X, Zhang X. 2016. Effect of intermittent oven drying on lipid oxidation, fatty acids composition and antioxidant activities of walnut. *LWT-Food Sci. Technol.* **65**, 1126-1132. https://doi.org/10.1016/j.lwt.2015.10.002

Garbe LA, Würtz A, Piechotta CT, Tressl R. 2010. The peptide-catalyzed Maillard reaction: characterization of 13C reductones. *Ann. Ny. Acad. Sci.* **1126**, 244-247. https://doi.org/ 10.1196/annals.1433.046

Gunn BF, Aradhya M, Salick JM, Miller AJ, Yongping Y, Lin L, Xian H. 2010. Genetic variation in walnuts (*Juglans regia* and *J. sigillata*; Juglandaceae): Species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. *Am. J. Bot.* **97**, 660-671. https://doi.org/10.3732/ajb.0900114

Hao YB, Wang KJ, Wang SL, Zhuang YL. 2002. Analysis of the composition of protein and fatty acid in several early walnut nuts. *Food Sci.* **23**, 123-125. https://doi.org/10.3321/j.issn:1002-6630.2002.10.033

Hodge JE. 1953. Dehydrated foods. Chemistry of browning reactions in model systems. *J. Agric. Food Chem.* **1**, 625-651. https://doi.org/10.1021/jf60015a004

Mao XY, Hua YF, Chen GG. 2014. Amino Acid Composition, Molecular Weight Distribution and Gel Electrophoresis of Walnut (*Juglans regia* L.) Proteins and Protein Fractionations. *Int. J. Mol. Sci.* **15**, 2003-2014. https://doi.org/10.3390/ijms15022003

Martins SIFS, Jongen WMF, Boekel MAJSV. 2000. A review of Maillard reaction in food and implications to kinetic modelling. *Trends Food. Sci. Tech.* **11**, 364-373. https://doi.org/ 10.1016/s0924-2244(01)00022-x

Olajide TM, Pasdar H, Weng, XC. 2018. A novel antioxidant: 6,6'-(butane-1,1-diyl) bis (4-methylbenzene-1,2-diol). *Grasas Aceites* **69** (3), e269. https://doi.org/10.3989/gya.0344181

Olajide TM, Liu T, Liu HA, Weng XC. 2020. Antioxidant properties of two novel lipophilic derivatives of hydroxytyrosol. *Food Chem.* **315**, 126197. https://doi.org/10.1016/j.foodchem.2020.126197

Rabrenovic B, Dimic E, Maksimovic M, Sobajic S, Gajickrstajic L. 2011. Determination of fatty acid and tocopherol compositions and the oxidative stability of walnut (*Juglans regia* L.) cultivars grown in Serbia. *Czech. J. Food Sci.* **29**, 74-78. https://doi.org/10.2217/ cer.14.74

Santos J, Alvarez-Ortí M, Sena-Moreno E, Rabadán A, Pardo JE, Oliveira MBPP. 2017. Effect of Roasting Conditions on the Composition and Antioxidant Properties of Defatted Walnut Flour. *J. Sci. Food Agric.* **98**, 1813-1820. https://doi.org/10.1002/jsfa.8657

Szetao KWC, Sathe SK. 2000. Walnuts (*Juglans regia* L): proximate composition, protein solubility, protein amino acid composition and protein in vitro digestibility. *J. Sci. Food Agric.* **80**, 1393–1401. https://doi.org/10.1002/1097-0010(200007)80:93.0.CO;2-F

Vaidya B, Eun JB. 2013. Effect of roasting on oxidative and tocopherol stability of walnut oil during storage in the dark. *Eur. J. Lipid Sci. Tech.* **115**, 348-355. https://doi.org/10.1002/ejlt.201200288

Verardo V, Riciputi Y, Sorrenti G, Ornaghi P, Marangoni B, Caboni MF. 2013. Effect of nitrogen fertilisation rates on the content of fatty acids, sterols, tocopherols and phenolic compounds, and on the oxidative stability of walnuts. *LWT-Food Sci. Tech.* **50**, 732-738. https://doi.org/10.1016/j.lwt.2012.07.018

Wang W, Wang H, Chen W, Rong R. 2015. Effects of Different Drying Methods on Quality of Walnuts and Stability of Unsaturated Fatty Acids. *J. Food Sci. Technol*. **33**, 59-65. https://doi.org/10.3969/j.issn.2095-6002.2015.01.011

Zhang J, Pan X, Mu Q, Xi C, Wang Z, Yuan S. 2012. Research about the Baking Process of 'Lvling No.1' Thin Shelled Walnut. *Chinese Agric. Sci. Bulletin.* **28**, 237-240. https://doi.org/10.3969/j.issn.1000-6850.2012.06.044

Zhao JJ, Zhang RG, Ma YJ. 2014. Optimization of Protein Extraction from Walnut Dregs. *Food Sci.* **35**, 18-19. https://doi.org/10.7506/spkx1002-6630-201418008

Ziaolhagh SH. 2017. Roasting Process Optimization of Walnut Kernels for the Preparation of Walnut Cream Using Response Surface Methodology. *J. Nuts.* **8**, 31-40. https://doi.org/10.22034/JON.2017.530390

GRASAS Y ACEITES 72 (2)
April-June 2021, e407
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.1259192

Inoculation with acetic acid bacteria improves the quality of natural green table olives

M. Mounir[a, ✉], J. Hammoucha[a], O. Taleb[a], M. Afechtal[b], A. Hamouda[c] and M. Ismaili Alaoui[a]

[a]Department of Food Science and Nutrition, Hassan II Institute of Agronomy and Veterinary Medicine, PB Box 6202, Rabat, Morocco

[b]Laboratory of Virology, National Institute for Agricultural Research (INRA), Kénitra, Morocco

[c]Department of Applied Statistics, Hassan II Institute of Agronomy and Veterinary Medicine, PB Box 6202 Rabat, Morocco.

✉Corresponding author: mounirmajid@gmail.com

Submitted: 22 December 2019; Accepted: 29 April 2020; Published online: 07 June 2021

SUMMARY: This study aims to develop a method for the preparation of natural table olives using locally selected microorganisms and without resorting to the usual techniques which employ lye treatment and acids. The effects of parameters, such as lye treatment, inoculation with yeasts, substitution of organic acids with vinegar and/or acetic acid bacteria, and finally alternating aeration have been assessed. Four different combinations were applied to the "*Picholine marocaine*" olive variety using indigenous strains, namely *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 and *Acetobacter pasteurianus* KU710511 (CV01) isolated respectively from olive brine, *Bouslikhen* dates and Cactus. Two control tests, referring to traditional and industrial processes, were used as references. Microbial and physicochemical tests showed that the L3V combination (inoculated with *A. pasteurianus* KU710511 and *L. plantarum* S1 under the optimal growth conditions of the Acetic Acid Bacteria (AAB) strain with 6% NaCl) was found to be favorable for the growth of the Lactic Acid Bacteria (LAB) strain which plays the key role in olive fermentation. This result was confirmed by sensory evaluation, placing L3V at the top of the evaluated samples, surpassing the industrial one where a chemical debittering treatment with lye was used. In addition, alternating aeration served to increase the microbial biomass of both AAB and LAB strains along with *Saccharomyces cerevisiae* LD01 strain, but also to use lower concentration of NaCl and to reduce the deterioration of olives compared to the anaerobic fermentation process. Finally, a mixed starter containing the three strains was prepared in a 10-L Lab-fermenter from the L3V sample in order to improve it in subsequent studies. The prepared starter mixture could be suitable for use as a parental strain to prepare table olives for artisan and industrial application in Morocco.

KEYWORDS*: Acetobacter pasteurianus; Aerobic fermentation; Lactobacillus plantarum; Mix starter; Natural table olives; Saccharomyces cerevisiae*

RESUMEN: ***La inoculación con bacterias del ácido acético mejora la calidad de las aceitunas de mesa verdes naturales.*** Este estudio tiene como objetivo desarrollar un método para la preparación de aceitunas de mesa naturales utilizando microorganismos seleccionados localmente y sin recurrir a las técnicas habituales que utilizan el tratamiento con lejía y ácidos. Se han evaluado los efectos de parámetros como el tratamiento con lejía, la inoculación con levaduras, la sustitución de ácidos orgánicos por bacterias de vinagre y/o ácido acético, y finalmente la aireación alterna. Se ensayaron cuatro combinaciones diferentes en la variedad de aceituna «*Picholine m arocaine*» utilizando cepas autóctonas, como *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 y *Acetobacter pasteurianus* KU710511 (CV01) aisladas respectivamente de salmuera de aceitunas, *Bouslikhen* y Cactus. Se utilizaron como referencia dos pruebas de control, referidas a procesos tradicionales e industriales. Las pruebas microbianas y fisicoquímicas mostraron que la combinación L3V (inoculada con A. *pasteurianus* KU710511 y L. *plantarum* S1 en las condiciones óptimas de crecimiento de la bacteria del ácido acético (AAB) con NaCl al 6%) resultó ser favorable para el crecimiento del Láctico. Las cepas de bacterias ácidas (LAB) juegan un papel clave en la fermentación de las aceitunas. Este resultado fue confirmado por la evaluación sensorial colocando L3V en la parte superior de las muestras evaluadas, superando a la industrial, donde se usó un tratamiento de eliminación química con lejía. Además, la aireación alterna permitió aumentar la biomasa microbiana de las cepas AAB y LAB junto con la cepa *Saccharomyces cerevisiae* LD01, también usar una concentración más baja de NaCl y reducir el deterioro de las aceitunas, en comparación con la operación de fermentación anaerobia. Finalmente, se preparó un iniciador mixto que contenía las tres cepas en un fermentador de laboratorio de 10-L a partir de la muestra L3V con el objeto de mejorarlo en los estudios posteriores. El iniciador de mezcla preparado podría ser adecuado para usarse como una cepa parental para preparar aceitunas de mesa para aplicaciones artesanales e industriales en Marruecos.

PALABRAS CLAVE**:** *Aceitunas de mesa naturales; Acetobacter pasteurianus; Fermentación aerobia; Lactobacillus plantarum; Mezcla iniciadora; Saccharomyces cerevisiae*

Citation/Cómo citar este artículo: Mounir M, Hammoucha J, Taleb O, Afechtal M, Hamouda A, Ismaili Alaoui M. 2021. Inoculation with acetic acid bacteria improves the quality of natural green table olives. *Grasas Aceites* **72** (2), e407. https://doi.org/10.3989/gya.1259192

1. INTRODUCTION

The olive tree (*Olea europaea* L.) constitutes an economic and social resource for the development of the populations of the zones where it is cultivated. The growing demand for olive oil and table olives has created favorable conditions for a more efficient expansion of its cultivation, accompanied in many countries by specific development programs (Fernandez Escobar *et al.,* 2013). In Morocco, the olive sector is supported by the Green Morocco Plan launched in 2008. Recent statistics provided by the Moroccan agriculture department (2019) show that olive orchards cover about 1.1 million ha, distributed in irrigated lowlands at 39%, mountain areas at 36% and rainfed areas at 25%. Moreover, Morocco occupies one of the top positions among exporters of table olives in the world. According to the Ministry of Agriculture and Maritime Fisheries, during the 2013/2014 season, the domestic production was in the order of 1,6 million tons of olives, including 120,000 tons of table olives for the same period (7.6% total olive production) (Anon, 2016).

Table olives, which are the most widespread fermented vegetables in Mediterranean countries, have a great economic significance as a food commodity. Their high nutritional value, the content in bioactive compounds, dietary fibers, fatty acid composition and antioxidants make table olives a valuable functional food (Campus *et al.,* 2018). Green table olives are considered the most popular fermented vegetable commodity in Morocco. They constitute a major agro-industrial sector in the country's economic development (Rokni *et al.,* 2015).

Olives are usually harvested at different stages of maturity and then processed to eliminate the bitter taste due to the glucoside named oleuropein. The green table olive de-bittering process can be accomplished in two ways: a) The Spanish-style method, i.e. using a lye treatment prior to fermentation, and b) the natural method (Sánchez Gómez *et al.,* 2006). The Spanish style (the most common conventional preparation), consists of an alkaline treatment with sodium hydroxide, followed by several water washings of the fruits to remove residual lye. Then, the fermentation is continued in the brine from an initial salt concentration of 10-11% to reach the value of 5-6% of NaCl at equilibrium. The Californian style is quite distinct from that used for any other style of table olive, as there is no fermentation step. In this method, olives are subjected to a lye treatment (0,5 to 2% NaOH), followed by darkening with ferrous gluconate or ferrous lactate. Then, the olives are bottled or canned in brine and pasteurized or sterilized (Colmagro *et al.*, 2001; Marsilio *et al.*, 2001). In the other hand, the natural method, also known as "Greek style", does not require any lye treatment of the fruit, which is placed directly into the brine (8-14% NaCl and pH between 4.0 and 4.2), where fermentation takes place (Colmagro *et al.*, 2001). In this case, the hydrolysis of oleuropein is attributed to the enzymatic reaction of ß-glucosidase and esterase produced by the microorganisms (Amer *et al.,* 2017).

Among the main lactic acid fermented vegetable products, i.e. cucumbers, cabbages and olives, Spanish-style green-olives are the most economically important in Morocco. This method is the most widely used for making green olives on an industrial scale. It is based on the alkaline treatment of olives to eliminate oleuropein, the agent responsible for the bitterness of the fruits of the olive tree (Ramírez *et al.,* 2017). After de-bittering, the olives undergo successive washes to remove residual lye. The treatment consists generally of (a) lye treatment 1.5 à 2% NaOH for 8-12 h at 24-25 °C; (b) washing by tap water for 14-15 h in two or three soakings, and (c) brining in 10-12% NaCl (El-Khaloui and Nouri, 2007; Chemonics International, 2007).

Although the Spanish-style method is a fast process, it presents some drawbacks as well for the producer (lye and washing water expenses),

for the consumer (risk of the chemical residues and the deterioration of the nutritional value of the treated olives) (Shahidi and Kiritsakis, 2017) and for the environment (releases of high quantities of wastewater of high organic matter, high phenolic content, high salinity and conductivity). Indeed, this process causes a strong degradation of oleuropein, which constitutes a significant loss in the nutritional value of de-bittered olives (Rokni *et al.,* 2015). Recently, several sources reported the possibility of use of unauthorized acids by producers such as sulfuric acid to quickly neutralize the lye residue after the de-bittering of olives. This is done in order to reduce the time and cost of washing and obtain more profit (Faid, 2018). Hence, it could be important to further develop the sector by using natural table olives to overcome the drawbacks mentioned above. Natural table olives are harvested at the green-yellow stage, or fully ripened, previously washed and graded, then submerged into NaCl solutions (6–10% w/v), where fermentation takes place, mainly due to the metabolism of autochthonous microbiota (Campus *et al.,* 2018). Yeasts and LAB control the progress of fermentation since they are more sensitive to salt concentration and the acidification of brines which are determined by metabolic activity exerted mainly by LAB (Campus *et al.,* 2018). On the other hand, acetic acid bacteria (AAB) can also be associated with olives and olive products, in which they can play several roles (Kavroulakis and Ntougias, 2011; Valero *et al.*, 2017). *Acetobacter pasteurianus* is one of the acetic acid bacteria (AAB) which have the capacity to incompletely oxidize ethanol as a substrate to produce acetic acid. For this reason, AAB are often involved, either directly or indirectly, in the fermentation of food and drinks (Cleenwerck and De Vos, 2008). It has been perfectly established that producers of table olives use organic acids, such as lactic, citric and also acetic acid, in order to favor the rapid start of fermentation and avoid the excessive increase in pH at the beginning of fermentation (Degirmencioglu, 2016). It has also been reported that vinegar may be added to the brine solution to preserve the olives, adjust the pH, and impart a particular flavor (Colmagro, 2001). Thus, following our research, it seemed appropriate to consider three species of microorganisms: *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 and *Acetobacter pasteurianus* KU710511 CV01 isolated from Morocco (Mounir *et al.,* 2016a; Mounir *et al.,* 2016b; Mounir *et al.,* 2016c; Mounir *et al.,* 2018)

The overall objective of our work was to develop a method for the preparation of natural table olives using locally selected microorganisms and without resorting to the usual techniques. This objective involves optimizing the synergy conditions between the selected microorganisms. To do this, ten tests were carried out (on washed and sorted olives) so that we could study the effect of the de-bittering, the effect of inoculation by yeasts, the effect of the substitution of organic acids with vinegar, the effect of the substitution of organic acids with acetic bacteria and finally the effect of alternating aeration. The monitoring of physicochemical and microbiological parameters and organoleptic evaluation were carried out to judge the quality of the olives produced.

2. MATERIALS AND METHODS

2.1. Olives and microorganisms

The olives used in our study were turning color olives of the *Picholine Marocaine* variety, collected from the region of Beni Mellal-Khenif (central Morocco), renowned for olive growing. The olives were kept in tap water during transport from the places where they were collected to the laboratory to avoid degradation. The microorganisms chosen to carry out this study were: a) *Acetobacter pasteurianus* KU710511 (CV01) isolated by Mounir *et al.,* (2016c) from cactus; b) *Lactobacillus plantarum* S1 isolated by Mounir *et al.,* from olive brine in 2016; and c) *Saccharomyces cerevisiae* LD01 isolated from Bouslikhen variety dates by Mounir *et al.,* (2016a).

2.2. Criteria for physico-chemical parameters

In order to compare the effect of aeration on the quality and duration of the fermentation process, two types of fermentation (aerobic and anaerobic) were studied. In addition, optimal conditions for the synergy among the three strains selected for this study (LAB, yeast and AAB) were assessed. Aerobic fermentation was achieved by introducing a central column into the vessel containing the brine and olives and through which the air bubbles were introduced to evacuate the CO_2 produced during fermentation (Sánchez Gómez *et al.,* 2006). According to Sánchez Gómez *et al.,*

(2006), LAB grow under these conditions only when the salt concentration is less than 8%.

Due to bubbling air, the flow was controlled by a flowmeter adapted to the air inlet of a fermenter. Usually, the flow is fixed on the basis of past experience. When the active fermentation is complete (3-4 months), aeration is necessary only if the CO_2 concentration and brine volume increase during the treatment (Sánchez Gómez *et al.,* 2006).

2.3. Implementation of the tests

The olives were first washed and sorted to remove any damaged fruits. The experimental design was set up to study the effect of de-bittering, the effect of inoculation by yeasts, the effect of acid substitution with vinegar, the effect of acid substitution with acetic bacteria, and finally the effect of alternating aeration. The tests were compared to the conditions used in the industry and to those of artisanal fermentations in order to have a basis for comparison as described by (Kailis and Harris, 2007; El-Khaloui and Rahmani, 2012) (Table 1). The tests were carried out separately in glass vessels with one kg of olives brined in 2 liters of brine. Before inoculation into pilot fermentations, *S. cerevisiae* LD01 were grown in YPD broth at 30 °C aerobically for 48 h and *L. plantarum* S1 were grown in MRS broth at 30 °C aerobically for 24-36 h. YPD had the following composition: glucose 20 g; casein peptone 10 g; yeast extract 5 g and 1000 ml of distilled water. 100 mg of chloramphenicol were added to the medium to inhibit bacterial growth (Beuchat 1992). GYEA medium was used to cultivate the AAB strain and was composed of 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone of casein, 3% (w/v) ethanol and 1% (w/v) acetic acid (Mounir *et al.,* 2016c). The cells were then collected by centrifugation at 13000 rpm at 4 °C and washed twice in saline solution (0.85% NaCl) (Zaragoza *et al.,* 2017). The yeast and bacteria cells were adjusted to 10^9 cells/ml prior to inoculation into the olive brines. Then, the brine was inoculated to reach an estimated 10^5-10^6 cells/mL of each strain (inoculation with about 0.75% of seed cultures).

2.3.1. Effect of de-bittering and vinegar suppletion on the fermentation process

In this part of the experiment, the aim was to study the effect of lye treatment under anaerobic conditions as well as the effect of the acidification of the medium by the addition of 6% date vinegar. Samples were incubated at 30 °C. The lye treatment consisted of immersing the olives in a NaOH solution (2%–3.5% w/v), which penetrates up to two-thirds of the olive flesh (Fan and Hansen 2012). The lye treatment was followed by several water washings of the fruits with tap water to remove the residual lye and then the fermentation continued in the brine with 10.6% of NaCl under a pH adjusted to 3.5 using vinegar. Then the samples were divided into two groups: a group seeded both by *L. plantarum* S1 LAB strain (0.75%) and *S. cerevisiae* LD01 strain (0.75%) (Zaragoza *et al.,* 2017) and a group seeded solely by the LAB strain (0.75%). A control test was designed by reproducing the same conditions described previously except for the lye treatment.

2.3.2. Effect of inoculation with Acetobacter pasteurianus KU710511 AAB strain under alternated aeration conditions

The purpose of this part of the study is to highlight the effect of alternating aeration (aeration by air bubbling for eight hours a day) on the synergy among the three microorganisms used (LAB, AAB and yeast), investigated in vitro (in 6% brine) with different fermentation media (Table 1). The first assay was conducted under the optimal conditions of *Acetobacter pasteurianus* KU710511 AAB strain, namely: ethanol: 28.18 g/L acetic acid: 10.12 g/L, glucose 15.15 g/l and pH: 5.33 as reported by Mounir *et al*., (2016c). The second assay was designed to ensure the optimal growth conditions of the *Saccharomyces cerevisiae* LD01 strain. The growth of the yeast strain was favored by the supplementation of the culture medium with glucose at a rate of 3 g/L.

2.4. Fermentation monitoring

2.4.1. Physico-chemical analysis

pH: was determined using a pH-meter "Eutech pH 1500", next to a benzene beak. The pH was measured directly in the fermentation brine by rinsing the electrode with sterile distilled water between each of two determinations.

Titratable acidity: It was expressed as a percentage of lactic acid in the broth. It was determined by acid

Table1. Class codes for each sample name and fermentation conditions

Code	Test name	Conditions	References
A	Industrial test	- De-bittering (1.3-2.0% NaOH (w/v)) 5-10h; - First wash (2-3h), 2nd wash (10-20h); - Brining (10.5-11.5%NaCl w/v); - Acidification (citric acid, pH 4.0); - 2 hours maturation. (Anaerobic condition)	Kailis and Harris 2007 Sánchez-Gómez *et al.,* 2006
B	Traditional test	- Washing; - Brining (12.0-15.0% NaCl w/v); - Renewing the brine every two to three days. (Anaerobic condition)	El-Khaloui and Rahmani 2012
C	L1VS	- De-bettering (2% NaOH) - Washing (2h-12h); - Brining (10.6%NaCl w/v); - pH adjustment (3.5); - Seeding with *L. plantarum* S1 (0.75%); (Anaerobic condition)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017
D	L1V	- De-bettering (2% NaOH) - Washing (2h-12h); - Brining (10.6%NaCl w/v); - pH adjustment (3.5); - Seeding with *L. plantarum* S1 (0.75%) and *S. cerevisiae* LD01 (0.75%). (Anaerobic condition)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017
E	L2V	- Washing; - Brining (10.6% NaCl w/v); - Adding vinegar to pH = 3.5; - Seeding with *L. plantarum* S1 (0.75%); (Anaerobic condition)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017
F	L2VS	- Washing; - Brining (10.6% NaCl w/v); - Adding vinegar to pH = 3.5; - Seeding with *L. plantarum* S1 (0.75%) and *S. cerevisiae* LD01 (0.75%). (Anaerobic condition)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017
G	L3V	- Washing; - Brining (10.6% NaCl w/v); - Seeding with *A. pasteurianus* KU710511 (0.75%) in its optimal growth conditions; - Seeding with *L. plantarum* S1 (0.75%); (Alternated aeration condition: 0.2*V/h; 8h/day)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017 Mounir *et al.,* 2016b Pino *et al.,* 2018
H	L3VS	- Washing; - Brining (10.6% NaCl w/v); - Seeding with *A. pasteurianus* KU710511 (0.75%) in its optimal growth conditions; - Seeding with *L. plantarum* S1 (0.75%) and *S. cerevisiae* LD01 (0.75%). (Alternated aeration condition: 0.2*V/h; 8h/day)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017 Mounir *et al.,* 2016b Pino *et al.,* 2018
I	L4V	- Washing; - Brining (10.6% NaCl w/v); - Seeding with *L. plantarum* S1 (0.75%) and *A. pasteurianus* KU710511 (0.75%); (Alternated aeration condition: 0.2*V/h; 8h/day)	Bousmaha *et al.*, 2010 Zaragoza , *et al.*, 2017 Pino *et al.,* 2018
J	L4VS	- Washing; - Brining (10.6% NaCl w/v); - Seeding with *L. plantarum* S1 (0.75%) and *A. pasteurianus* KU710511 (0.75%); - Seeding with *S. cerevisiae* LD01 (0.75%) in its optimal growth conditions x; (Alternated aeration condition: 0.2*V/h; 8h/day)	Bousmaha *et al.*, 2010 Zaragoza *et al.*, 2017 Mounir *et al.,* 2016c Pino *et al.,* 2018

base titration using N/9 sodium hydroxide solution in the presence of phenolphthalein as a colored indicator.

Determination of chloride: NaCl content was determined by titration of the concentration of the chloride ion in the brine according to Mohr's method (Sheen and Kahler 1938).

Determination of oleuropein: Prior to chromatographic analysis, the samples were prepared as follows: 1.5 g of olive flesh were mixed with 20 ml of an 80:20 (v/v) solution of methanol/water. The mixture was homogenized and then centrifuged at 3000 rpm for 5 min. The supernatant was then filtered through a filter paper and then passed through a 0.2 µm supplementary filter. 20 µl of the extract were then injected into the HPLC following a method inspired by Tayoub *et al.,* (2012). Briefly, chromatographic separation was carried out with an LC system coupled to a diode array detector (DAD), using a reversed phase C18 column (250 × 4.6, 3.5 µm) at 35 °C. The mobile phase was a mixture of water, acetonitrile and formic acid in the following proportions, respectively (84.6: 15: 0.4). The flow rate of the mobile phase was 1 ml/min for an injection volume of 20 µL, with UV detection at 240 nm.

2.4.2. Microbiological analysis

LAB, yeasts and AAB were counted using MRS (De MAN *et al.,* 1960), YPD (Mounir *et al.,* 2016a) and GYEA (Mounir *et al.,* 2016c) culture media, respectively. Enumeration was done after 24-48 hours incubation, by counting petri dishes with a number of colonies between 30 and 300.

2.5. Sensory evaluation of olives

Sensory analysis was carried out on olive samples three days after the end of the experiment in order to classify them. Criteria such as external appearance, texture, odor and flavor were evaluated (Table 2). The sensory evaluation was carried out by a panel of 15 tasters composed of professors and students initiated into sensory food analysis (9 females, 6 males at 23–51 years of age), who consumed fermented table olives frequently. Before starting the evaluation, the panelists received a 2-h training session for the sensory evaluation of table olive products and the necessary explanations to carry out this part of the study. All samples were coded with random 3-digit numbers. Pieces of bread were used to restore the taste by reducing the bitter and acidic sensations between two tastings. Each consumer was provided with thirty plates (10 trials with 3 repetitions each) individually and separately, containing five fruits of each treatment of table olive (Rababah *et al.*, 2019). The sensory evaluation was facilitated using the answer sheet presented in the supplementary file 1.

2.6. Statistical analysis of the results

XLSTAT software (2018.3 version) was used to perform a Factorial Correspondence Analysis (FCA) on the sensory analysis results. FCA aims to study the connection or the correspondence between the qualitative characteristics of a contingency table through graphical representations. To do this, ten products noted A to J were prepared using different

Table 2. Class codes for attributes used in sensory evaluation of fermented olives

Attribute	Level	Code *
External appearance	Good	AEB
	Normal	AEM
	Bad	AEV
Firmness	Good	FM
	Normal	FN
	Bad	FD
Odor	Good	OB
	Normal	OM
	Bad	OV
Salty flavor	Good	SB
	Normal	SM
	Bad	SV
Bitterness	Good	AmB
	Normal	AmM
	Bad	AmV
Altered flavor	Good	AlB
	Normal	Alm
	Bad	AlV

* The code represents an abbreviation taking into account the nature of the sensory evaluation attribute and its level: AEB, AEM and AEV, good, normal and bad external appearance, respectively; FM, FN and FD, good, normal and bad firmness, respectively; OB, OM and OV, good, normal and bad odor, respectively; SB, SM and SB, good, normal and bad salty flavor, respectively; AmB, AmM and AmV, good, normal and bad bitterness, respectively; AlB, Alm and AlV, slightly, moderately and formally altered flavor.

combinations (Table 1). To evaluate the quality among these products six attributes were used with three levels for each one (good, medium and bad) as depicted in Table 2.

3. RESULTS

From more than 60 isolates, three microbial strains were selected to carry out experiments aimed at the preparation of natural table olives. These strains, namely : *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 and *Acetobacter pasteurianus* KU710511 (CV01) were identified using biochemical and molecular techniques and characterized in previous studies (Mounir *et al.,* 2018; Mounir *et al.,* 2016a; Mounir *et al.,* 2016b; Mounir *et al.,* 2016c) The determination of chloride, titratable acidity, pH, and counts of LAB, yeasts and AAB were carried out using the methods described in the Material and methods section. Then, samples were presented to tasters to evaluate their organoleptic quality.

The Figure 1 shows that the pH of the traditional test remained between 5 and 5.5 throughout the experiment, with three relatively high values observed between the 40th and 55th day of the experiment. On the other hand, the titratable acidity, expressed in grams of lactic acid per liter started with an acidity of 1.7 at the beginning of the experiment, and gradually decreased to 0.09 g/l, as noted in the 40th day. Then it started to increase continuously to reach 1.75 in the last days of the experiment.

Figure 2 shows the evolution of the number of Lactic Acid Bacteria and Yeast in the brine during the

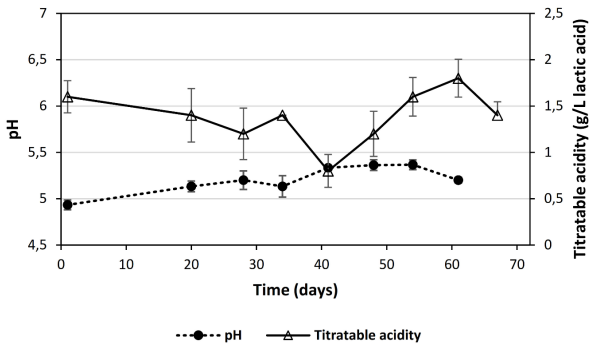


FIGURE1. Changes in the pH (circles) and titratable acidity (triangles) in g/L lactic acid of the brine in traditional tests (brining with 12.0-15.0% NaCl w/v and renewing the brine every two to three days). Data points are mean values ± standard deviation of triplicate samples. Error bars represent standard deviations of values and each value is significantly different at $p < 0.05$ according to the Tuky test.

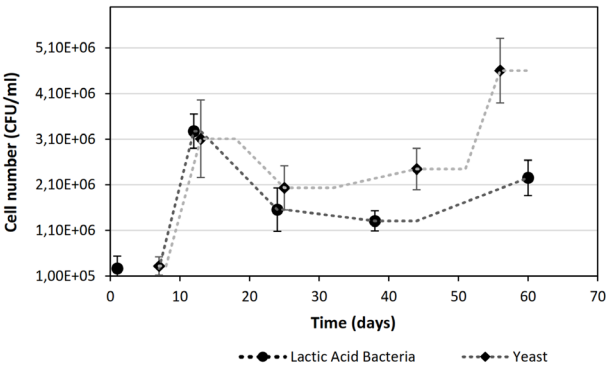


FIGURE 2. Evolution of the number of Lactic Acid Bacteria (circles) and Yeast (diamonds) in the brine during the spontaneous fermentation process of the traditional assay. Each value is an average of three determinations, mean ± SD. Error bars represent standard deviations of values and each value is significantly different at $p < 0.05$ according to the Tuky test. Enumeration was carried out using the plate count method using the MRS and YPD media, respectively, for LAB and Yeast.

spontaneous fermentation process of the traditional assay. This figure reveals that the evolution pattern of the concentration of the two types of microorganisms was similar. The two groups of microorganisms had an average value of $3.2 \cdot 10^5$ CFU/ml during the first days of the experiment. The graph also shows that the concentration of the two groups changed by increasing to exceed $3 \cdot 10^6$ CFU/mL on the 12th day, and decreased to around $2 \cdot 10^6$ CFU/mL between the 12th and 23th days. After that, the number of cells continued to increase slightly for the LAB strains; while the increase was much more significant for the yeast strains on the last 10 days to reach $4.7 \cdot 10^6$ CFU/mL at the end of the experiment.

3.1. Effect of de-bittering and vinegar suppletion on the fermentation process

In this part of the study, de-bettering was performed with sodium hydroxide and two successive washes. After that, date vinegar, produced with the *S. cerevisiae* LD01 yeast strain along with the *L. plantarum* S1 LAB strain, was added to the 10.6% (w/v) NaCl brine, all anaerobically. Then, three flasks were inoculated with the LAB strain only and three others were inoculated both with the LAB and yeast strains. This test was not seeded by the AAB strain, so we did not count it.

Figure 3 shows the effect of de-bittering on fermentation process parameters with and without inoculation with the *S. cerevisiae* strain. As can be seen from the graphs in Figure 3.A, the titratable acidity for samples not seeded by the *S. cerevisiae*

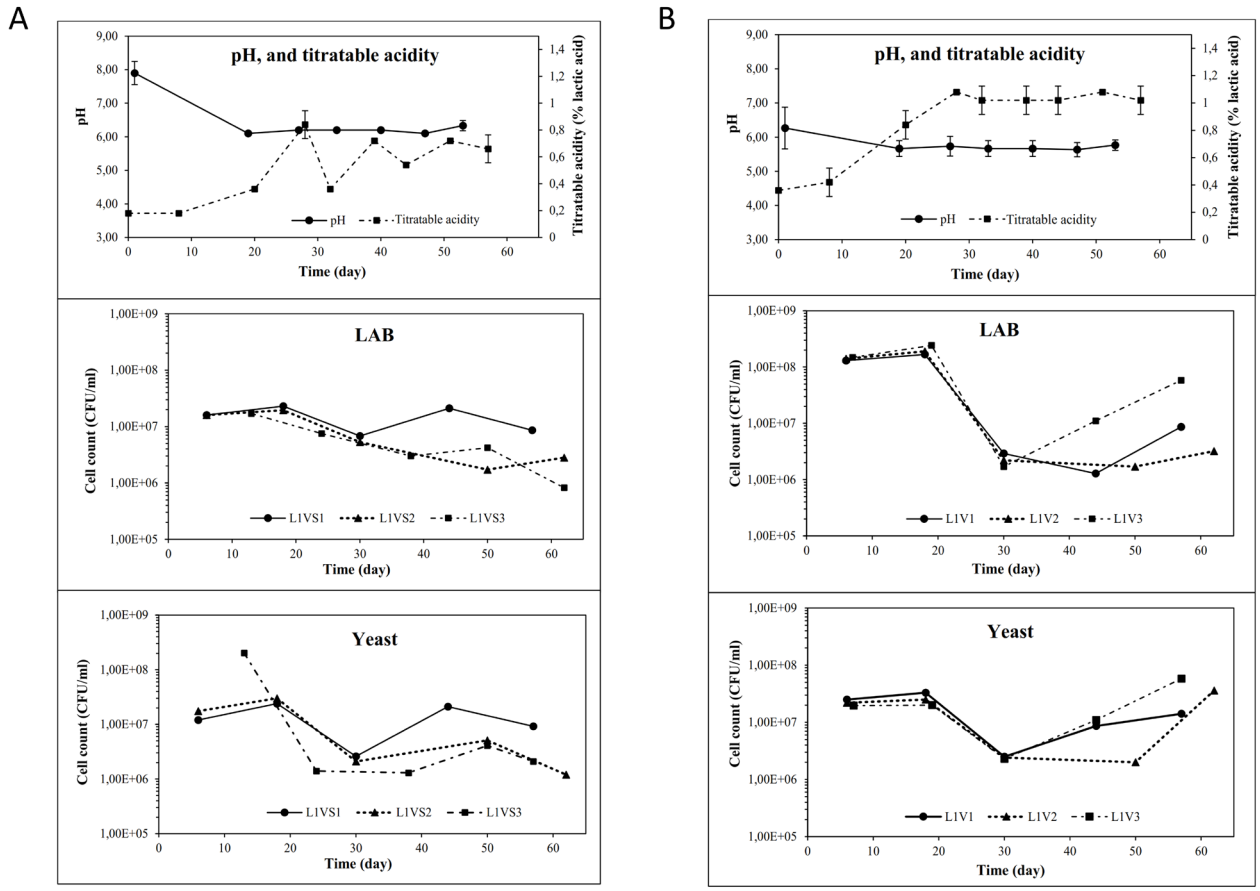


FIGURE 3. Fermentation process parameters in de-bittered samples using 2–3.5% w/v NaOH. Three replicates (L1V1, L1V2 and L1V3) were inoculated with 0.75% of *S. cerevisiae* LD01 strain (B), and three others were not (A) used as reference (L1VS1, L1VS2 and L1VS3). All samples were inoculated with 0.75% of *L. plantarum* S1 LAB strain. The samples were prepared according to the descriptions given in Table 1 (test L1V and L1VS, respectively). The tests were carried out anaerobically with one kg of olives brined in 2-liter bottles. Cell numbers were determined by the plate count method using MRS and YPD media, respectively, for LAB and Yeast.

strain was 0.2 g/L at the beginning of the experiment, then gradually increased to 0.8 g/L lactic acid on the 27th day and then varied at around 0.67 g/l until the end of the experiment. For inoculated samples with the *S. cerevisiae* strain (Figure 3.B), the pH started with a value of 6.2 at the beginning of the fermentation cycle, then the pH stabilized at 5.8. The titratable acidity started with a value of 0.4 g/L lactic acid and it then gradually increased from the 8th day to around 1 g/L to be stabilized at this value afterwards. In this assay, the microbial count of the three repetitions monitored separately showed the same trend, especially during the first thirty days. The average of the initial values for the LAB strain in inoculated samples (Figure 3.B) was quite high ($1.4 \cdot 10^8$ CFU/mL), and a very slight increase was observed up to the 18th day followed by a sudden drop until the 30th day to be close to a concentration of 10^6 CFU/ml. A slight increase occurred during the last 30 days to reach a maximum value of $5.8\ 10^7$ CFU/ml for the third repetition and around $5.9 \cdot 10^6$ CFU/mL for the other two replicates (Figure 3.B).

On the other hand, Figure 3.B shows that the *S. cerevisiae* LD01 strain experienced similar growth for the 3 replicates, with an average concentration of $2.2 \cdot 10^7$ CFU/mL, which remained more or less stable until the 20th day, followed by a decrease in concentration to arrive at $2.4 \cdot 10^6$ CFU/mL. After that, the three repetitions experienced an increase in concentration with slopes that differed between each repetition to reach the end of the experiment at an average value of $3.6\ 10^7$ CFU/mL. However, Figure 3.A shows that the LAB strain had an average growth value of 10^7 CFU/mL at the beginning of the experiment, with a concentration of $2 \cdot 10^8$ CFU/mL on the 13th day for the third repetition. The

concentration remained approximately at an average of 5 10^6 CFU/mL, then dropped slightly for the 3 samples to reach $9 \cdot 10^6$, $1.2 \cdot 10^6$ and $2.1 \cdot 10^6$ CFU/mL for the three repetitions, respectively. Figure 3.A also shows that the yeast concentration of the first repetition showed an overall decrease from $1.65 \cdot 10^7$ CFU/mL to reach $8.6 \cdot 10^6$, $2.80 \cdot 10^6$ and $8.2 \cdot 10^5$ CFU/mL, repetitively, for repetitions 1, 2 and 3.

The second experiment was conducted with acidification of the brine with 10.6% (m/v) date vinegar produced in the lab using the selected yeast and AAB strains. The fermentation was carried out under anaerobic conditions. Three flasks were inoculated with the LAB strain (L2V1, L2V2 and L2V3) and three others were inoculated with the LAB and yeast strains (L2VS1, L2VS2, L2VS3). Microbiological counting of this test gave no results for lactic acid bacteria and yeasts during the first days of the experiment.

In samples not inoculated with *S. cerevisiae* (Figure 4.A), the starting pH was 3.9, which was adjusted by the addition of vinegar, then the pH increased to an average value of 4.5 on the 19th day. Then, from the 20th day, the pH fluctuated between 4.5 and 5 until the end of the experiment. For titratable acidity, the average starting value was 3.18 g/L lactic acid, and it then slightly decreased until the 28th day with 3.00 g/l and then stabilized at an average value of 3.50 g/l. For microbial growth, the curves obtained for the three repetitions did not keep the same pace. However, we noticed that there was a slightly positive effect on the growth of LAB but an insignificant effect on the growth of endogenous yeasts (Figure 4).

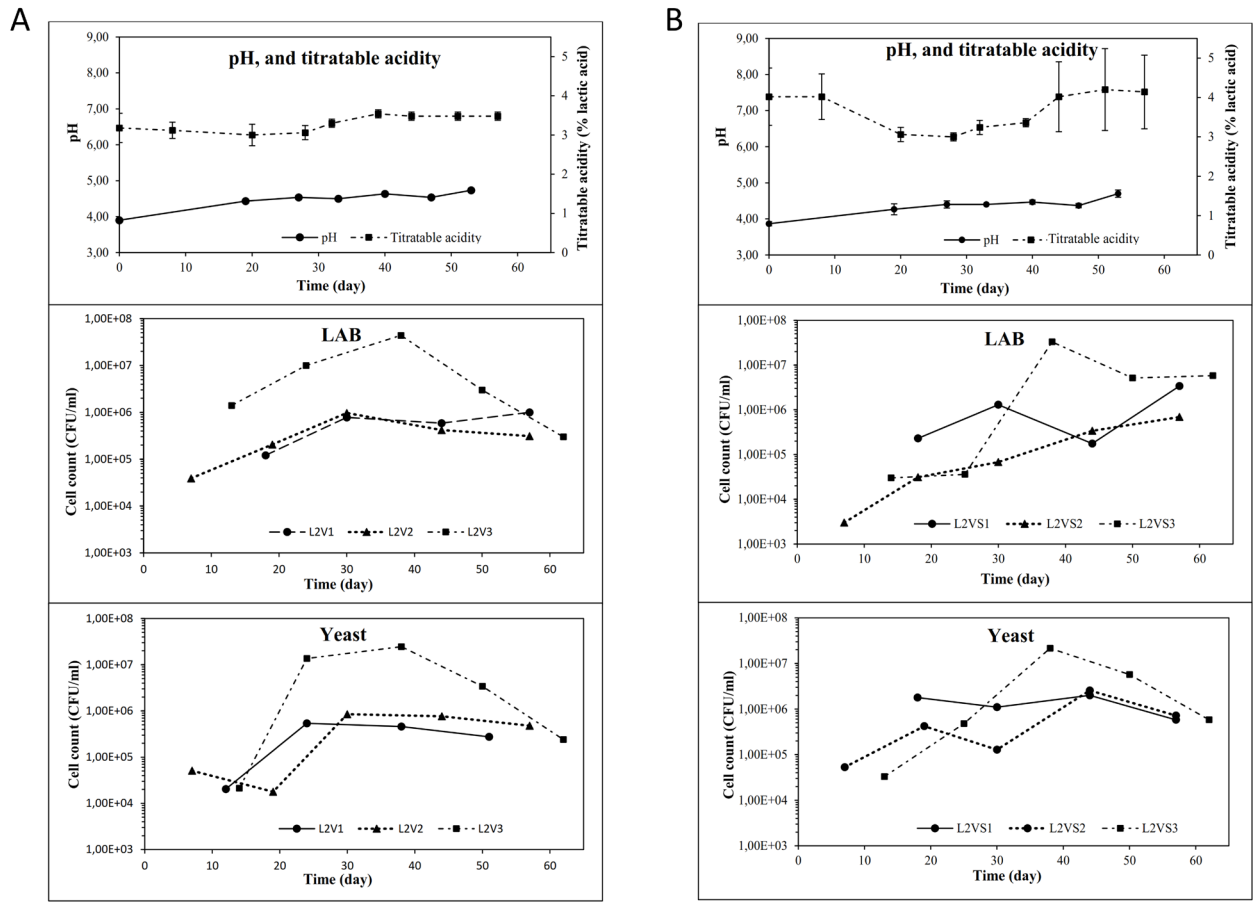


FIGURE 4. Fermentation process parameters in non de-bittered samples with acidification of the brine with 10.6% (m/v) date vinegar. Three replicates (L2VS1, L2VS2, L2VS3) were inoculated with 0.75% of *S. cerevisiae* LD01 strain (B), and three others were not (A) used as reference (L2V1, L2V2 and L2V3). All samples were inoculated with 0.75% of *L. plantarum* S1 LAB strain. The samples were prepared according to the description provided in Table 1 (test L2VS and L2V respectively). The tests were carried out anaerobically with one kg of olives brined in 2-liter bottles. Cell numbers were determined by the plate count method using MRS and YPD media, respectively for LAB and Yeast.

3.2. Alternated aeration effect on microbial growth in the brine of naturally fermented olives

The olives of this test were prepared in brine at 6% (w/v) NaCl under the optimal conditions of *A. pasteurianus* KU710511 strain with alternating aeration at a rate of 0.2xV/h and 8 hours per day. Three flasks were seeded with LAB and AAB strains (L3V1, L3V2, L3V3) and three others with LAB, AAB and the yeast strains (L3VS1, L3VS2, L3VS3).

A shown in Figure 5A, the pH was around 3.9 during the first 28 days, and increased substantially to be stabilized at around 5.2 from day 40. The acidity started with an average value of 13.5 g/L of lactic acid and dropped significantly and stabilized at an average of 3 g/L. The pH was around 3.9 during the first 28 days for samples that were inoculated with the yeast strain (Figure 5.B), and increased significantly to about 5.5 by the end of the experiment. The acidity started with an average value of 16 g/L of lactic acid and dropped significantly and stabilized at an average value of 2.9.

We also noted that during the first days of the experiment, the yeasts showed an average growth of $8.35 \cdot 10^5$ CFU/mL and then increased to around $8 \cdot 10^9$ CFU/mL. Subsequently, the yeasts of L3V1 fell to

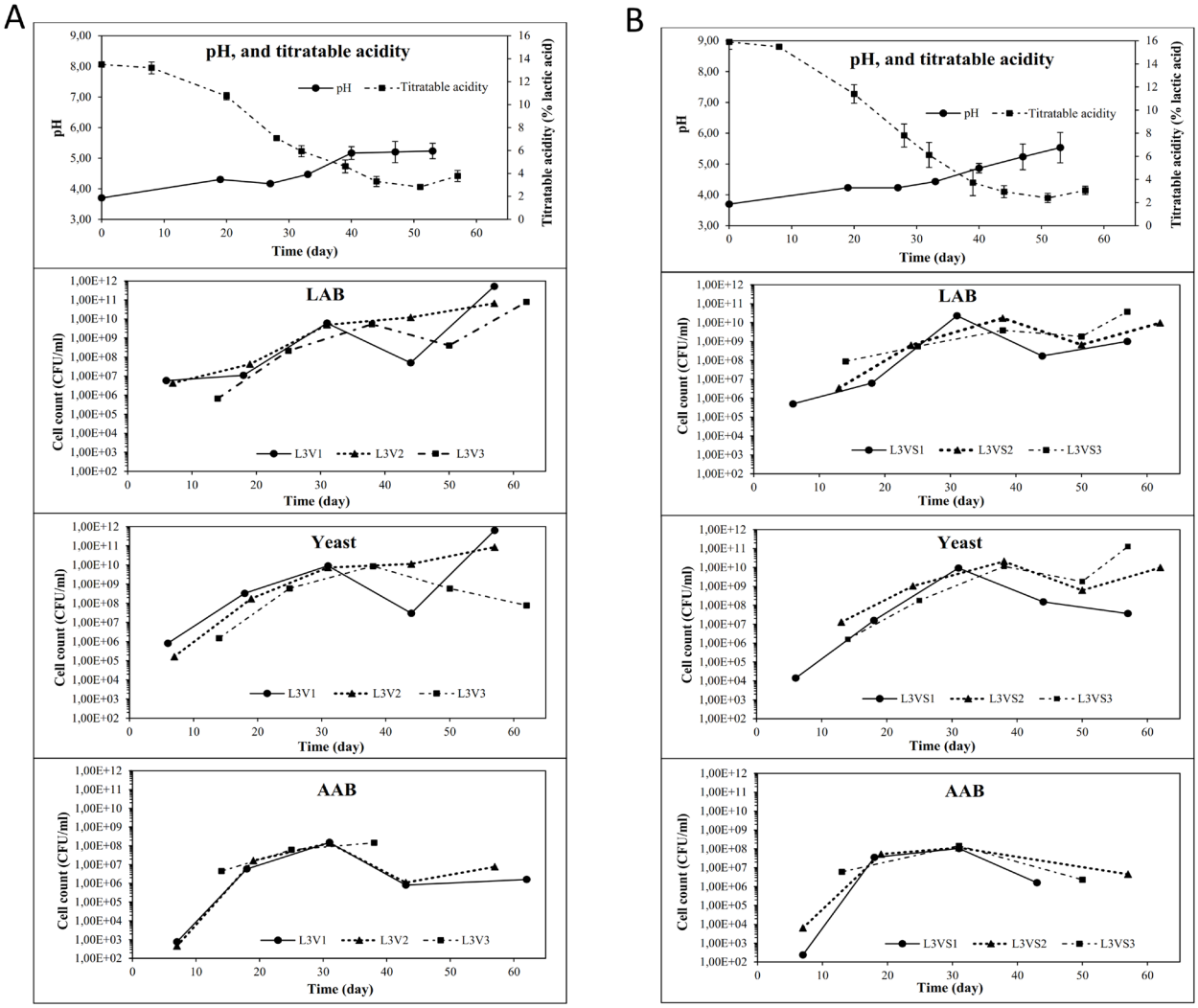


FIGURE 5. Fermentation process parameters of naturally fermented samples under alternating aeration and inoculation by AAB strain conditions. Three replicates (L3VS1, L3VS2 and L3VS3) were inoculated with 0.75% of *S. cerevisiae* LD01 strain (B), and three others were not (A) used as reference (L3V1, L3V2 and L3V3). All samples were inoculated with *A. pasteurianus* KU710511 (0.75%) and with *L. plantarum* S1 LAB strain (0.75%). The samples were prepared according to the description provided in Table 1 (test L3VS and L3V, respectively). Cell numbers were determined by the plate count method using MRS and YPD media, respectively, for LAB and Yeast.

$3 \cdot 10^7$ CFU/mL on the 44th day, increasing to $6.4 \cdot 10^{11}$ CFU/mL 13 days later. Those of L3V2 continued their progressive rise to arrive at 8.5 10^{10} UFC/mL towards the end (57th day). L3V3 peaked on the 38th day at $8.7 \cdot 10^9$ and dropped to $7.8 \cdot 10^7$ CFU/mL on day 62. Whereas, the LAB strain exhibited a growth of approximately $5 \cdot 10^6$ CFU/ml at the beginning of the experiment, then gradually increased to an average value of $2.21 \cdot 10^{11}$ CFU/mL towards the end of the experiment with an intermediate increase at around $5.8 \cdot 10^9$ CFU/mL on day 31 for L3V1 and on day 38 for L3V3. On the other hand, the AAB strain started at very low concentrations (about 10^3 CFU/mL), and subsequently increased to around 10^8 CFU/mL by the 30th day. Subsequently, the concentration decreased to be stable at $5 \cdot 10^6$ CFU/mL from the 42nd day to the end of the experiment.

However, Figure 5B shows that the yeast strain showed a growth of $1.4 \cdot 10^4$ CFU/mL for the L3VS1 test at day 6. As for the L3VS2 and L3VS3 repetitions, the first determination made on the 13th day gave an average of $7.2 \cdot 10^6$ CFU/mL. Subsequently, the L3VS2 and L3VS3 replicates ranged from around $5 \cdot 10^9$ CFU/mL; while L3VS1, after reaching a maximum of 9.3 10^9 CFU/ml, gradually decreased to $3.7 \cdot 10^7$ CFU/mL at the 57th day. The LAB strain had a cell concentration of approximately $5 \cdot 10^5$ CFU/mL at the beginning of the experiment, then gradually increased to an average value of approximately $1.62 \cdot 10^{10}$ CFU/mL towards the end of the experiment with an intermediate increase to around $1.5 \cdot 10^{10}$ CFU/mL on the 36th day. Finally, the AAB strain started at very low concentrations (about 10^3), and subsequently increased to around 10^8 by the 30th day. Subsequently the concentration decreased to be stable at $3.5 \cdot 10^6$ CFU/mL towards the end of the experiment.

3.3. Organoleptic evaluation of produced olives

The contingency table (supplementary file 2) contains the frequencies of the tasters who noted the different quality criteria of the samples by classifying them as "good", "medium" or "bad". Criteria were then subjected to Factorial Correspondence Analysis. The calculation of eigenvalues indicates that the variation shares explained by axis 1 and axis 2 were 50.1% and 22.6%, respectively. The main plane constituted by these two axes therefore alone accounted for 72.7% of the total variation of the cloud of points (Figure 6). The projection of the line points (10 samples) and the column points (quality criteria) in the main plane is shown in Figure 6. Examination of this figure indicates that:

The vertical axis F2 opposes the group formed by the samples C, D, A, G and H characterized by good appearance, good bitterness and quite normal firmness to the samples B, E and F, which have opposite characteristics, namely a normal to bad external appearance, a hard firmness and a quite pronounced bitterness. The horizontal axis F1 opposes the group formed by the samples G and H, characterized by a good smell and a normal salty flavor compared to the group formed by A, C and D, which is characterized by a normal to bad odor and a medium to bad salty flavor. Samples I and J were close to the center and are therefore not very well presented by this plan. However, it can be said that they have good appearance with a good smell to bad, fairly strong bitterness and hard firmness.

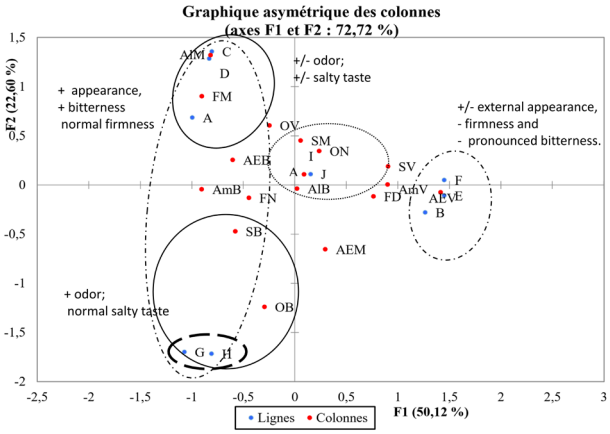


Figure 6. Graphical representation of the results of a Factorial Correspondence Analysis (FCA) of the sensory evaluation of the fermented olives: projection in factorial axes 1 and 2 of fermented olives groups (A, B, C, D, E, F, G, H, I and J (Table 1)), regarding classes of the qualitative attributes (External appearance, firmness, odor, salty flavor, bitterness and altered flavor (Table 2)). The vertical axis opposes the group formed by the samples C, D, A, G and H, which are characterized by good appearance, good bitterness and normal firmness as compared to the samples B, E and F, which had opposite characteristics (dash-point). The horizontal axis opposes the group formed by the samples G and H, which are characterized by good attributes compared to the group formed by A, C and D (solid line). G and H samples were expected to be the most highly appreciated ones.

4. DISCUSSION

In this study, four different combinations were applied to the "*Picholine marocaine*" olive variety

using indigenous strains, namely *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 and *Acetobacter pasteurianus* KU710511 (CV01).

First, we noted that the greater or lesser variability between the repetitions of the same test at the level of the microbiological characteristics can be due to the moment of analysis. Since these were not carried out at the same time for the three repetitions (considering our human and material resources), we opted for the separation of repetitions over time by taking all the necessary precautions (operator, material and environment effects).

The traditional test was the only one where the brine was changed regularly (every three days), which may explain the variability in the microbiological results of the three repetitions, and also the fact that the pH alternatively varied from around 5.3 throughout the experiment. This behavior was also observed for titratable acidity. We also noted that the increase in microbial count, both for LAB and yeast strains, was not very pronounced since after a first value of about $2 \cdot 10^5$ CFU/mL there was an increase to reach 10^6 CFU/mL, then there was stagnation around this value throughout the experiment.

Results found by Zaragoza *et al.,* (2017) revealed that the introduction of exogenous spoilage yeast and LAB into olive fermentations caused significant but distinct alterations in the numbers and diversity of microbes associated with the olives and brines. According to the microbial evolution of tests 1 and 2 (Table 1), we noted that the acidity had a more severe effect on the microbial concentration than the basicity, indeed this concentration was very weak or even null in the first days of experiment for test 2 (supplemented by vinegar). Whereas for test 1, where pH values were around 6 to 7 (because of NaOH residues), the population was relatively high and even reached 10^8 CFU/mL at the beginning of the experiment but dropped gradually after a few days.

We also noted that alternate aeration increased the concentration of the LAB and the yeast strains, since for the anaerobic tests, it did not exceed 10^7 CFU/ml; whereas for tests 3 and 4 (alternating aeration) it reached 10^{11} CFU/mL. A possible relationship between LAB and yeasts during the fermentation of olives in brine was reported by (Heperkan 2013). In addition to the LAB usually used in this kind of fermentation, the use of *Saccharomyces cerevisiae* yeast as a starter is justified by the role that it can play in the fermentation of olives by producing ethanol, ethylacetate, acetaldehyde and other compounds responsible for the development of olive flavors (Ciafardini and Zullo, 2019; Fernandez Escobar *et al.,* 2013).

On the other hand, it was demonstrated that fermentation in altered aeration mode, served to modify the metabolic pathway of *L. plantarum* by promoting the conversion of lactate into acetate (Bobillo and Marshall 1991). Indeed, acetic acid can inhibit the growth of gram-negative strains which are responsible for the degradation of table olives during storage (Makras and De Vuyst 2006). In addition, this process allowed us to use lower concentrations of NaCl (6% w/v) compared to the concentrations used in anaerobic fermentations (10 to 15% w/v). Reducing salt in the processing of olives is always a goal. In fact, a diet that is low in sodium and high in potassium and calcium is recommended to lower blood pressure and to protect against osteoporosis, colon cancer, and cardiovascular diseases (Degirmencioglu, 2016).

The combination of alternating aeration, the use of optimal growth conditions of the AAB strain and the chosen NaCl concentration served to create a synergy among the three strains which led to the elaboration of table olives with very good organoleptic quality and in a fairly short time (40 days).

On the other hand, for test 4, the use of alternating aeration with the same NaCl concentration as test 3 was not sufficient to obtain good olives after 60 days of fermentation. This finding suggests that this trial required more time, despite the high LAB and yeast populations (of the same order of magnitude as in test 3), suggesting that the AAB strain plays a vital role in the success of fermentation by creating the synergy necessary to obtain table olives with good sensory quality in a relatively short time. Indeed, aerobic fermentation is generally used to avoid the appearance of a deterioration called "gaseous pockets" which is manifested by the swelling of the skin caused by accumulation of gas under the epidermis of the olive (Lanza 2013). In addition, AAB strain could adjust the acidity of the medium and thus promote the onset of fermentation by providing yeasts and lactic acid bacteria with favorable conditions for their development by continuously eliminating the ethanol produced by the yeast and thus maintaining an acceptable level of pH by producing acetic acid (Hammoucha and Taleb, 2017).

Concerning the industrial test, lye de-bettering resulted in olives without bitterness and a good appearance, although the texture was judged as normal to soft. On the other hand, the L3V test led to both a very good taste, a normal texture and a salinity which was very much appreciated compared to the industrial test.

5. CONCLUSION

The objective of the work was to contribute to the development of a method for the preparation of natural table olives using locally selected microorganisms and without resorting to chemical additives. The effects of the parameters, essentially inoculation with *Lactobacillus plantarum* S1, *Saccharomyces cerevisiae* LD01 and *Acetobacter pasteurianus* KU710511 strains and alternating aeration were assessed. The samples were inoculated with pure suspensions of these microorganisms and then monitored over time using the plate count method. Only molecular analyses could provide accurate data on the survival of the selected starters during fermentation, but the inoculated tests by selected strains most likely contributed to reach those CFU/ml numbers as they were inoculated in a high number at the beginning of the fermentations.

It has been reported previously that acetic acid, the main product of oxidative fermentation by AAB in ethanol, is a compound that has a positive correlation with the growth of yeasts and could have an accelerating effect on LAB growth (Pino *et al.,* 2018). The results obtained served to confirm that the aerobic fermentation normally used for the elimination of the deterioration of table olives due to "gas pockets" can also be used to create the conditions necessary for the introduction of the acetic acid bacteria which are strict aerobes in the fermentation process of the olives. In addition, alternated aeration conditions allowed for increasing the cell biomass during fermentation and the use of a lower concentration of NaCl compared to anaerobic fermentation.

The microbiological, physicochemical and sensory analyses carried out demonstrated the possibility of introducing a new process for producing natural table olives. In this method, we used a starter composed of two strains, namely *Acetobacter pasteurianus* KU710511 (CV01) and *Lactobacillus plantarum* S1 under the optimal conditions of AAB growth as reported by Mounir *et al.,* (2016b) with 6% NaCl and alternating aeration of 8 hours/day.

ACKNOWLEDGMENTS

We would like to express our sincere gratitude to Mostafa Cheheb, laboratory technician, for his valuable help and constructive suggestions during the planning and development of this work.

REFERENCES

Amer A, Faiz ul-Hassan N, Kashfa B, Aasia B. 2017. Microbial β-Glucosidase: Sources, Production and Applications. *J. Appl. Env. Microbiol* **5**, 31-46. https://doi.org/10.12691/JAEM-5-1-4

Anon. 2016. International olive council. *Conclusions IOC Conf. COP22*. Available at: http://www.internationaloliveoil.org/news/view/686-year-2016news/787-conclusions-of-the-ioc-conference-at-cop22?lang=fr_FR [Accessed July 20, 2017].

Beuchat LR. 1992. Media for detecting and enumerating yeasts and moulds. *Int. J. Food Microbiol.* **17**, 145-158. https://doi.org/10.1016/0168-1605(92)90112-G

Bobillo M, Marshall VM. 1991. Effect of salt and culture aeration on lactate and acetate production by *Lactobacillus plantarum*. *Food Microbiol.* **8**, 153-160. https://doi.org/10.1016/0740-0020(91)90008-P

Bousmaha L, El Yachioui M, Ouhssine M. 2010. Amélioration du procédé de fermentation traditionnelle des olives vertes. *Afrique Sci. Rev. Int. des Sci. Technol.* **5**, 114–125.

Campus M, Degirmencioglu N, Comunian R. 2018. Technologies and trends to improve table olive quality and safety. *Front. Microbiol.* **9**. https://doi.org/10.3389/fmicb.2018.00617

Chemonics International, Inc. 2007. Guide De Bonnes Pratiques De Fabrication Des Olives De Table, *Agriculture and Agrobusiness Intégrés,* USAID-Maroc, Repport n. 071, pp.1-42.

Ciafardini G, Zullo BA. 2019. Use of selected yeast starter cultures in industrial-scale processing of brined Taggiasca black table olives. *Food Microbiol.* **80**, 103250. https://doi.org/10.1016/j.fm.2019.103250

Cleenwerck I, De Vos P. 2008. Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology. *Int. J. Food Microbiol.* **125**, 2-14

Colmagro S, Collins G, Sedgley M. 2001. Processing Technology of the Table Olive.

in Jules J, (Ed.) *Horticultural Reviews*. John Wiley and Sons, Inc, pp. 235-242. https://doi.org/10.1002/9780470650783.ch5

Degirmencioglu N. 2016. Modern Techniques in the Production of Table Olives. in Boskou D, Clodoveo ML, (Ed.) *Products from Olive Tree*. IntechOpen, pp. 215–234. https://doi.org/10.5772/64988

El-Khaloui M, Nouri A. 2007. Procédés d'élaboration des olives de table à base des variétés Picholine marocaine et Dahbia. *Transfert de Technologie en Agriculture* **037**, 77–80.

El-Khaloui M, Rahmani M. 2012. Ypicité des préparations traditionnelles d'olives de table dans la province d'Ouazzane. *Transfert de Technologie en Agriculture* **197**, 1–5.

Fan L, Hansen LT. 2012. Fermentation and biopreservation of plant-based foods with lactic acid bacteria. *Handb. Plant-Based Fermented Food Beverage Technol. Second Ed.*, 35-48. https://doi.org/10.1201/b12055-5

Faid M. (n.d.). *Beware of eating red olives / Mohamed Faid - YouTube*. Retrieved April 6, 2020, from https://www.youtube.com/watch?v=yJoUDDsy-c0&t=75s

Fernandez Escobar R, de la Rosa R, Leon L. 2013. Evolution and sustainability of the olive production systems. *Options Méditerranéennes. Séries A Mediterr. Semin.* **106**, 11-41.

Hammoucha J, Taleb O. 2017. *Contribution à l'amélioration des conditions de fermentation des olives de table*. Institut Agronomique et Vétérinaire Hassan II, Rabat. Maroc

Heperkan D. 2013. Microbiota of table olive fermentations and criteria of selection for their use as starters. *Front. Microbiol.* **4**, 1-11. https://doi.org/10.3389/fmicb.2013.00143

Kailis S, Harris DJ. 2007. *Producing table olives*, Landlinks Press. https://doi.org/10.1071/9780643094383

Kavroulakis N, Ntougias S. 2011. Bacterial and β-proteobacterial diversity in *Olea europaea* var. mastoidis- and *O. europaea* var. koroneiki-generated olive mill wastewaters: Influence of cultivation and harvesting practice on bacterial community structure. *World J. Microb. Biotechnol.* **27** (1), 57-66. https://doi.org/10.1007/s11274-010-0426-3

Lanza B. 2013. Abnormal fermentations in table-olive processing: Microbial origin and sensory evaluation. *Front. Microbiol.* **4**, 1-7. https://doi.org/10.3389/fmicb.2013.00091

Makras L, De Vuyst L. 2006. The *in vitro* inhibition of Gram-negative pathogenic bacteria by bifidobacteria is caused by the production of organic acids. *Int. Dairy J.* **16**, 1049-1057. https://doi.org/10.1016/j.idairyj.2005.09.006

Man JC de, Rogosa M, Sharpe ME. 1960. A edium for the cultivation of Lactobacilli. *J. Appl. Bacteriol.* **23**, 130-135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.x

Marsilio V, Campestre C, Lanza B. 2001. Phenolic compounds change during California-style ripe olive processing. *Food Chem.* **74**, 55-60. https://doi.org/10.1016/S0308-8146(00)00338-1

Mounir M, Fauconnier ML, Afechtal M, Thonart P, Ismaili Alaoui M, Delvigne F. 2018. Aroma profile of pilot plant-scale produced fruit vinegar using a thermo-tolerant *Acetobacter pasteurianus* strain isolated from Moroccan cactus. *Acetic Acid Bact.* **7**, 1-11. https://doi.org/10.4081/aab.2018.7312

Mounir M, Belgrire M, Lahnaoui S, Hamouda A, Thonart P, Delvigne F, Ismaili Alaoui M. 2016a. Maîtrise de la fermentation alcoolique sous stress éthanolique, thermique et osmotique de la souche *Saccharomyces cerevisiae* YSDN1 en vue de la préparation du vinaigre de fruits. *Rev. Marocaine Sci. Agron. Vétérinaires* **4**, 86-95.

Mounir M, Shafiei R, Zarmehrkhorshid R, Hamouda A, Thonart P, Delvigne F, Ismaili Alaoui M. 2016b. Optimization of biomass production of *Acetobacter pasteurianus* KU710511 as a potential starter for fruit vinegar production. *African J. Biotechnol.* **15**, 1429-1441. https://doi.org/10.5897/AJB2016.15323

Mounir M, Shafiei R, Zarmehrkhorshid R, Hamouda A, Ismaili Alaoui M, Thonart P. 2016c. Simultaneous production of acetic and gluconic acids by a thermotolerant *Acetobacter strain* during acetous fermentation in a bioreactor. *J. Biosci. Bioeng.* **121**, 166-171. https://doi.org/10.1016/j.jbiosc.2015.06.005

Pino A, De Angelis MD, Todaro A, Van Hoorde KV, Randazzo CL, Caggia C. 2018. Fermentation of Nocellara Etnea table olives by functional starter cultures at different low salt concentrations. *Front. Microbiol.* **9**. https://doi.org/10.3389/fmicb.2018.01125

Rababah T. 2019. Sensory properties of green table olives prepared by different fermentation processes. *CYTA - J. Food* **17**, 997-1005. https://doi.org/10.1080/19476337.2019.1693430

Ramírez E, Medina E, García P, Brenes M, Romero C. 2017. Optimization of the natural debittering of table olives. *LWT - Food Sci. Technol.* **77**, 308-313. https://doi.org/10.1016/j.lwt.2016.11.071

Rokni Y, Ghabbour N, Chihib NE, Thonart P, Asehraou A. 2015. Physico-chemical and microbiological characterization of the natural fermentation of moroccan picholine green olives variety. *J. Mater. Environ. Sci.* **6**, 1740-1751.

Rincón-Llorente B. 2018. Table olive wastewater: Problem, treatments and future strategy. A review. *Front. Microbiol.* **9**. https://doi.org/10.3389/fmicb.2018.01641

Sánchez Gómez AH, García García P, Rejano Navarro L. 2006a. Trends in table olive production Elaboration of table olives. *Grasas Aceites* **57** (1), 86-94. https://doi.org/10.3989/gya.2006.v57.i1.24

Shahidi F, Kiritsakis A eds. 2017. *Olives and Olive Oil as Functional Foods*, John Wiley & Sons, Ltd, Chichester, UK. https://doi.org/10.1002/9781119135340

Sheen HT, Kahler HL. 1938. Effect of ions on Mohr method for chloride determination. *Ind. Eng. Chem. Anal. Ed.* **10**, 628-629. https://doi.org/10.1021/ac50127a004

Tayoub G, Sulaiman H, Hassan AH, Alorfi M. 2012. Determination of Oleuropein in leaves and fruits of some Syrian olive varieties. *Int. J. Med. Aromat. Plants* **2**, 428-433.

Valero A, Medina E, Arroyo-López FN. 2017. Microbial hazards and their implications in the production of table olives. in Singh O V, (Ed.) *Foodborne Pathogens and Antibiotic Resistance*. John Wiley & Sons, Inc, pp. 119-138. https://doi.org/10.1002/9781119139188.ch5

Zaragoza J, Bendiks Z, Tyler C, Kable ME, Williams TR, Luchkovska Y, Chow E, Boundy-Mills K, Marco ML. 2017. Effects of Exogenous Yeast and Bacteria on the Microbial Population Dynamics and Outcomes of Olive Fermentations. *MSphere* **2**, 1-14. https://doi.org/10.1128/mSphere.00315-16

GRASAS Y ACEITES 72 (2)
April-June 2021, e408
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0326201

Determination of Hansen solubility parameters for sugarcane oil. Use of ethanol in sugarcane wax refining

E. Hernández✉, M. Díaz and K. Pérez

Cuban Research Institute for Sugarcane Derivatives
✉Corresponding author: ehr930815@gmail.com

Submitted: 03 March 2020; Accepted: 15 May 2020; Published online: 21 June 2021

SUMMARY: Currently, the refining of sugarcane wax is carried out with 95% v/v ethanol as solvent. This process has a high rate of ethanol consumption thus, the main objective of this work was to corroborate the feasibility of 95% v/v ethanol as a solvent in the refining of sugarcane wax. The suitability of its performance over other organic solvents was evaluated through the relative energy difference (RED) determined from Hansen solubility parameters (HSPs) of sugarcane oil, which were calculated using HSPiP software. HSPs turned out to be δ_D = 16.24 MPa^{½}, δ_P = 3.21 MPa^{½} and δ_H = 10.34 MPa^{½}, similar to those reported for pine resin and castor oil. The best solvent was 1-decanol. 95% v/v ethanol turned out to be a bad solvent. Absolute ethanol had a RED value of 0.993, which made it a better candidate since it could reduce consumption rates, and constituted an eco-friendly solvent produced in Cuba for the refining process.

KEYWORDS*: Ethanol; Hansen solubility parameters; Sugarcane oil; Sugarcane press mud*

RESUMEN: ***Determinación de los parámetros de solubilidad de Hansen para el aceite de caña de azúcar. Uso del etanol para la refinación de la cera de caña de azúcar.*** La refinación de la cera cruda de caña se realiza con etanol a 95% v/v, incurriendo en altos índices de consumo, por lo que el objetivo principal de este trabajo es determinar si el etanol a 95% v/v es un solvente adecuado. Su uso respecto a otros solventes orgánicos es evaluado por la diferencia de energía relativa (RED) determinada a partir de los parámetros de solubilidad de Hansen (HSPs), se calcularon usando el programa HSPiP. Los HSPs fueron δD = 16.24 MPa^{½}, δP = 3.21 MPa^{½} and δH = 10.34 MPa ½, siendo similares a los reportados para la resina de pino y el aceite de ricino. El mejor solvente fue 1-decanol, el etanol del 95% v/v fue un mal solvente; el etanol absoluto con un RED= 0.993 es un mejor candidato, ya que podría reducir las tasas de consumo, manteniendo un solvente ecológico y producido en Cuba para el proceso de refinación.

PALABRAS CLAVE*: Aceite de caña de azúcar; Cachaza; Etanol; Parámetros de solubilidad de Hansen*

Citation/Cómo citar este artículo: Hernández E, Díaz M, Pérez K. 2021. Determination of Hansen solubility parameters for sugarcane oil. Use of ethanol in sugarcane wax refining. *Grasas Aceites* **72** (2), e408. https://doi.org/10.3989/gya.0326201

1. INTRODUCTION

The press mud, obtained during the purification of sugarcane juice, is mainly used as an organic fertilizer in sugarcane fields, as well as in biogas production. The mud contains sugarcane wax at 3% w/w of the cake (ICIDCA, 2000). During the second half of the last century in Cuba the American Sugar Co. Swenson (1947) developed and introduced a technology for the production of sugarcane wax by extraction with heptane, which was further exported to the United States for refining. During the 90s a wax refining technology was developed, and a wax refining plant was built in Cuba due to the interest in refined wax (WR) as raw material for the production of some pharmaceutical products.

Sugarcane wax has a dry base average composition of 68.53% wax, 28.61% oil and 2.47% impurities, also called resins. The sugarcane wax refining technology consists of a solvent extraction process with 95% v/v ethanol. The refining process begins by bringing the sugarcane wax into contact with cold 95% v/v ethanol for eight hours in a stirring tank. Then the dispersed solid is centrifuged and the heavy phase, rich in refined wax, is subsequently extracted with hot ethanol in an extractor-decanter centrifuge; the refined wax is cooled and separated in a disk centrifugal bowl. After that, the refined wax is dried and packaged for later commercialization. This technology uses 95% v/v ethanol as solvent because it is a renewable solvent of national production. There are several reports about the use of ethanol for vegetable oil extraction, but there is no evidence about how useful it is compared to other solvents.

The extraction of vegetable oils with ethanol is not an exclusive feature of sugarcane wax refining, since literature reports several works about the solubility degree of this solvent with several vegetable oils. During the first half of the last century, Taylor *et al.,* (1936) investigated the solubility of 14 different vegetable oils in ethanol, including corn, olive, peanut, peach, soybean and sunflower oils, among others. This study showed that they have different solubility degrees according to their composition and that they vary from 1 to 10 kg of oil per 100 kg of ethanol at 90% w/w and between 5 to 15 kg of oil for 100 kg of absolute ethanol. Measurements were made at 25 ˚C. The results showed the influence of small amounts of water on the solubility of vegetable oils in this solvent. In their studies Kaparthi *et al.,* (1955) concluded that even in the case of absolute ethanol, this solvent is not a useful solvent for vegetable oils, such as sunflower, peanut and cottonseed because concentrations higher than 10 kg of oil per 100 kg of ethanol at temperatures lower than 30 ˚C are not achieved, although at 70 ˚C the sunflower, peanut and cottonseed oils are soluble.

Rao *et al.,* (1955) carried out research in the extraction of vegetable oils from cotton, peanut, sesame and soybean seeds using ethanol of various alcoholic degrees and under several temperature conditions. For all the oils, they determined solubility curves and showed that there was a linear relationship between the "critical temperature", defined as the temperature at which the alcohol solution reached saturation for the oil studied, and the alcohol concentration used.

Given the importance of ethanol in Brazil, Freitas *et al*., (2010) showed the advantages of the use of ethanol instead of hexane for the extraction of coffee and sunflower oil, owing to the ecological nature of ethanol. With its use for the extraction of coffee and sunflower oil, they obtained results of 11.4 and 18.6%, respectively, during their extraction of seeds, although these results are lower than those obtained with hexane. A similar study was developed by Rodrigues *et al*., (2010) for soybean oil and concluded that the use of ethanol for this process is feasible; while Rodrigues *et al*., (2010) evaluated it in the extraction of rice oil. Both studies used experimental designs for their respective purposes and concluded that temperature and the alcoholic degree were the main independent variables, which agreed with the results reported in previous works on the use of ethanol as a vegetable oil solvent.

The solubility of canola, maize, jatropha and maracauba oils in ethanol at temperatures of 25-60.15 ˚C was investigated by Da Silva *et al.*, (2010); while Shariati *et al.*, (2013) determined the solubility curves of sunflower oil in ethanol.

There are only a few studies in which Hansen's solubility theory was used as a tool to determine the HSPs of vegetable oils to know which would be the most suitable solvent for these oils. Batista *et al.*, (2015) determined the HSPs of used frying oil, coconut oil, palm oil and biodiesel. They confirmed that used frying oil and biodiesel–diesel blends were feasible alternatives that could be used as additives for diesel fuel.

A study by De la Peña-Gil *et al*., (2016) to develop a specific method to determine the HSPs of complex vegetable oils used group contribution methods with two approaches: the first assumed that the contributions of the 3 forces could be subdivided into larger functional groups (i.e. fatty acids and fatty acid methyl esters), which are additives; while the second approach assumed that vegetable oils are composed of simple triglycerides mixtures in the same mass fractions as fatty acids. They concluded that the HSPs calculated for vegetable oils with the second assumption provided similar values to the HSPs obtained from the HSPiP software.

During studies developed by The Cuban Research Institute of Sugarcane Derivatives (ICIDCA) in the 90s about sugarcane oil extraction from sugarcane wax with ethanol at 18-20 ˚C, only 17.9% efficiency was achieved with ethanol at 93% v/v and 42.9% with absolute ethanol. Therefore, the use of absolute alcohol in the refining process of sugarcane wax was suggested, but investigating the possibility of using other more efficient solvents for this purpose were recommended, topics which are investigated in this work.

In the current refined wax plant, low efficiencies and high 95% v/v ethanol consumption rates are reported during sugarcane oil extraction. The objective of this work was to verify whether ethanol 95% v/v is a suitable solvent for the sugarcane wax refining process using Hansen's solubility theory, as well as to determine other solvents that can be suitable for this process.

2. MATERIALS AND METHODS

2.1. Oil purification

Sugarcane oil obtained from the sugarcane wax refining factory annexed to the Majibacoa sugar mill in Las Tunas province, Cuba, was used for this study. HPLC grade acetone at 25 ˚C was used for the purification process, according to factory laboratory standards. This impure oil obtained in the factory, with an average dry base composition of 78.9% oil, 15.78% wax and other impurities, was subsequently dissolved in acetone at 25 ˚C with an acetone-oil ratio of 4:1 and filtrated under vacuum with a Buchner funnel and fast filtration paper to be purified. Acetone with oil was evaporated in a vacuum rotary evaporator and then the oil was dried in an oven at 100 ˚C. The oil obtained was subjected to a second purification step.

2.2. Hansen Solubility Parameters determination (HSPs)

2.2.1. Fundamental

HSPs determination is a useful tool for solvent selection in the extraction of natural chemical products because it expresses the affinity degree between a solute and any solvent based on the Hansen theory, which stipulates that "likes attract likes". There are two important groups of methods for HSPs determination, experimental and theoretical methods, but only experimental methods are evaluated in this work for the HSPs determination of sugarcane oil.

The basic equation governing the assignment of Hansen parameters establishes that the total cohesion energy between two molecules, E, must be the sum of the individual energies: non-polar interactions or atomic dispersion (ED), molecular di-polar interactions (EP) and hydrogen-bonding interactions (EH) (Hansen C.M. 2007), so**:**

$$E = ED + EP + EH \quad (1)$$

Dividing this by the molar volume gives the square of the Hildebrand solubility parameter as the sum of the squares of the Hansen D, P, and H components: dispersion (δ_D), polar (δ_P), and hydrogen bonding (δ_H).

$$\frac{E}{V} = \frac{ED}{V} + \frac{EP}{V} + \frac{EH}{V} \quad (2)$$

$$\delta_T^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \quad (3)$$

The Hansen parameters of different solutes and solvents may be plotted in a tridimensional plot x,y,z (δ_D,δ_P ,δ_H). The closer the solute-solvent pair is in the three-dimensional space, the more soluble they are. The distance between the solvent (a) and solute (b) is usually called R_a and can be determined by the following expression:

$$R_a = [4*(\delta_{Da} - \delta_{Db})^2 + (\delta_{Pa} - \delta_{Pb})^2 + (\delta_{Ha} - \delta_{Hb})^2]^{1/2} \quad (4)$$

Experimental evaluation of a solute with various solvents, generally between 40-50 different solvents, and its classification into "good" or "bad" solvents by visual inspection, helps us to determine the HSPs of the solute (solute's coordinates within Hansen space).

Considering that the "good solvents" surround the solute forming a hypothetical sphere, called "Hansen's Sphere", good solvents can also be found on the surface of the Hansen sphere, so for them Ra is equal to the radius of the sphere (R_0). By defining the Ra/R_0 ratio as the relative energy difference (RED), the good solvents will obtain RED values less than 1 and the bad solvents higher. Both the radius of the sphere and the center (HSPs) are determined by the use of appropriate multi-response optimization algorithms. (Abbott and Yamamoto, 2015; Díaz and Hernández, 2020).

$$RED = \frac{R_a}{R_o} \quad (5)$$

2.2.2. Hansen solubility parameters determination through classic Hansen method

The determination of Hansen's parameters was executed with 48 different analytical degree solvents as shown in Table 1. Solvent-oil samples in a 9:1 ratio were placed in duplicate test tubes and shaken for 24 hours at 30 ^{0}C and then allowed to rest 72 h for a visual inspection of the presence of phase separation (Batista *et al.*, 2015).

Visual inspection was performed by each author separately and individual ratings were compared. The samples were divided into three groups: soluble (without phase separation), not soluble (phase separation) and doubtful. The doubtful group included the samples in which their evaluation differed for each author and also those classified directly as such. Due to the nature of the oil used, the solution tended to darken and it was sometimes difficult to visually determine whether or not there was phase separation. Therefore, tests classified as "doubtful" were observed under the microscope or a drop of sample was placed in a filter paper; if a black spot was formed at the sample application point, then it was classified as not soluble, on the other hand, if the sample formed a ring of uniform color, it was soluble according to the established procedure (Redelius, 2004).

Samples in which the solvent solubilized the solute received a score of 1 and samples in which the solvents did not solubilize the solute the score were labelled 0. Both the HSPiP software using the genetic algorithm and the improved Microsoft Excel workbook from the authors were used for data processing to define the HSPs for sugarcane oil (Abbott and Yamamoto, 2015; Díaz and Hernández, 2020).

2.2.3. Fit improvement of Hansen solubility parameters

The reliability of the parameters was mainly based on the fact that the solvents used in the study would cover the largest possible area of the three-dimensional space in such a way that the edge of the sphere and its center could be correctly defined.

To check the quality of fit, the values called "Core" by HSPiP software were used. This option showed how much the solute´s HSPs could vary in different directions without too high a penalty. Clearly the larger the "Core" values, the less the Sphere was defined, so some additional solvents may be needed to redefine it.

One way to improve the fit is to use the "Sphere Radius Chk option" in HSPiP software. It goes through a list of green solvents that can be edited and finds any that are near the boundary of the Sphere (i.e. they have a RED of 0.9 to 1.1). It then checks each of those solvents for closeness to solvents in your test list. If they are close, then you already have enough data in that area. But if no solvent is nearby then it is likely that this test solvent would provide useful new information to improve the quality of the fit.

The suggested solvents by the software to achieve a better fit were: 2-nitropopane, 1-bromonaphthalene, 1,3-butanediol, butyronitrile and chlorobenzene. Since they were not available the "Solvent Optimizer" option was used. This option calculates solvent mixtures capable of achieving similar characteristics to recommended solvents. The mixtures were named Somix, tested, classified and scored according to the procedure described above. To determine the HSPs of sugar cane oil, a total of 53 solvents were tested, including mixtures.

2.3. Intrinsic viscosity measurements method

One of the more promising methods to evaluate polymer HSPs for limited data is by using intrinsic viscosity (Hansen, 2007).

In this technique, intrinsic viscosities determined with different solvents are normalized by dividing each one by the highest intrinsic viscosity value. These normalized data (≤ 1) are then used in a weighted averaging technique, according to the following equations, to determine the HSPs of the solute (Hansen, 2007).

TABLE 1. **Good and bad solvents for sugarcane oils to determine the Hansen solubility parameters**

No[a].	Solvent	Dispersion component (δ_D) $MPa^{1/2}$	Polar component (δ_P) $MPa^{1/2}$	Hydrogen bonding component (δ_H) $MPa^{1/2}$	Score[h]	Relative Energy Difference (RED)	Molecular volume (MVol) cm^3/mol
197	1-Decanol	16.0	4.7	10.5	1	0.146	191.8
542	1-Octanol	16.0	5.0	11.2	1	0.189	158.2
375	Ethylene Glycol Monobutyl Ether	16.0	5.1	12.3	1	0.256	131.8
306	1,4-Dioxane	17.5	1.8	9.0	1	0.295	85.7
930	1-Hexanol	15.9	5.8	12.5	1	0.319	125.2
617	Tetrahydrofuran (Thf)	16.8	5.7	8.0	1	0.334	81.9
429	Isoamyl Acetate	15.3	3.1	7.0	1	0.359	150.2
328	Ethyl Acetate	15.8	5.3	7.2	1	0.362	98.6
182	Cyclohexanol	17.4	4.1	13.5	1	0.371	105.7
545	Oleic Acid	16.0	2.8	6.2	1	0.392	319.7
552	1-Pentanol	15.9	5.9	13.9	1	0.418	108.6
732	2-Methyl-2-Butanol	15.3	6.1	13.3	1	0.422	109.6
93	2-Butanol	15.8	5.7	14.5	1	0.457	92.0
524	Methylene Dichloride (Dichloromethane)	17.0	7.3	7.1	1	0.505	64.4
156	Chloroform	17.8	3.1	5.7	1	0.520	80.5
92	1-Butanol	16.0	5.7	15.8	1	0.558	92.0
649	Trichloroethylene	18.0	3.1	5.3	1	0.572	90.1
58	Benzyl Alcohol	18.4	6.3	13.7	1	0.581	103.8
431	Isobutyl Alcohol	15.1	5.7	15.9	1	0.604	92.9
5	Acetic Acid	14.5	8.0	13.5	1	0.624	57.6
570	2-Propanol	15.8	6.1	16.4	1	0.628	76.9
255	Diethyl Ether	14.5	2.9	4.6	1	0.628	104.7
254	Diethyl Carbonate	15.1	6.3	3.5	1	0.732	121.7
569	1-Propanol	16.0	6.8	17.4	1	0.736	75.1
7	Acetone	15.5	10.4	7.0	1	0.751	73.8
698	o-Xylene	17.8	1.0	3.1	1	0.762	121.1
999	So Mix[b]	18.7	3.8	3.7	1	0.770	86.0
367	Ethylene Dichloride	18.0	7.4	4.1	1	0.772	79.4
398	Formic Acid[g]	14.6	10.0	14.0	0	0.779	37.9
999	So Mix[c]	18.5	4.2	2.8	1	0.823	103.0
598	Pyridine	19.0	8.8	5.9	1	0.838	80.9
187	Cyclopentane	16.4	0.0	1.8	1	0.851	94.6
637	Toluene	18.0	1.4	2.0	1	0.859	106.6
52	Benzene	18.4	0.0	2.0	1	0.924	89.5
11	Acetophenone	18.8	9.0	4.0	1	0.929	117.4
325	Ethanol	15.8	8.8	19.4	1	0.992	58.6
181	Cyclohexane	16.8	0.0	0.2	1	0.996	108.9
122	Carbon Tetrachloride (0 Dipole Moment)	17.8	0.0	0.6	1	0.998	97.1
999	So Mix[d]	15.3	12.4	5.1	1	1.000	98.0
999	So Mix[e]	16.2	12.1	4.3	1	1.000	76.0
297	Dimethyl Formamide (Dmf)	17.4	13.7	11.3	0	1.002	77.4
540	Octane	15.5	0.0	0.0	0	1.019	163.4
409	Heptane	15.3	0.0	0.0	0	1.024	147.0
417	Hexane	14.9	0.0	0.0	0	1.040	131.4
999	So Mix[f]	16.5	8.1	20.9	0	1.081	82.0
670	2,2,4-Trimethylpentane	14.1	0.0	0.0	0	1.086	165.5
437	Isopentane	13.8	0.0	0.0	0	1.107	117.1
303	Dimethyl Sulfoxide (Dmso)	18.4	16.4	10.2	0	1.289	71.3

No[a].	Solvent	Dispersion component (δ_D) MPa$^{1/2}$	Polar component (δ_P) MPa$^{1/2}$	Hydrogen bonding component (δ_H) MPa$^{1/2}$	Score[h]	Relative Energy Difference (RED)	Molecular volume (MVol) cm^3/mol
456	Methanol	14.7	12.3	22.3	0	1.425	40.6
10	Acetonitrile	15.3	18.0	6.1	0	1.442	52.9
368	Ethylene Glycol	17.0	11.0	26.0	0	1.631	55.9
406	Glycerol	17.4	11.3	27.2	0	1.750	73.4
696	Water	15.5	16.0	42.3	0	3.203	18.0

[a] Solvent identification number in the HPSiP program.
[b] Solvent mixture (57% v/v benzene, 43% v/v pyridine) representing 1-Bromonaphthalene
[c] Solvent mixture (acetophenone 43% v/v, benzene 35% v/v, toluene 22% v/v) representing Chlorobenzene
[d] Solvent mixture (acetonitrile 54% v/v, diethyl carbonate 39% v/v, oleic acid 7% v/v) representing Butyronitrile
[e] Solvent mixture (acetonitrile 60% v/v, cyclohexane 25% v/v, acetophenone 15% v/v) representing 2-Nitropropane
[f] Solvent mixture (glycerol 50% v/v, isobutyl alcohol 40% v/v, 1,4 Dioxane 10% v/v) representing 1,3 Butanediol
[g] Bad solvent within the Hansen sphere
[h] Score 1: good solvents
Score 0: bad solvents

$$\delta_{D2} = \sum(\delta_{Di} * [\eta]_i) / \sum[\eta]_i \quad (6)$$

$$\delta_{P2} = \sum(\delta_{Pi} * [\eta]_i) / \sum[\eta]_i \quad (7)$$

$$\delta_{H2} = \sum(\delta_{Hi} * [\eta]_i) / \sum[\eta]_i \quad (8)$$

The members on the left side of each equation represents dispersion, polar and hydrogen bonding forces respectively; the subscript 2 represents the solute. On the right side of each equation is, as appropriate, weighted averaging of the component of the force of attraction according to normalized intrinsic viscosity ($[\eta]_i$); the subscript i represents the solvent.

2.3.1. Determination of Hansen solubility parameters using the intrinsic viscosity method

To determine the HSPs using the intrinsic viscosity method, 7 solvents were chosen with the criterion of expanding the exploration zone for the calculation of the HSPs of sugarcane oil. Among them were: isopropanol, 1,4 Dioxane, Ethyl acetate, 1 Decanol, 1 Octanol, 2- Methyl -2- Butanol and o-Xylene.

To calculate the intrinsic viscosity, sugarcane oil solutions were prepared for each solvent chosen from 10; 15; 20; 25; 30 kg / m^3. A ubbelohde viscometer was used to measure the relative viscosity (η_{re}) for each concentration. Each measurement was performed in triplicate while maintaining a temperature of 25 ^{0}C (Stanciu I, 2009).

A linear regression of the inherent viscosity was adjusted against the concentrations, where the intercept is the value of the intrinsic viscosity:

$$\eta_{inh} = \frac{\ln \eta_r}{c} \quad (9)$$

Where η_r is the reduced viscosity which was calculated by:

$$\eta_r = \eta_{re} - 1 \quad (10)$$

With the HSPs values of the solvents studied and those of the normalized intrinsic viscosity, the HSPs of the solute was estimated.

3. RESULTS AND DISCUSSION

3.1. Hansen solubility parameter determination

The HSPs obtained for sugarcane oil using HSPiP software were δ_D = 16.56 MPa$^{1/2}$, δ_P = 2.78 MPa$^{1/2}$ and δ_H = 10.54 MPa$^{1/2}$. Of all solvents analyzed, 14 were alcohols, among which 12 were aliphatic with two aromatics. For this particular case, methanol was the only one that had a RED greater than 1; other alcohols were good solvents for sugarcane wax oil. 1-decanol was the best of all the solvents analyzed. Usually, according to the international chemical safety sheet, it is used in the manufacture

of plasticizers, lubricants, surfactants and as solvent, but its use in this application requires a technical-economic feasibility analysis.

When running the HSPiP program, the HSPs of the sugarcane oil varied their values, thus slightly moving the center of the Hansen sphere in three-dimensional space according to an indicator of the program, mentioned above as the "Core". At times. when the program was run, the absolute ethanol did not fit inside the sphere, leaving it outside with a RED of 1,009, which is incorrect, since it had been classified as a good solvent. In addition, other solvents that were classified as bad remained within the sphere, including Dimethyl Formamide (Dmf), formic acid, among others, depending on where the algorithm had defined the center of the sphere.

The HSPs presented at the beginning of this section were the best fit that was achieved, with 97.9% fit, in which all the solvents classified as good were within the sphere and only formic acid, which turned out to be classified as a bad solvent was wrongly situated inside.

Of course, accuracy depends on the reliability of the data used, which is indicated by the fit of the Hansen sphere "Core" Values. According to Abbott (2013), if errors were made during sample evaluation, many solvents that do not dissolve the solute would fall inside the sphere or many that dissolve the solute would be outside. Figure 2 shows that only one solvent that does not dissolve the solute is within the sphere, which validates the reliability of the input data.

3.1.1. Fit improvement in Hansen solubility parameters

For the first fitting, the "Core" was 1.30, 1.60, 1.35. In the program it is defined that if, for the parameter analyzed, the core is greater than 1, it is possible that the adjustment was inaccurate. This tells us that the amount of solvents analyzed was not sufficient to guarantee a result with high reliability.

Table 1 shows the classification of solvents as Good and Bad. Recalculating the HSPs provided: δ_D = 16.24 $MPa^{½}$; δ_P = 3.21 $MPa^{½}$; and δ_H = 10.34 $MPa^{½}$ with a "Core" of 1.00, 0.50, 0.85, respectively. This indicates a more reliable result, although the parameter δ_D could still be investigated. The results obtained from the use of the GRG nonlinear optimization method of Microsoft Excel Workbook were 16.38, 3.10 and 10.25 for δ_D, δ_P and δ_H, respectively, not quite different from the use of HSPiP software.

Absolute ethanol was located at the limit of the Hansen solubility sphere with a RED = 0,993. this means that any mixture of ethanol-water that is used for the extraction of this oil would not be good. Absolute ethanol is produced in Cuba and is an environmentally friendly solvent capable of decreasing the plant's consumption rates and increasing extraction efficiency. If used, the technology would not change much, making it a possible substitute for 95% v / v ethanol currently used.

Table 2 compares different types of vegetable oils according to their HSPs and it is observed that absolute ethanol is only reported as a good solvent for sugar cane oil, being the only one with a RED less than 1. In order to know why this oil has a larger δ_H, an infrared spectroscopy was performed to identify its main structural groups.

Figure 1a shows the infrared spectrum of crude wax oil, which was very similar to the infrared spectrum of castor oil and pine resin (Figures 1b and 1c, respectively) (Institute of Chemistry University of Tartu, 2019). A wide band of low intensity between 3200-3650, bands between 1540-1850, 2200-3000 and signals in 1200-1420 suggest the presence carboxylic groups. This result allows us to corroborate a possibility that δ_H is influenced by the presence of carboxylic groups.

Figure 2 shows the plotted Hansen sphere using 3-D axes for sugarcane oil and solvents as shown in Table 1. Blue spheres represent good solvents and red cubes represent bad solvents. The unfilled cube with the red edges (formic acid) indicate a bad solvent that fell inside the Hansen sphere. It could be because the program needs more information for a better fit, such as the evaluation of more solvents. When running the program for different times, the HSPs values of the sugarcane oil did not vary much and reached an adjustment of 98.1%.

According to RED values, sugarcane oil is soluble in 39 of the solvents tested. For Batista *et al.*, (2015) the best solvents for the vegetable oils analyzed were n-butyl acetate, ethyl acetate and o-xylene. In the case of ethyl acetate and o-xylene, which were tested in the present study, they were good solvents, but they were not among the best. Contrary to the results obtained by Batista *et al.*, (2015), who exposed 1-decanol as a poor solvent, in the current work 1-decanol was the best solvent

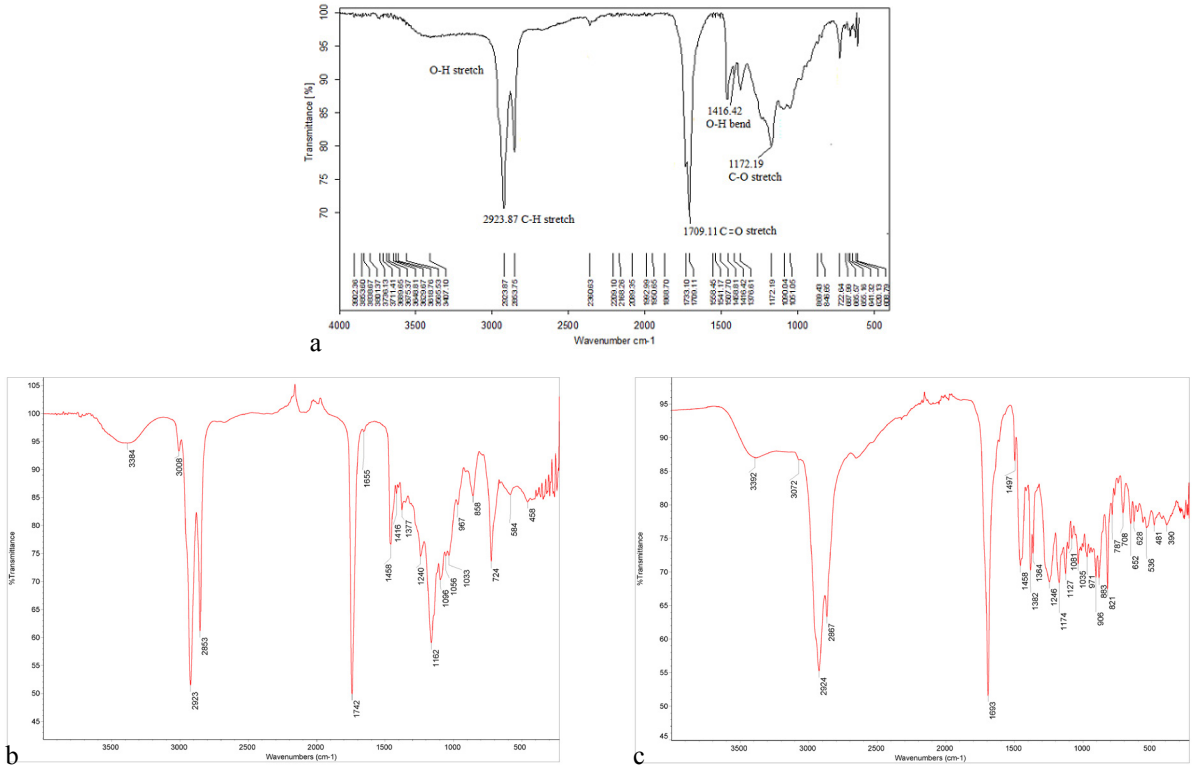
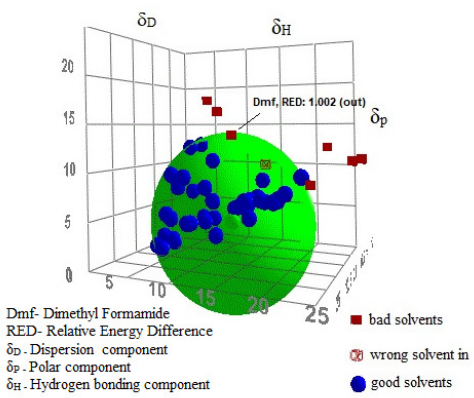


FIGURE 1.- Infrared spectra of different oils. (a) Raw sugarcane wax; (b) castor seed oil and (c) pine resin. (b) and (c) source: Institute of Chemistry University of Tartu

TABLE 2. **Parameters of different types of vegetable oils found in the literature**

Solute	Dispersion component (δ_D) MPa$^{1/2}$	Polar component (δ_P) MPa$^{1/2}$	Hydrogen bonding component (δ_H) MPa$^{1/2}$	Distance between solute-ethanol (R_a)	Hansen sphere radius (R_0)	Relative Energy Difference (RED)	References
Sugarcane oil	16.24	3.21	10.34	10.72	10.80	0.993	This work
used frying oil	15.35	3.77	6.87	13.52	9.57	1.413	Batista *et al.*, 2015
Coconut oil	14.95	4.63	6.98	13.22	9.80	1.349	Batista *et al.,* 2015
Palm oil	17.54	3.34	4.08	16.62	7.48	2.222	Batista *et al*., 2015
Soybean oil	16.50	2.00	2.70	18.08	NR	NR	De la Peña *et al.,* 2016
Canola oil	16.50	2.10	2.60	18.14	NR	NR	De la Peña *et al.,* 2016
Coconut oil	16.20	2.50	2.80	17.77	NR	NR	De la Peña *et al*., 2016
Cocoa butter	16.20	2.10	2.20	18.47	NR	NR	De la Peña *et al*., 2016
Soybean oil (tgas + glycerol)	16.80	1.90	9.60	12.15	NR	NR	De la Peña *et al*., 2016
Canola oil (tgas + glycerol)	16.80	1.90	9.60	12.15	NR	NR	De la Peña *et al*., 2016
Coconut oil (tgas + glycerol)	16.40	2.30	11.40	10.37	NR	NR	De la Peña *et al.,* 2016
Cocoa butter (tgas + lycerol)	16.50	1.90	9.50	12.14	NR	NR	De la Peña *et al*., 2016
Pine oil	15.60	3.00	9.80	11.22	NR	NR	Hansen, 2007
Olive oil	15.90	1.20	5.40	15.93	12.00	1.328	Hansen, 2007
Castor oil	15.90	4.60	12.00	8.51	NR	NR	Hansen, 2007
Palm oil	17.70	3.50	3.70	17.00	4.70	3.617	Hansen, 2007
Linseed oil	14.17	3.65	3.65	16.89	NR	NR	Burke, 1984
Cottonseed oil	15.14	3.39	4.07	16.31	NR	NR	Burke, 1984

NR Not reported



Solvent	Score[g]	Solvent	Score[g]	Solvent	Score[g]	Solvent	Score[g]
1-Decanol	1	Dichloromethane	1	Ethylene Dichloride	1	Dimethyl Formamide	0
1-Octanol	1	Chloroform	1	Formic Acid[f]	0	Octane	0
Ethylene Glycol Monobutyl Ether	1	1-Butanol	1	So Mix[b]	1	Heptane	0
		Trichloroethylene	1	Pyridine	1	Hexane	0
1,4-Dioxane	1	Benzyl Alcohol	1	Cyclopentane	1	So Mix[a]	0
1-Hexanol	1	Isobutyl Alcohol	1	Toluene	1	2,2,4-Trimethylpentane	0
Tetrahydrofuran	1	Acetic Acid	1	Benzene	1	Isopentane	0
Isoamyl Acetate	1	2-Propanol	1	Acetophenone	1	Dimethyl Sulfoxide	0
Ethyl Acetate	1	Diethyl Ether	1	Ethanol	1	Methanol	0
Cyclohexanol	1	Diethyl Carbonate	1	Cyclohexane	1	Acetonitrile	0
Oleic Acid	1	1-Propanol	1	Carbon Tetrachloride (0 Dipole Moment)	1	Ethylene Glycol	0
1-Pentanol	1	Acetone	1			Glycerol	0
2-Methyl-2-Butanol	1	o-Xylene	1	So Mix[c]	1	Water	0
2-Butanol	1	So Mix[a]	1	So Mix[d]	1		

[a] Solvent mixture (57% v/v benzene, 43% v/v pyridine) representing 1-Bromonaphthalene
[b] Solvent mixture (acetophenone 43% v/v, benzene 35% v/v, toluene 22% v/v) representing Chlorobenzene
[c] Solvent mixture (acetonitrile 54% v/v, diethyl carbonate 39% v/v, oleic acid 7% v/v) representing Butyronitrile
[d] Solvent mixture (acetonitrile 60% v/v, cyclohexane 25% v/v, acetophenone 15% v/v) representing 2-Nitropropane
[e] Solvent mixture (glycerol 50% v/v, isobutyl alcohol 40% v/v, 1,4 Dioxane 10% v/v) representing 1,3 Butanediol
[f] Bad solvent within the Hansen sphere
[g] Score 1: good solvents
Score 0: bad solvents

FIGURE 2. Hansen spheres plotted using 3-D axes for the sugarcane oil and the solvents shown in Table 1

for crude wax oil, given its similar δ_H and δ_P values. This could be due to a different composition between the oils analyzed by Batista *et al.,* (2015) and the current work, which were expressed in Hansen's parameters.

A large amount of good solvents was consistent with the R_0 calculated by the HSPiP program which adjusted R_0 = 10.7. Solvents close to RED = 1, such as benzene, cyclohexane, N, N-dimethylformamide, ethanol, carbon tetrachloride, octane, acetophenone and the Somix used to improve HSPs of sugarcane oil, are those that improved the Hansen sphere by its influence to R_0 value.

3.2. Calculation of Hansen's solubility parameters using the intrinsic viscosity method

This method was used to verify the HSPs of the sugarcane oil obtained from the solvent testing method, since no information about them was found in the literature. The results are shown in Table 3. The HSPs calculated using the intrinsic viscosity method were: δ_D = 16.16 $MPa^{½}$; δ_P = 4.44 $MPa^{½}$; and δ_H = 10.40 $MPa^{½}$. The Results obtained by this method are compared with those reported by the HSPiP software in Table 4, where it may be observed that the values calculated by both methods are similar.

TABLE 3. **Intrinsic viscosity for each solvent**

Solvent	Intrinsic viscosity $[\eta]_i$, kg /m^3	Normalized intrinsic viscosity$[\eta]_i$
Isopropanol	0.0105 ± 0.0026	0.203
1.4 Dioxane	0.0107 ± 0.0018	0.211
Ethyl Acetate	0.0034 ± 0.0041	0.065
1 decanol	0.0494 ± 0.0008	0.974
1-Octanol	0.0018 ± 0.0005	0.036
2-Methyl-2-Butanol	0.0507 ± 0.0009	1.000
o-Xylene	0.0260 ± 0.0115	0.513

TABLE 4. **Comparison of HSPs values for sugarcane oil**

Method	Dispersion component (δ_D) $MPa^{1/2}$	Polar component (δ_P) $MPa^{1/2}$	Hydrogen bonding component (δ_H) $MPa^{1/2}$
Hansen	16.24	3.21	10.34
Intrinsic viscosity	16.16	4.44	10.40

In the intrinsic viscosity method, some inaccuracies in the results may arise due to the choice of solvents for the study, more in this case, where the HSPs of sugarcane oil were unknown; but the result obtained through the method of Intrinsic viscosity strengthens the one obtained using Hansen's Solubility Theory.

4. CONCLUSIONS

According to the crude wax oil, the HSPs obtained in this work indicated that absolute ethanol could be used as a solvent for its extraction. Although it is not the best solvent, it has the advantages of being of national production, environmentally-friendly with very low toxicity. The good solvents determined in this work must be evaluated from the economic point of view. Sugarcane oil HSPs resulting from the analysis are: δ_D = 16.24 $MPa^{1/2}$; δ_P = 3.21 $MPa^{1/2}$: δ_H = 10.34 $MPa^{1/2}$.

ACKNOWLEDGMENTS

We wish to express our gratitude to Professor Steven Abbott for his help, as well as to Sandra Fanego for her support in the realization of the infrared spectra.

REFERENCES

Abbott S. 2013. www.hansen-solubility.com

Abbott S. and Yamamoto H. (2015) HSPiP Software, 5th edn. 5.2.05

Batista MM, Guirardello R, Krähenbühl MA. 2015. Determination of the Hansen Solubility Parameters of Vegetable Oils, Biodiesel, Diesel, and Biodiesel–Diesel Blends. *J. Am. Oil. Chem. Soc.* **92**, 95–109. https://doi.org/10.1007/s11746-014-2575-2

Burke, J. 1984. The Book and Paper Group Annual, vol.3, https://cool.culturalheritage.org.bpg

Díaz de los Ríos M, Hernández Ramos E. 2020. Determination of the Hansen solubility parameters and the Hansen sphere radius with the aid of the solver add-in of Microsoft Excel, SN Applied Sciences **2**, 676, https://doi.org/10.1007/s42452-020-2512-y

Freitas SP, Lago RCA. 2007. Equilibrium Data for the Extraction of Coffee and Sunflower Oils with Ethanol. *Braz. J. Food. Technol.* **10**, 220-224.

Hansen CM. 2007. Solubility parameters-an introduction. *Hansen solubility parameters: a user's handbook*. CRC, Boca Raton, 43-66. ISBN 978-0-9551220-2-6

ICIDCA. 2000. Producción de cera y grasas a partir de cachaza. *Manual de los Derivados de la Caña de Azúcar*. Editorial científico-técnica, La Habana, 572-596.

Institute of Chemistry University of Tartu, Estonia http://lisa.chem.ut.ee/IR_spectra/paint/binders/castor-oil/ ½, 11/3/2019.

Kaparthi R, Charti KS. 1955. Solubilities of Vegetable Oils in Aqueous Ethanol and Ethanol-Hexane Mixtures. *J. Am. Oil. Chem. Soc.* **36**, 77-80. https://doi.org/10.1007/BF02540248

Peña-Gil A de la, Toro-Vázquez JF, Rogers MA. 2016. Simplifying Hansen Solubility Parameters for Complex Edible Fats and Oils. *Food. Biophys.* **3**, 283-291. https://doi.org/10.1007/s11483-016-9440-9

Rao RK, Krishna MG, Zaheer RH, Arnold LK. 1955. Alcoholic Extraction of vegetable oils. Part I. Solubilities of Cottonseed, Peanut, Sesame and Soybean Oils in Aqueous Ethanol. *J. Am. Oil. Chem. Soc.* **32**, (7) 420-423. https://doi.org/10.1007/BF02639700

Redelius P. 2004. Bitumen Solubility Model Using Hansen Solubility Parameter. *Energ. Fuel* **18**, 1087-1092. https://doi.org/10.1021/ef0400058

Rodrigues ChEC, Oliveira R. 2010. Response surface methodology applied to the analysis of rice bran oil extraction process with ethanol. *Int. J. Food. Sci. Tech*. **45**, 813–820. https://doi.org/10.1111/j.1365-2621.2010.02202.x

Shariati A, Azaribeni A, Hajighahramanzadeh P, Loghmani Z. 2013. Liquid Liquid Equilibria of Systems Containing sunflower. Oil, Ethanol and Water. *APCBEE. Procedia* **5**, 486 – 490. https://doi.org/10.1016/j.apcbee

Silva CAS da, Sanaiotti G, Lanza M, Follegatti-Romero LA, Meirelles AJA, Batista EAC. 2010. Mutual Solubility for Systems Composed of Vegetable Oil + Ethanol + Water at Different Temperatures. *J. Chem. Eng. Data* **55**, 440-447. https://doi.org/10.1021/je900409p

Stanciu I. 2009. Study solubility of two copolymers used as viscosity improvers for multi-grade oil. AUDJG – Food Technology, 323-326

Swenson OJ. 1947. Method of extracting wax from cachaza. US2508002A.

Taylor TI, Larson L, Johnson W. 1936. Miscibility of Alcohol and Oils. *Ind. Eng. Chem.* **28**, 616-618. https://doi.org/10.1021/ie50317a030

Villanueva RG, Feyt LR. 2011. Transesterificación de la cera de caña de azúcar usando etanol. *Centro Azúcar* **38**, 46-50.

GRASAS Y ACEITES 72 (2)
April-June 2021, e409
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0442201

Passion fruit seed oil: extraction and subsequent transesterification reaction

T.B. Massa[a], I.J. Iwassa[a], N. Stevanato[a], V.A.S. Garcia[b] and C. Silva[a,c,✉]

[a]Programa de Pós-graduação em Engenharia Química, Universidade Estadual de Maringá (UEM), Avenida Colombo, 5790, D-90, CEP 87020-900, Maringá, PR, Brasil.

[b]Departamento de Engenharia (FAEN), Engenharia de Alimentos, Universidade Federal da Grande Dourados (UFGD), 79804-970, Dourados, MS, Brasil.

[c]Departamento de Tecnologia, Universidade Estadual de Maringá (UEM), 87506-370, Umuarama, PR, Brasil.

✉Corresponding author: camiladasilva.eq@gmail.com

Submitted: 06 April 2020; Accepted: 25 May 2020; Published online: 03 June 2021

SUMMARY: This work aims to remove the oil from passion fruit seeds using ethanol as solvent and then to carry out the transesterification of the product from the extraction step (oil + ethanol). The effects of operational variables in the ultrasound-assisted extraction (UAE) were evaluated and traditional extraction was performed for comparison. The extraction product was directed to the reaction step using an enzymatic catalyst. UAE provided oil yield from 12.32 to 21.76%, and the maximum value (73.7% of the traditional extraction yield) was obtained at 60 °C and 50 min using a solvent-to-seed ratio of 4. Oil removal was favored by increases in the investigated variables. γ-tocopherol, δ-tocopherol and a high concentration of polyunsaturated fatty acids were identified in the oils. The oil obtained by UAE presented higher phytosterol contents. From the reaction step, samples were obtained with higher concentrations of ethyl esters, in addition to emulsifiers (diglycerides and monoglycerides).

KEYWORDS*: Emulsifiers; Ethanol; Ethyl esters; Oil yield; Ultrasound*

RESUMEN: ***Aceite de semilla de maracuyá: extracción y posterior reacción de transesterificación.*** Este trabajo tiene como objetivo la extracción del aceite de la semilla de maracuyá utilizando etanol como disolvente y posteriormente llevar a cabo la transesterificación del producto obtenido (aceite + etanol). Se evaluaron los efectos de las variables operativas en la extracción asistida por ultrasonido (EAU) y se realizó la extracción clásica a efectos comparativos. El producto de extracción se dirigió a la etapa de reacción usando catalizador enzimático. EAU proporcionó un rendimiento de aceite de 12,32 a 21,76%, y el valor máximo (73,7% del rendimiento de extracción clásico) se obtuvo a 60 °C durante 50 min usando una relación de solvente a semilla de 4. La extracción de aceite se favorece con el aumento de las variables investigadas. Se identificaron en los aceites γ- y δ-tocoferol y una alta concentración de ácidos grasos poliinsaturados. El aceite obtenido por los EAU presentó un mayor contenido de fitosteroles. De la etapa de reacción, se obtuvieron muestras con concentraciones más altas de ésteres etílicos, además de emulsionantes (diglicéridos y monoglicéridos).

PALABRAS CLAVE*: Emulsionantes; Ésteres etílicos; Etanol; Rendimiento de aceite; Ultrasonido*

Citation/Cómo citar este artículo: Massa TB, Iwassa IJ, Stevanato N, Garcia VAS, Silva C. 2021. Passion fruit seed oil: extraction and subsequent transesterification reaction. *Grasas Aceites* **72** (2), e409. https://doi.org/10.3989/gya.0442201

1. INTRODUCTION

Passion fruit is a tropical fruit which is popular worldwide and is commonly used in juice production, with Brazil accounting for ~80% of world production ("Embrapa", 2017). Bark and seeds are the main agro-industrial residues from the fruit-crushing process to obtain the juice and they cause problems for the industry due to the waste generated, whose volume amounts to many tons (Oliveira *et al.*, 2013). Passion fruit seeds represent ~12% of the fruit and can be considered good sources of oils, besides containing high amounts of fiber, which supports their use as a complementary source of these nutrients in the diet (Chau and Huang, 2004).

Passion fruit seeds have 18 to 30% oil in their composition (Malacrida and Jorge, 2012; Piombo *et al*., 2006; Santana *et al*., 2017). High levels of linoleic acid can be found in this oil (Malacrida and Jorge, 2012; Oliveira *et al*., 2013), in addition to phytosteroids and tocopherols, which give the oil high antioxidant activity (Lee *et al*., 2015; Pereira *et al.*, 2017; Santana *et al*., 2017). In relation to the tocopherols present in the oil of passion fruit seeds, α-tocopherol, γ-tocopherol, and δ-tocopherol have been identified (Pereira *et al*., 2017; Pereira *et al*., 2018). In addition, Silva and Jorge (2017) reported the presence of phytosterols in this oil, highlighting β-sitosterol in higher concentrations.

Ultrasound-assisted extraction (UAE) stands out as a technique for removing oil from oilseeds since it is considered cheap, simple, efficient (Khoei and Chekin, 2016), and selective when compared to traditional extraction (Luque-García and Luque de Castro, 2003), besides being conducted with less contact time and demanding lower consumption of solvent (Perrier *et al*., 2017). These characteristics are attributed to the cavitation process, which causes changes in the cell membrane of the sample, aiding in the process of extracting the target compound from the intracellular to the extracellular media (Koubaa *et al*., 2016). Zhong *et al*., (2018) evaluated the surface morphology of samples after extraction of the oil by ultrasound and observed drastic changes in the surface of the vegetal tissue, since the ultrasonic waves that pass through the solvent create cavities, which, when they come into contact with the surface of the solid, cause the formation of pores that facilitate the entrance of the solvent.

A variety of studies available in the literature report the achievement of higher oil yields with the use of the ultrasound-assisted process for a wide range of plant matrices. For example, Rodrigues *et al.,* (2017), and Stevanato and Silva (2019) reported that the use of ultrasound allowed increases of ~40 and ~70% in the oil yield of macauba pulp and radish seeds, respectively, when compared to the yield obtained without ultrasound.

Oil extraction from the passion fruit seeds using ultrasound is reported in previous works (Lee *et al.*, 2015; Oliveira *et al*., 2013; Oliveira *et al*., 2014; Oliveira *et al*., 2016; Pereira *et al*., 2017; Pereira *et al*., 2018); however, the evaluation of the effect of the process variables performed in these works is limited and suggests that a detailed study with the aim of optimizing the extraction process is required.

Keeping in line with the efficiency of the extraction technique, environmental concerns, health, and safety, the use of ethanol has gained interest because it is a biodegradable solvent recognized as GRAS (generally recognized as safe) due to its low toxicity and lower volatility. In addition, this alcohol is obtained from renewable sources and is readily available (Neto *et al*., 2018; Plotka-Wasylka *et al.*, 2017).

Extraction with ethanol is an appropriate method when the oil obtained is to be used for the synthesis of higher added value products such as fatty acid esters, di- and monoglycerides. Considering that ethanol acts as a solvent for oil extraction and a reagent in the transesterification reaction, the sequential process can be applied. In such a process, the product from the extraction step (oil + solvent) is directed to the reaction step, without the need for solvent removal or oil treatment steps (Stevanato and Silva, 2019).

The objective of this work was to determine the maximum oil yield that can be obtained from passion fruit seeds using UAE with ethanol as solvent, and to use the extraction product (oil + ethanol) in its maximum condition for the reaction step, thereby simulating a sequential process. The effects of the main variables affecting the extraction were evaluated (temperature, sample/solvent ratio, and time) by means of an experimental Box–Behnken design, as well as the effect of ultrasound on the removal of oil. The maximum yield obtained by UAE and the composition of the oil obtained under these conditions were compared with those obtained from

traditional extraction. In the product (oil + ethanol) from UAE (maximum oil yield) an enzymatic catalyst was added and the transesterification reaction was conducted, with subsequent determination of the composition of the product obtained.

2. MATERIALS AND METHODS

2.1. Materials

In the present work, ethanol (99.75%, JT Baker) and *n*-hexano (98.5%, Anidrol) were used as solvents and the oil was obtained from the seeds of passion fruit (purchased in the local market of Umuarama, PR). Methyl heptadecanoate (> 99%, Sigma-Aldrich), 5α-cholestane (> 99%, Sigma-Aldrich), a FAME (fatty acid methyl esters) mixture (Supelco), heptane and the derivatization reagent Boron trifluoride-methanol solution (14%, Sigma Aldrich) were used for the characterization of the oil, and *N,O* bis (trimethylsilyl) trifluoroacetamide (1% TMCS, Fluka). Novozym® 435 lipase (*Candida Antarctica* immobilized), heptane (Anidrol) and methyl heptadecanoate (Sigma-Aldrich, 99.9% purity) were used to determine the ethyl ester contents in the samples, and *N*-Methyl-*N*- (trimethylsilyl) trifluoroacetamide (MSTFA) was used to determine the mono-, di- and triglyceride contents.

2.2. Preparation of the raw material

The fruits were cut in half in the horizontal direction, and their seeds were removed manually. The seeds were then washed under running water, the surface water was removed, and the seeds were put in the oven (Marconi, MA 035) at 70 ºC to obtain samples with a moisture content of 2.34 ± 0.69%. After drying, the aryl was removed completely and the material was triturated and passed through granulometric grading to separate the 0.638-mm fraction for the experiments.

2.3. Oil extraction

UAE was conducted using ethanol as a solvent. In each experimental run, the flask containing the passion fruit seeds and ethanol was placed in the center of an ultrasound bath (Ultronique, Q 5.9/40A), previously heated to the experimental temperature, and ultrasonic irradiation was immediately initiated (power of 165 W and frequency of 25 kHz). To avoid the loss in solvent, the flask was connected to the condenser which was held at 10 °C.

In order to determine the effect of the process variables and the experimental conditions that maximize the removal of the oil from the seeds, the following levels for each variable were evaluated: (a) times (X_1) of 10, 30, and 50 min; (b) temperatures (X_2) of 30, 45, and 60 ºC; and solvent-to- seed ratios (X_3) of 2, 3, and 4 ($mL \cdot g^{-1}$), which were combined by means of a Box–Behnken design. The experimental range for process variables was defined based on previous studies: time (Pereira *et al.*, 2017), temperature (Rodrigues *et al*., 2017; Rosa *et al*., 2019; Xu *et al*., 2016) and solvent-to-seed ratios (Oliveira *et al.* 2014).

Analysis of variance was carried out to evaluate the effect of the variables on the oil yield with a 95% confidence interval using Statistica 8.0 software (StatSoft™, Inc.). In addition, the experimental data were fitted to a second-order polynomial model, which was used to determine the condition of maximum oil extraction. In this condition, the effect of ultrasound on the removal of oil from passion fruit seeds was evaluated, with extractions performed without ultrasound in an orbital shaker (Marconi, MA 839/A) at 40 rpm as described above.

For comparative purposes, the traditional extraction was performed in a Soxhlet extractor using the *n*-hexane a at a sample ratio of 30 ($mL \cdot g^{-1}$) for 480 min.

For each extraction method used, after the duration of the experimental run, the seeds were separated and the solvent was removed until constant weight was achieved, thereby obtaining the oil. The oil yield was determined as the ratio between the mass of oil extracted and the mass of seeds used.

2.4. Oil characterization

The methodology reported by Gonzalez *et al*., (2013) was used in the preparation of the samples to determine the fatty acid composition. In this methodology, the samples were derivatized with BF3-methanol and subsequently methyl heptadecanoate diluted in heptane was added to quantify the compounds. The analysis was performed in a gas chromatograph (GC–MS QP2010 SE, Shimadzu) equipped with Rtx – Wax (Shimadzu, 30 m x 0.32 mm id x 0.25 μm) capillary column and flame ionization detector, using the

chromatographic conditions reported by Stevanato and Silva (2019). The components present in the samples were identified through comparison with authentic standards of fatty acid methyl esters.

In the determination of phytosterol and tocopherol contents, 5α-cholestane as internal standard and thereafter 40 μL of *N,O*-bis (trimethylsilyl) trifluoroacetamide were added to 40 mg of sample. The samples were maintained for 60 min at 60 °C and after dilution in heptane to obtain a volume of 1000 μL of solution, they were analyzed on an GC–MS QP2010 SE (Shimadzu) with SH-RTx-5MS column (Shimadzu, 5% phenyl–methylsiloxane, 30 m × 0.25 mm id x 0.25 μm). The column temperature was initially programmed at 50 °C, increasing to 230 °C at a rate of 10 °C·min^{-1} and then to 280 °C at a rate of 15 °C·min^{-1}, which was held for 22 min. Helium was used as the carrier gas at a 1.0 mL·min^{-1} flow rate with split ratio of 10 and the amount of injected sample was 1 μL. Mass spectra were recorded at 70 eV with mass range from m/z 50 to 550 amu. Compounds were identified through the comparison of spectrum data to those presented in the NIST14.lb and NIST14.lbs spectral libraries.

2.5. Sequential reaction

The UAE product (oil + ethanol) was added again in an Erlenmeyer flask and received the enzymatic catalyst in the proportion of 40% (relative to oil mass) as defined by Stevanato and Silva (2019). The enzyme was activated for 1 h at 40 °C prior to use. The flask was incubated in orbital agitation (Marconi MA 830/A) at 150 rpm by applying reaction times of 12 and 24 h. The experiments were carried out under the temperature of maximum oil yield determined for the UAE (60 ºC). At the end of each reaction, the enzymes and seeds were separated by filtration and the excess ethanol in the samples was evaporated until reaching constant weight.

2.6. Characterization of the reaction product

The analysis of the chemical composition of the reaction product was carried out in a gas chromatograph (Shimadzu, GC-2010 Plus) equipped with a flame ionization detector and capillary columns SH-Rtx-Wax™ (Shimadzu, 30 m × 0.32 mm × 0.25 μm) and ZB-5HT inferno™ (Zebron, 10 m × 0.32 mm × 0.10 μm) to determine the content of methyl esters and acylglycerols, respectively. The chromatographic conditions for both analyses were previously established (Stevanato and Silva, 2019).

To quantify the content ethyl esters, the injected sample was prepared with 100 μL of a solution of oil in heptane (C = 30 mg·mL^{-1}), 80 μL of internal standard (methyl heptadecanoate, C = 12.5 mg·mL^{-1}) and 820 μL of heptane. For simultaneous determination of the contents of glycerol, mono-, di- and triglycerides, MSTFA was used as a silylating agent. Glycerol, monolein, diolein and triolein were used as external standard to construct the calibration curve ($R^2 > 0.99$).

The free fatty acid (FFA) content in the reaction product was analyzed in a gas chromatograph (Shimadzu, GC-2010 Plus) and the samples were prepared and analyzed according to the methodology for determining phytosterols and tocopherols as described in section 2.4, using methyl heptadecanoate as an internal standard.

2.7. Analysis of data

The data were subjected to ANOVA using Excel® 2010 software and the Tukey test (p=0.05) to evaluate differences between the results. The experiments and analyses were performed in duplicate, so four observations were used to calculate each mean value.

3. RESULTS AND DISCUSSION

3.1. Ultrasound-assisted extraction

3.1.1. Effect of experimental variables

The experimental results for the oil yield obtained from passion fruit seeds in each experimental run of the experimental design are presented in Table 1. Table 2 shows the effect of the independent variables on oil yield, considering the linear, quadratic, and interaction effects among the variables.

Equation 1 shows the correlation between oil yield and the experimental variables obtained from regression analysis data (Table 2). From the F-test, the validation of the predictive equation was verified, with values of 101.15 and 3.73 for F_{CAL} and F_{TAB}, respectively. Thus, the equation was capable of representing the experimental data for the range of variables investigated, because $F_{CALC} > F_{TAB}$ (calculated from the ANOVA table and tabulated, respectively).

$$\text{Oil yield (\%)} = 18.06 + 1.62X_1 + 2.44X_2 + 2.03X_3 - 0.86X_1^2 - 0.93X_3^2 - 0.42X_1X_2 - 0.32X_3X_2 \quad (1)$$

From the data presented in Table 1, it can be verified that high yields (20.82 and 21.12%) were obtained at the highest level (60 ºC) of the temperature variables (runs 4 and 12), which corroborates the effect of this variable presented in Table 2, which indicates that in the experimental range evaluated, the oil removal from passion fruit seeds was favored by the increase in temperature.

Baümler *et al.,* (2016) determined the equilibrium constant (K) of the oil extraction of sunflower collets and reported a decrease in this parameter with increasing temperature and a greater oil extraction capacity. According to Stamenković *et al.*, (2018) and Zhong *et al*., (2018) the increase in temperature may cause increased solubility of the oil in the solvent and diffusivity of the oil from the sample to the solvent, since the properties of ethanol are drastically affected by the increase in temperature. According to Granero *et al*., (2014), the increase in temperature from 20 to 50 °C results in a reduction in the density and dynamic viscosity of ethanol from 789.48 to 763.22 kg·m^{-3} and 1.24 to 0.72 mPa s, respectively. Pereira *et al*., (2017) reported higher solubility of passion fruit oil in ethanol with increasing temperature and obtained better oil extraction at 60 ºC.

Table 1. Experimental conditions applied and oil yield obtained in experiments to assess the effects of the operating variables using a Box-Behnken design.

Run	X_1[1]	X_2[2]	X_3[3]	Oil yield (%)
1	10	30	1:3	12.69
2	50	30	1:3	16.39
3	10	60	1:3	18.79
4	50	60	1:3	20.82
5	10	45	1:2	12.75
6	50	45	1:2	16.06
7	10	45	1:4	16.16
8	50	45	1:4	20.09
9	30	30	1:2	12.32
10	30	60	1:2	17.48
11	30	30	1:4	17.37
12	30	60	1:4	21.25
13-16	30	45	1:3	18.06[4] ±0.08

[1]X_1: time (min); [2]X_2: temperature (ºC); [3]X_3: solvent to seed ratio (mL·g^{-1}); [4]Average of four experiments.

Table 2. Model coefficients and *p*-value of the model for the extraction of passion fruit seed oil by UAE.

Variables	Effect [a]	*p*-value [b]	Coefficient [c]
Mean/Interaction	16.85	< 0.0001	18.06
X_1 (L)	3.24	0.0003	1.62
X_1 (Q)	0.86	0.0026	-0.86
X_2 (L)	4.89	0.0001	2.44
X_2 (Q)	0.02	0.6470	-0.02
X_3 (L)	4.06	0.0002	2.03
X_3 (Q)	0.93	0.0023	-0.93
X_1 x X_2	-0.83	0.0103	-0.42
X_1 x X_3	0.31	0.0685	0.15
X_2 x X_3	-0.64	0.0175	-0.32

[a] Effect of the independent variable on the dependent variable; [b] statistical significance $p < 0.05$; [c] coefficients of second-order polynomial model (Equations 1 and 2).

The increase in oil yield with the increase in the solvent to seed ratio is due to the higher dissolution capacity, which leads to changes in the thermodynamic and mass transfer properties of the extraction process. Stamenković *et al*., (2018) performed thermodynamic analyses of oil extraction of white mustard (*Sinapis alba* L.) seeds, respectively, and found that the spontaneity of the process was favored by increases in the volume of solvent used. Meziane and Kadi (2008) and Toda *et al.,* (2016) reported higher values for the mass transfer coefficients with the use of higher amounts of solvent.

In addition, during ultrasonic treatment, the collapse between the cavitation bubbles generated high-speed solvent microjets that caused damage to the plant matrix cell wall, increasing the contact area between the matrix and solvent and, consequently, increasing the efficiency of oil extraction (Vinatoru *et al*., 2017). Other authors have reported an effect similar to that observed in this study for the experimental interval evaluated; for example, Mabayo *et al*., (2018) reported that an increase in the ratio of *n*-hexane to rubber seeds from 2 to 4 (mL·g^{-1}) led to an increase in the oil yield from 21.3 to 30.7%, respectively.

On analyzing the interaction between the variables solvent-to-seed ratio and time, it was verified that they had no effect on oil yield. This can be explained on the basis of the results of Meziane and Kadi (2008), who determined the mass transfer coefficients for the extraction stages characterized by washing and diffusion, respectively, using a different solvent-

to-sample ratio and obtaining higher mass transfer coefficients with increasing amounts of solvent used, with higher coefficients in the initial stage.

Extraction occurs in two phases: the first corresponds to rapid extraction, with the removal of the surface constituents of the particle, followed by a slower second phase called diffusion, in which the inner components of the matrix are removed (Chanioti and Tzia, 2018; Stamenković *et al*., 2018; Toda *et al*., 2016). In the initial stage, there is a greater extraction of the oil. This effect is observed in the comparison of the results presented in Table 1 for runs 5–6 and 7–8, for example, where it is possible to observe the removal of ~80% of the oil obtained in 50 min (runs 6 and 8) in an extraction time of only 10 min (runs 5 and 7). However, the use of longer extraction times contributes to further rupture of the cell walls, resulting in greater penetration of the solvent (Balachandran *et al.,* 2006; Kazemi *et al.,* 2016) and consequently an increase in oil yield ($p < 0.05$).

Other studies report the best removal of the oil by subjecting the sample to a longer ultrasound treatment time, among which we can mention the works of Mello *et al*., (2015) and Rodrigues *et al*., (2017), who obtained higher oil yields with increases in the time of treatment with ultrasound from 20 to 60 min.

The effect of time is more pronounced at lower temperatures, as can be seen in the comparison of the data from runs 1–2 and 3–4, since the equilibrium is reached more rapidly with the rise in temperature.

3.1.2. Maximizing oil yield

From the predictive equation, Equation 1, the maximum oil yield that could be obtained within the experimental range evaluated was 21.76% at 50 min and 60 °C with a solvent-to-seed ratio of 4 ($mL \cdot g^{-1}$). Additional experiments were performed in this predicted experimental condition, in triplicate, providing an oil yield of 21.39 ± 0.7%.

The oil yield obtained is close to those reported in other studies that evaluated oil removal from passion fruit seeds using UAE; however, it is worth noting the lower volume of solvent used in this work. Oliveira *et al*., (2013) obtained an oil yield of 19.5% through extraction conducted for 60 min using an ethanol-to-seed ratio of 6 ($mL \cdot g^{-1}$). Pereira *et al*., (2017) reported an oil yield of 20.96% in extraction conducted for 60 min with an ethanol-to-seed ratio of 50 ($mL \cdot g^{-1}$).

The results obtained by Liu *et al*., (2009) and Oliveira *et al*., (2016) showed higher yields of 25.98 and 27%, respectively, with supercritical CO_2 extraction; however, they used high operating pressures (25 MPa) and longer extraction times (150 and 180 min).

3.2. Effect of ultrasound on extraction

Extraction performed without the use of ultrasound, provided an oil yield of 15.82 ± 0.8% under the conditions of maximum oil yield obtained by Equation 1, which represents 75% of the yield obtained with ultrasound. The advantages and characteristics of ultrasound have already been discussed and justify the result obtained.

3.3. Comparison between UAE and classical extraction

Table 3 shows the results of the comparison between traditional extraction and UAE in terms of oil yield, fatty acid composition, and phytosterol and tocopherol contents.

In the traditional extraction (in Soxhlet), the passion fruit seeds used in this work presented oil yields of 29.02 ± 1.61%. This result is close to that reported in the works of Oliveira *et al*., (2013), Pereira *et al*., (2017) and Santana *et al*., (2017), who presented oil yields of 26.4, 28.33, and 28.87%, respectively.

UAE provided 73.7% of the yield obtained from traditional extraction; however, with this method the oil is obtained in a shorter time and mainly using a smaller volume of solvent, since a solvent-to-seed ratio of 4 ($mL \cdot g^{-1}$) is required, while in traditional extraction a solvent-to-seed ratio of 30 ($mL \cdot g^{-1}$) is used. In UAE, the cavitation of the medium favors mass transfer and thus a smaller volume of solvent is necessary; whereas in the classic method the extraction process is governed by mass diffusion, requiring a high consumption of solvent (Menezes *et al.*, 2018).

The results presented in Table 3 show that there is no difference between the fatty acid compositions of the oils obtained with the techniques used. Passion fruit seed oil contains a high concentration of PUFAs (59.28 to 61.19), with linoleic acid forming the major part (59.13 to 61.07%). Another acid that stands out is oleic acid (17.45%). Other studies have also reported that these were the main fatty acids found in passion fruit seeds (Pereira *et al*., 2018; Silva and Jorge, 2017).

Table 3. The fatty acid profile of the passion fruit seed oil.

Parameter evaluated		Extraction method	
		CE	UAE[1]
Oil yield (%)		29.02±1.61[a]	21.39±0.7[b]
Fatty acid (g per 100 g of oil)	Palmitic	12.25±0.15[a]	12.31±0.01[a]
	Palmitoleic	0.17±0.05[a]	0.19±0.05[a]
	Stearic	2.48±0.04[a]	2.44±0.14[a]
	Oleic	17.45±1.25[a]	17.03±0.28[a]
	Linoleic	59.13±1.39[a]	61.07±0.98[a]
	Linolenic	0.15±0.04[a]	0.12±0.03[a]
	SFA	14.73	14.75
	MUFA	17.62	17.22
	PUFA	59.28	61.19
Phytosterol (mg per 100 g of oil)	Stigmasterol	74.29±2.17	75.67±0.05
	Campesterol	19.20±1.42	21.55±0.30
	β-Sitosterol	54.26±4.91	69.11±3.12
	Total	147.75±4.16[a]	166.34±3.36[b]
Tocopherol (mg per 100 g oil)	γ-tocopherol	7.94±1.20	7.63±1.30
	δ-tocopherol	26.00±4.83	37.02±5.36
	Total	33.94±6.03[a]	44.65±6.65[a]

[1]obtained in the condition of maximum oil yield in oil: 50 min, 60 °C and solvent-to-seed ratio of 4 ($mL \cdot g^{-1}$). (CE) Conventional extraction, (UAE) Ultrasound-assisted extraction (UAE) (SFA) Saturated fatty acids, (MUFA), Monounsaturated fatty acids and (PUFA) polyunsaturated fatty acids. Experiments and analyses conducted in duplicate. Means followed by same letters (on each line) did not differ statistically ($p > 0.05$) by the Tukey test.

As can be seen in Table 3, stigmasterol and δ-tocopherol are the predominant phytosterols and tocopherols, respectively, in the composition of passion fruit seed oil.

In the extraction of tocopherols, the evaluated methods provided similar levels of these compounds. The values obtained (33.94 to 44.64 mg per 100 g of oil) were higher than those reported by Pereira *et al*., (2017) in the extraction using pressurized propane (9.87 to 11.14 mg per 100 g of oil) and UAE with ethanol (10.10 mg per 100 g of oil). However, they were lower than the value of 49.9 mg per 100 g of oil as reported by Malacrida and Jorge (2012) with the traditional extraction using petroleum ether.

The UAE provided an extract with a higher content in phytosterols of 166.64 mg per 100 g of oil. The content obtained in this study was lower than that obtained by Piombo *et al*., (2006) and Silva and Jorge (2017), who reported values of 209.74 and 274.67 mg per 100 g of oil, respectively.

The differences found in relation to the literature are related to the nature of the raw material used, as well as the solvent extractor and the efficiency of the extraction method used.

3.4. Sequential reaction

Table 4 shows the results of the chemical composition of the samples obtained after conducting the reaction step in 12 and 24 h. The analysis of the data must be carried out by observing which compound is of most interest in the product, which must be directed to the separation step aiming at the concentration of the target compound. It should be noted that in addition to the compounds identified (Table 4), these samples contain phospholipids, waxes and sugars (Baümler *et al.,* 2016), compounds identified in vegetable oils obtained using ethanol as a solvent.

From the data presented in Table 4, it appears that in 12 h of reaction the product obtained contains ethyl esters, diglycerides (DG) and monoglycerides (MG) in higher concentrations. The high triglyceride conversion in the reaction transesterification products

Table 4. Chemical composition of reaction products obtained from transesterification reaction of product of extraction step (oil + ethanol).

Reaction time (h)[1]	Component (wt%)					
	Ethyl esters	TG	DG	MG	GLY	FFA
12	42.30±0.52	4.82±0.05	22.26±0.27	21.80±0.30	0.314±0.01	0.063±0.001
24	55.58±0.26	0.35±0.01	6.71±0.05	25.63±0.06	0.549±0.01	0.055±0.004

[1]Experiments and analyses conducted in duplicate. (TG) Triglycerides, (DG) Diglycerides, (MG) Monoglycerides, (GLY) glycerol, (FFA) free fatty acid.

after 24 h of reaction was observed, obtaining a sample with a predominance in ethyl esters and MG under these conditions.

Ethyl esters can be directed to use as biofuels (Gonzalez *et al*., 2013; Stevanato and Silva, 2019), in compliance with current legislation. Bitonto *et al*., (2019) reported that these compounds are considered non-hazardous organic compounds, in addition to biofuels, and have industrial applications such as solvent, fragrances and cosmetics.

MG and DG have applications as emulsifiers in the food and pharmaceutical industry (Hartel *et al*., 2018). These emulsifiers are frequently used in bakery products, frozen desserts, and sauces/dressings (Nicholson *et al*., 2019). According to Ferreti *et al*., (2018) diglycerides are widely used for foods such as mayonnaise, salad dressings and in the confectionery industry, as they combine a hydrophilic head and a hydrophobic tail in the molecule that help hydrophilic and lipophilic substances to mix.

Thus, it was found that the sequential process provided a high-quality oil, eliminating the solvent removal step, which favors its application in different products and because it is a food residue, the oil obtained from the seeds Passion fruit can be applied to food products that require emulsifiers during their production process.

4. CONCLUSIONS

This study presents the use of UAE to obtain the oil of passion fruit seeds. Through the use of ethanol with solvent, it was verified that temperature was the variable with the greatest influence on the extraction, and high yields of oil (21.7%) were obtained using a low solvent volume (a solvent-to-seed ratio of 4 $mL \cdot g^{-1}$). High yields can be obtained at low extraction times (10 min), representing ~80% of the maximum yield obtained in 50 min. The extraction of the oil is favored by the application of ultrasound, with which it was possible to obtain 73.7% of the yield provided by traditional extraction. Oleic and linoleic acids represent ~80% of the oil composition. The evaluated techniques provided oil with a similar tocopherol content; however, higher levels were obtained through extraction by ultrasound. Conducting the sequential reaction to the extraction process provided products with different applications, eliminating the solvent removal step.

ACKNOWLEDGMENTS

The authors thank CNPq and the Araucaria Foundation for financial support.

REFERENCES

Balachandran S, Kentish S, Mawson R, Ashokkumar, M. 2006. Ultrasonic enhancement of the supercritical extraction from ginger. *Ultrason. Sonochem.* **13**, 471-479. https://doi.org/10.1016/j.ultsonch.2005.11.006

Baümler ER, Carrin ME, Carelli AA. 2016. Extraction of sunflower oil using ethanol as solvent. *J. Food. Eng.* **178**, 190-197. https://doi.org/10.1016/j.jfoodeng.2016.01.020

Bitonto Luigi, Menegatti S, Pastore C. 2019. Process intensification for the production of the ethyl esters of volatile fatty acids using aluminium chloride hexahydrate as a catalyst. *J. Clean. Prod.* **239**, 118122. https://doi.org/10.1016/j.jclepro.2019.118122

Chanioti S, Tzia C. 2018. Processing parameters on the extraction of olive pomace oiland its bioactive compounds: a kinetic and thermodynamic study. *J. Am. Oil Chem. Soc.* **95**, 371-382. https://doi.org/10.1002/aocs.12021

Chau CF, Huang YL. 2004. Characterization of passion fruit seed fibres: a potential fibre source. *Food Chem.* **85**, 189-194. https://doi.org/10.1016/j.foodchem.2003.05.009

EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária, 2017. Retrieved from http://www.cnpmf.embrapa.br/Base_de_Dados/index_pdf/dados/brasil/maracuja/b1_maracuja.pdf

Ferretti CA, Spotti ML, Di Cosimo, JI. 2018. Diglyceride-rich oils from glycerolysis of edible vegetable oils. *Catal. Tod.* **302**, 233-241. https://doi.org/10.1016/j.cattod.2017.04.008

Gonzalez SL, Sychoski MM, Navarro-Díaz HJ, Callejas N, Saibene M, Vieitez I, Jachmanián I, Silva C, Hense H, Oliveira JV. 2013. Continuous catalyst-free production of biodiesel through transesterification of soybean fried oil in supercritical methanol and ethanol. *Energ. Fuel.* **27**, 5253-5259. https://doi.org/10.1021/ef400869y

Hartel RW, von Elbe JH, Hofberger R. 2018. Fats, oils and emulsifiers. In: Confectionery Science and Technology, Springer, Cham., pp.85-124. https://doi.org/10.1007/978-3-319-61742-8_4

Kazemi M, Karim R, Mirhosseini H, Hamid AA. 2016. Optimization of pulsed ultrasonic-assisted technique for extraction of phenolics from pomegranate peel of *Malas* variety: punicalagin and hydroxybenzoic acids. *Food Chem.* **206**, 156-166. https://doi.org/10.1016/j.foodchem.2016.03.017

Khoei M, Chekin F. 2016. The ultrasound-assisted aqueous extraction of rice bran oil. *Food. Chem.* **194,** 503-507. https://doi.org/10.1016/j.foodchem.2015.08.068

Koubaa M, Mhemdi H, Barba FJ, Roohinejad S, Greiner R, Vorobiev E. 2016. Oilseed treatment by ultrasounds and microwaves to improve oil yield and quality: An overview. *Food Res. Int.* **85**, 59-66. https://doi.org/10.1016/j.foodres.2016.04.007

Lee SY, Fu SY, Chong GH. 2015. Ultrasound-assisted extraction kinetics, fatty acid profile, total phenolic content and antioxidant activity of green solvents extracted passion fruit oil. *Int. J. Food Sci. Tech.* **50**, 1831-1838. https://doi.org/10.1111/ijfs.12844

Li T, Qu XY, Zhang QA, Wang ZZ. 2012. Ultrasound-assisted extraction and profile characteristics of seed oil from *Isatis indigotica* Fort. *Ind. Crop. Prod.* **35**, 98-104. https://doi.org/10.1016/j.indcrop.2011.06.013

Liu S, Yang F, Zhang C, Ji H, Hong P, Deng C. 2009. Optimization of process parameters for supercritical carbon dioxide extraction of *Passiflora* seed oil by response surface methodology. *J. Supercrit. Fluids* **48**, 9-14. https://doi.org/10.1016/j.supflu.2008.09.013

Luque-García J, Luque de Castro M. 2003. Ultrasound: a powerful tool for leaching. *Trac-Trend Anal. Chem.* **22**, 41-47. https://doi.org/10.1016/S0165-9936(03)00102-X

Mabayo VIF, Aranas JRC, Cagas VJB, Cagas DPA, Ido AL, Arazo RO. 2018. Optimization of oil yield from *Hevea brasiliensis* seeds through ultrasonic-assisted solvent extraction via response surface methodology. *Sustain. Environ. Res.* **28**, 39-46. https://doi.org/10.1016/j.serj.2017.08.001

Malacrida CR, Jorge N. 2012. Yellow passion fruit seed oil (*Passiflora edulis* f. *flavicarpa*): physical and chemical characteristics. *Braz. Arch. Biol. Technol.* **55**, 127-134. https://doi.org/10.1590/S1516-89132012000100016

Mello BTF, Garcia VAS, Silva C. 2015. Ultrasound-Assisted Extraction of Oil from Chia (*Salvia hispânica* L.) Seeds: Optimization Extraction and Fatty Acid Profile. *J. Food Process Eng.* **40**, 1-8. https://doi.org/10.1111/jfpe.12298

Menezes ML, Johann G, Diório A, Pereira NC, Silva EA. 2018. Phenomenological determination of mass transfer parameters of oil extraction from grape biomass waste. *J. Clean. Prod.* **176**, 130-139. https://doi.org/10.1016/j.jclepro.2017.12.128

Meziane S, Kadi H. 2008. Kinetics and Thermodynamics of Oil Extraction from Olive Cake. *J. Am. Oil Chem.'Soc.* **85**, 391-396. https://doi.org/10.1007/s11746-008-1205-2

Neto OZS, Batista EAC, Meirelles AJA. 2018. The employment of ethanol as solvent to extract Brazil nut oil. *J. Clean. Prod.* **180**, 866-875. https://doi.org/10.1016/j.jclepro.2018.01.149

Nicholson RA, Marangoni AG. 2019. Diglycerides. In: Melton L, Shahidi F, Varelis P, *Encyclopedia of Food Chemistry* **1**, 70–73.

Oliveira CF, Giordani D, Lutckemier R, Gurak PD, Cladera-Oliveira F, Marczak LDF. 2016. Extraction of pectin from passion fruit peel assisted by ultrasound. *LWT – Food Sci. Technol.* **71**, 110-115. https://doi.org/10.1016/j.lwt.2016.03.027

Oliveira RC, Barros STD, Gimenes ML. 2013. The extraction of passion fruit oil with green solvents. *J. Food Eng.* **117**, 458-463. https://doi.org/10.1016/j.jfoodeng.2012.12.004

Oliveira RC, Guedes TA, Gimenes ML, Barros STD. 2014. Effect of process variables on the oil extraction from passion fruit seeds by conventional and non-conventional techniques. *Acta Sci. Technol.* **36**, 87-91. https://doi.org/10.4025/actascitechnol.v36i1.15217

Pereira MG, Hamerski F, Andrade EF, Scheer AP, Corazza ML. 2017. Assessment of subcritical propane, ultrasound-assisted and Soxhlet extraction of oil from sweet passion fruit (*Passiflora alata* Curtis) seeds. *J. Supercrit. Fluids* **128**, 338-348. https://doi.org/10.1016/j.supflu.2017.03.021

Pereira MG, Maciel GM, Haminiuk CWI, Bach F, Harmeski F, Scheer AP, Corazza ML. 2018. Effect of Extraction Process on Composition, Antioxidant and Antibacterial Activity of Oil from Yellow Passion Fruit (*Passiflora edulis* Var.

Flavicarpa) Seeds. *Waste Biomass Valori.* 1-15. https://doi.org/10.1007/s12649-018-0269-y

Perrier A, Delsart C, Bousseta N, Grimi N, Citeau M, Vorobiev E. 2017. Effect of ultrasound and green solvents addition on the oil extraction efficiency from rapeseed flakes. *Ultrason. Sonochem.* **39**, 58-65. https://doi.org/10.1016/j.ultsonch.2017.04.003

Piombo G, Barouh N, Barea B, Boulanger R, Brat P, Pina M, Villeneuve P. 2006. Characterization of the seed oils from kiwi (*Actinidia chinensis*), passion fruit (*Passiflora edulis*) and guava (*Psidium guajava*). *OCL* **13**, 195-199. https://doi.org/10.1051/ocl.2006.0026

Plotka-Wasylka J, Rutkowska M, Owczarek K, Tobiszewski M, Namieśnik J. 2017. Extraction with environmentally friendly solvents. *Trac-Trend Anal. Chem.* **91**, 12-25. https://doi.org/10.1016/j.trac.2017.03.006

Rodrigues GM, Mello BTF, Garcia VAS, Silva C. 2017. Ultrasound-assisted extraction of oil from macauba pulp using alcoholic solventes. *J. Food Process Eng.* **40**, 1-8. https://doi.org/10.1111/jfpe.12530

Rosa ACS, Stevanato N, Iwassa I, Garcia VAS, Silva C. 2019. Obtaining oil from macauba kernels by ultrasound-assisted extraction using ethyl acetate as the solvent. *Braz. J. Food Technol*. **22**, e2018195. https://doi.org/10.1590/1981-6723.19518

Santana FC, Torres LRO, Shinagawa FB, Silva AMO, Yoshime LT, Melo ILP, Marcellini PS, Mancini-Filho J. 2017. Optimization of the antioxidant polyphenolic compounds extraction of yellow passion fruit seeds (*Passiflora edulis* Sims) by response surface methodology. *Int. J. Food Sci. Tech.* **54**, 3552-3561. https://doi.org/10.1007/s13197-017-2813-3

Silva AC, Jorge N. 2014. Bioactive compounds of the lipid fractions of agro-industrial waste. *Food Res. Int.* **66**, 493-500. https://doi.org/10.1016/j.foodres.2014.10.025

Silva AC, Jorge N. 2017. Bioactive compounds of oils extracted from fruits seeds obtained from agroindustrial waste. *Eur. J. Lipid. Sci. Tech.* **119**, 1-5. https://doi.org/10.1002/ejlt.201600024

Stamenković OS, Djalović IG, Kostić MD, Mitrović PM, Veljković VB. 2018. Optimization and kinetic modeling of oil extraction from white mustard (*Sinapis alba* L.) seeds. *Ind. Crop. Prod.* **121**, 132-141. https://doi.org/10.1016/j.indcrop.2018.05.001

Stevanato N, Silva C. 2019. Radish seed oil: Ultrasound-assisted extraction using ethanol as solvent and assessment of its potential for ester production. *Ind. Crop. Prod.* **132**, 283-291. https://doi.org/10.1016/j.indcrop.2019.02.032

Toda TA, Sawada MM, Rodrigues CEC. 2016. Kinetics of soybean oil extraction using ethanol as solvent: Experimental data and modeling. *Food Bioprod. Process.* **98**, 1-10. https://doi.org/10.1016/j.fbp.2015.12.003

Vinatoru M, Mason TJ, Calinescu I. 2017. Ultrasonically assisted extraction (UAE) and microwave assisted extraction (MAE) of functional compounds from plant materials. *Trac-Trend Anal. Chem.* **97**, 159-178. https://doi.org/10.1016/j.trac.2017.09.002

Xu G, Liang C, Huang P, Liu Q, Xu Y, Ding C, Li T. 2016. Optimization of rice lipid production from ultrasound-assisted extraction by response surface methodology. *J. Cereal Sci*. **70**, 23-28. https://doi.org/10.1016/j.jcs.2016.05.007

Zhong J, Wang Y, Yang R, Liu X, Yang Q, Qin X. 2018. The application of ultrasound and microwave to increase oil extraction from *Moringa oleifera* seeds. *Ind. Crop. Prod.* **120**, 1-10. https://doi.org/10.1016/j.indcrop.2018.04.028

GRASAS Y ACEITES 72 (2)
April-June 2021, e410
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0219201

Effect of process parameters on emulsion stability and droplet size of pomegranate oil-in-water

A.H. Kori[a], S.A. Mahesar[a, ✉], S.T.H. Sherazi[a], U.A. Khatri[b], Z.H. Laghari[a] and T. Panhwar[a]

[a]National Centre of Excellence in Analytical Chemistry, University of Sindh,

Jamshoro-76080, Pakistan

[b]Metallurgy and Materials Engineering, Mehran University of Engineering and Technology,

Jamshoro-76090, Pakistan

Corresponding Author: sarfaraz.mahesar@usindh.edu.pk

Submitted: 19 February 2020; Accepted: 25 May 2020; Published online: 03 June 2021

SUMMARY: The development of efficient emulsion is essential and requires a good understanding of the parameters that govern the formation and stability of the emulsion. The droplet size significantly affects the stability of the emulsion. In this study, the stability of pomegranate oil-in-water emulsions (0.5 to 7.0% v/v) was investigated using various emulsifiers in terms of droplet size and instability index during 16 days of storage. The Mastersizer and Lumisizer were used to measure the droplet size and instability index. It was observed that the minimum droplet size was achieved by using 0.3% carboxy methyl cellulose (5.37 µm) and maximum with 1.0/2.5% whey protein/maltodextrin (24.26 µm). The Lumisizer results during storage revealed the higher emulsion stability of carboxy methyl cellulose due to smaller droplet size and high thickness as compared to other emulsions studied. The findings of the present study would

be useful for food applications to obtain fine and stable microcapsules.

KEYWORDS*: Emulsi iers; Emulsion; Optimization; Pomegranate oil; Stability*

RESUMEN: ***Efecto de los parámetros del proceso sobre la estabilidad de la emulsión y el tamaño de la gota de aceite de granada en agua.*** El desarrollo de una emulsión eficiente es esencial y requiere una buena comprensión de los parámetros que rigen la formación y la estabilidad de la emulsión. El tamaño de la gota afecta significativamente a la estabilidad de la emulsión. En este estudio, se investigó la estabilidad de las emulsiones de aceite de granada en agua (0,5 a 7,0% v/v) usando varios emulsionantes, en términos de tamaño de gota e índice de inestabilidad, durante 16 días de almacenamiento. El Mastersizer y el Lumisizer se usaron para medir el tamaño de gota y el índice de inestabilidad. Se observó que, el tamaño mínimo de gota se logró utilizando 0,3% de carboximetilcelulosa (5,37 µm) y el máximo (24,26 µm), con 1,0/2,5% de proteína de suero/maltodextrina Los resultados del Lumisizer, durante el almacenamiento, revelaron una mayor estabilidad de la emulsión de carboximetilcelulosa debido al tamaño de gota más pequeño y al alto espesor en comparación con otras emulsiones estudiadas. Los resultados del presente estudio se utilizarían en aplicaciones alimentarias para obtener microcápsulas finas y estables.

PALABRAS CLAVE: *Aceite de granada; Emulsión; Emulsionantes; Estabilidad; Optimización*

Citation/Cómo citar este artículo: Kori AH, Mahesar SA, Sherazi STH, Khatri UA, Laghari ZH, Panhwar T. 2021. Effect of process parameters on emulsion stability and droplet size of pomegranate oil-in-water. *Grasas Aceites* **72** (2), e410. https://doi.org/10.3989/

gya.0219201

1. INTRODUCTION

Emulsions are extensively utilized in various industrial applications but mainly used in the food industries. In the formulation of some food products such as yogurts, cream liqueurs, mayonnaise, ice cream, and salad dressings, emulsions play a very important role (Charcosset, 2009).

In general, emulsion is considered a heterogeneous composition, in which one immiscible liquid is dispersed into another liquid as droplets with diameters which surpass 0.1 mm (Bai *et al.,*2016). There are two predominant types of emulsions, one is oil-in-water (O/W) and the other is water-in-oil (W/O), while double or multiple emulsions types are O/W/O and W/O/W.

Emulsions are less thermodynamically stable; emulsions break up into two separate phases over time either quickly or slowly. The common mechanisms of the instability of emulsions are creaming, coalescence, Ostwald ripening, and flocculation (Tcholakova *et al.,* 2006). The shelf-life and quality of emulsions are highly affected by the aggregation of droplets. McClements, (2015) described that the properties of stable emulsions slowly change with time or show resistance to change. The increase in the droplet size of emulsions is one of the main reasons for the loss in stability (Silva *et al*., 2010). So, the instability of emulsions can be overcome by reducing the droplet size (McClements, 2015).

Krstonosic *et al.,* (2009) reported that the stability of the emulsion can also be increased with the addition of emulsifiers to decrease surface tension and avoid droplet flocculation by absorption on the surface of droplets. The main purpose of an emulsifier is to prevent the aggregation of newly formed droplets by forming a protective layer as well as decreasing interfacial tension. This results in stabilizing against coalescence (McClement, 2015).

Although the tools for characterizing emulsions are now well developed and the mechanisms of emulsification are reasonably understood, it is still difficult to predict the exact result of an emulsification process, since this is a combination of numerous parameters, including formulation and process variables.

Many micro molecule emulsifiers including Span, sodium dodecyl sulfate, Tweens, etc. and macromolecules of proteins and carbohydrates are added to foods and drugs to form stable emulsions (Hashtjin and Abbasi, 2015, Galooyak and Dabir, 2015). Forming a kinetically stable emulsion for a specific period to increase shelf-life is one of the main challenges of food product formulations.

Tween 20 is ester sorbitol and is widely used as an emulsifier in an oil-water emulsion with a hydrophile-lipophile balance (value 16) for food products. Usually, a low concentration of Tween 20 is considered safe while it is toxic at high concentrations. The carboxyl methyl cellulose (CMC) is taken as a thickener and emulsifier, as it imparts longer time emulsion stability and inhibits creaming by increasing the viscosity of the aqueous continuous phase (Dickinson, 2003). Whey protein (WP) is a dairy by-product and one of the most commonly studied emulsifiers used in polyunsaturated fatty acids (oils). Food proteins have excellent properties of foaming, gelling, and emulsifying as well as confering high nutrition to food products (Chen *et al*., 2006, Matalanis *et al*., 2011). Maltodextrin (MD) is also utilized with WP in combination as it is inexpensive, bland in flavor, and has low viscosity at high solids and prevents the emulsion from creaming or coalescence.

Many researchers have reported that a stable emulsion is a prerequisite for higher encapsulation efficiency, higher stability, smaller particles size, low surface oil and better retention of volatile components; while the opposite is true for the unstable emulsions (Minemoto, Hakamata, Adachi and Matsuno, 2002).

Over hundreds of years, the pomegranate has accompanied mankind as a symbol of life, longevity, health, knowledge, morality, and spirituality (Mackler *et al*., 2013). Pomegranate seeds contain around 3% of the total fruit weight and contain oil in the range of 12–20%. Pomegranate seed oil mainly consists of > 90% polyunsaturated fatty acids such as punicic acid (conjugated fatty acid), linolenic acid, and linoleic acid (Özgül-Yücel, 2005, Fadavi *et al*., 2006). As this oil is highly unsaturated, it easily oxidizes with heat, light, and air, which renders it unfit for edible consumption. It is feasible to add pomegranate oil into water emulsions for encapsulation to improve shelf-life. To the best of our knowledge no report has been published on the pomegranate oil-in-water emulsion.

Therefore, the present study aims at a better understanding of the effect of Tween 20, WP, and

MD as emulsifiers and stabilizers and CMC as a thickener, along with various parameters such as agitation speed, time, and concentration of pomegranate oil-in-water to prepare the most stable emulsion.

2. MATERIALS AND METHODS

2.1. Materials

Virgin pomegranate seed oil was purchased from Natural Source, USA. Various materials such as Tween-20, whey protein, maltodextrin DE=14, and carboxyl methylcellulose were purchased from Sigma Aldrich (Germany). Throughout the experimentations, deionized water from a Millipore (Leeds, MA) system was used.

2.2. Emulsion preparation

Four types of materials i.e. Tween 20, carboxyl methyl cellulose (CMC), whey protein (WP), and a combination of whey protein with maltodextrin (WP/MD) were selected as emulsifiers. To evaluate the effect of different parameters such as revolutions per minute (rpm) 5000 to 25000, time (1 to 5 min), Tween 20 concentration (0.5% to 7% w/v of 5 mL water) and amount of oil (0.5 to 7 g w/v), the emulsion stability and droplet size of pomegranate oil-in-water were optimized as shown in the flow chart in Figure 1. Optimized parameters were also further evaluated for the different concentrations of other emulsifiers on the basis of droplet size and instability index during 16 days of storage. First, the surfactant was dispersed into distilled water then the oil was mixed with magnetic agitation for 1 min. The mixture was then homogenized using a rotor-stator homogenizer.

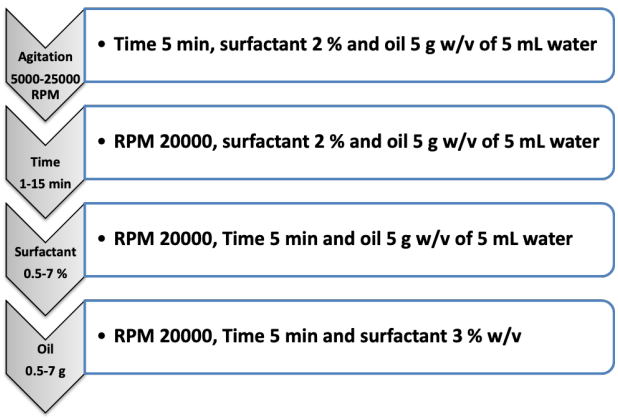


Figure 1. Flow chart of experimental design

2.3. Methods and instruments

A rotor-stator homogenizer, Ultra-Turrax type, IKA T 25 digital (Janke and Kunkel, Germany), with the following specification, was used for homogenization (25000 rpm, 800 W, Stator/rotor diameter: 25 mm (outside) and 18 mm (inside), the gap between rotor and stator 0.5 mm and immersion depth 40 – 165 mm).

2.4. Droplet size distribution

The mean droplet size was measured with a Mastersizer 2000 (Malvern, Worcestershire, UK) and expressed as the Sauter (surface average) diameter $d_{3,2}$. The homogenized samples were analyzed after 1 h prior to preparation following a relative refractive index (1.465) of the dispersed phase (water). After adding drops of the emulsion into dispersion liquid when the obscuration index reached 15%, then the surface mean diameter $d_{3,2}$ and droplet size distribution was measured.

2.5. Centrifugal separation analysis Lumisizer

The LUMiSizer LS650 (Photocentric) multiple samples (Malvern, Worcestershire, UK) was used to understand the storage stability of the pomegranate emulsion that acts on the accelerated centrifugal mechanism. The assessment of emulsion stability (kinetic) requires a longer time, therefore, accelerated tests are used to mitigate this issue. Many techniques have been reported such as ultrasound, centrifugation, and turbidimetry or light scattering, and ultrasonic (Curt, 1994, Horozov and Binks, 2004) but failed in sufficient specificity and reliability in order to clarify the cause of destabilization. The most reliable and effective technique is Lumisizer, which directly measures the stability of emulsion (transmitted light intensity) in neat form. This instrument uses the Space and Time-resolved Extinction Profiles (STEP) technology, which measures transmitted light intensity over the entire sample length in a cuvette during centrifugation as a function of time (Lerche, 2002).

The instability index describes the reason for transmission clarification based on the separation of layer and particle size by accelerated gravitational force at a given time. It is a dimensionless number

between 0 and 1 under the centrifugal field, where 0 (highest stability) represents no change in emulsion transmission or no separation, and 1 (lowest stability) indicates complete segregation of phases. Comparing instability indices of emulsion under accelerated gravitational field ultimately aids in a quick comparison of their shelf-life instead of waiting a long time at the earth's gravitation. A rectangular cuvette of polycarbonate of 298 mm was used to place 350 µL of sample and run on a centrifuge at 3000 rpm at 25 °C by applied laser wavelength (865 nm). The instability or separation index was calculated by SEPView software v 4.1.

2.6. Optical microscopy

The size of emulsions was observed with an optical microscope (BX41-Olympus, UK) connected to a digital camera (Cannon), Japan. One drop of the emulsion was kept on a glass slide and covered with a coverslip to avoid the mobilization of droplets. The droplet size was measured at room temperature with 20 x 100 magnification.

2.7. Statistical analysis

Statistical analysis of the data was carried out using Minitab16 USA software. Data were analyzed by analysis of variance (ANOVA) followed by the Tukey test ($p \leq 0.05$). Results are reported as mean ± (SD) of three replicates (each replicate corresponds to a different batch of emulsion).

3. RESULTS AND DISCUSSION

3.1. Optimization of agitation, time and amount of oil on emulsion droplet size

Initially, the effect of agitation (speed) on emulsion droplet size was carried out to get the smallest droplet size of emulsion on Mastersizer as shown in Figure 2a. According to our results, it was observed that at 20000 rpm, the smallest droplet size of emulsion was recorded at 20.61 µm. At higher agitation speed the size of emulsion droplet decreased with increasing agitation to break up droplets of oil from the large layer into smaller droplets that were coated with emulsifier or surfactant and formed stable or finer emulsion as reported by (McClements, 2015). It has been reported that high agitation speed promotes smaller oil droplet size with stable emulsion. Our results are also comparable to those of Li and Xiang (2019) for coconut oil, where a smaller droplet size of emulsion was found at 15000 rpm. Similarly, in another study, Yerramilli and Ghosh, (2017) observed similar results of the emulsion prepared with canola oil using 20000 rpm. Therefore, 20000 rpm was selected for further parameters.

After fixing or getting the smallest result for the droplet size with agitation at 20000, another variable factor effect of time on emulsion droplet size was examined. The effect of time was selected from 1 to 15 min from a total of five experiments (rpm 2000, Tween 20 2% w/v, oil 5g w/v of 5 mL water were constant) as shown in Figure 2b. With the smaller time period, larger emulsion droplets were produced from 1 to 2 min because of less time to break up oil layers into smaller droplets. With the greater time period (5 min) smaller droplets were obtained and beyond this, no significant effect on emulsion droplets size was observed as shown in Figure 2b. The same finding was reported by Bendjaballah *et al*., (2010) when mineral oil was homogenized at different periods to determine a smaller impact of emulsification time on finer droplet size.

According to McClement (2015), an emulsifier is a surface-active substance that is capable of adsorbing to an oil-water interface and protecting emulsion droplets from flocculation and/or coalescence. The effect of emulsifier on emulsion was checked by conducting 5 experiments using Tween 20 in the concentration range of 0.5% to 7% w/v in 5 mL water by keeping other parameters constant (oil 5 g, agitation 20000 rpm and time 5 min).

It was observed that the lower concentration of the surfactant could not form smaller droplets because it did not cover all oil droplets properly. Dickinson (2009) found that low concentrations of emulsifier (such as xanthan gum) had a destabilizing effect on emulsions due to a mechanism known as depletion flocculation. This mechanism is induced by the excess non-absorbing hydrocolloid and/or surfactant forming micelles as reported by Traynor *et al*., (2013). When surfactant concentration increased from 0.5% (29.38 µm) smaller droplets were obtained at 3% concentration and the smallest droplet size (17.25 µm) was found due to the structural forces inducing a repulsive energy barrier which enhances emulsion stability. At higher concentrations, 7% (18.36 µm) surfactant led to larger droplet size due to an increase

Fig 2a

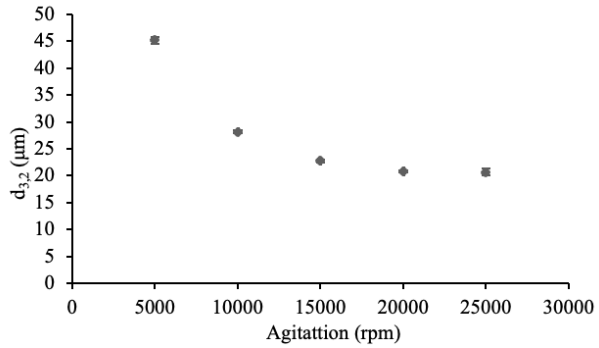


Fig 2b

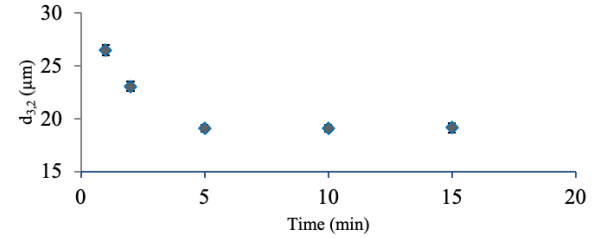


Fig 2c

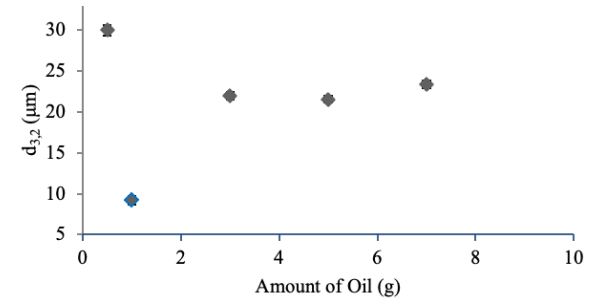


FIGURE 2. Optimization of process parameter on droplets size using Tween 20 as an Emusifier: (a) agitation, (b) time, (c) amount of oil. The values provided in the figures are the mean values of triplicate analyses with standard deviation.

in polydispersity in micellar size, which reduced emulsion stability and increased the size of droplets because of depletion flocculation, as reported earlier (Yerramilli and Ghosh, 2017). Our results also matched the findings of Jiao and Burgers (2003), who found that a higher concentration of Span 83 in mineral oil emulsion caused instability because of larger droplet size. Similarly, Bendjaballah *et al*., (2010) observed the same trend of droplet size on a concentration above 1%. In another study Traynor *et al*., (2013) reported that specific concentrations of xanthan gum with sunflower oil in emulsions had a stabilizing effect, but higher concentrations produced a destabilizing effect and instability due to an accelerated creaming process because of the promotion of droplet flocculation.

The oil volume ratio is very important for the size and stability of the droplets of emulsion. The high volume of oil ratio causes less oil to be entrapped by the emulsifier resulting in poor emulsion and larger droplets; while low oil concentrations were prone to destabilization by depletion flocculation due to the presence of non-adsorbing polysaccharides (Dickinson, 2003). As three variables were fixed, the most important factor, oil ratio with surfactant, was also analyzed. So, a different amount of oil from 0.5 g to 7 g was added to the water surfactant solution to check the droplet size. It was observed that at a lower level of oil such as 1 g smaller droplet size was produced, i.e. 16% of total 6 mL emulsion. On the other hand, higher oil concentrations led to higher mean diameters as shown in Figure 2c. The increase in oil concentration implied lower surfactant concentration since Tween 20 has emulsifying properties, therefore, a lower concentration of surfactant may have resulted in less efficient emulsification. Matalanis *et al*., (2011) found the finest droplet size at 20% of flaxseed oil emulsion, which is close to the present results. Similarly, the result of sunflower oil emulsion in xantham gum with a concentration of 19% oil showed finer droplet size as reported by Traynor *et al*., (2013). In contrast, Li and Xiang, 2019 observed smaller droplets at 5% coconut oil emulsion with ultrasound homogenization.

3.2. Use of different emulsifiers

After fixing all the parameters of pomegranate oil emulsion, different emulsifiers were used to obtain smaller droplet emulsion size, hence all the parameters were kept constant (such as rpm 2000, oil 1 g w/v of 5 mL distilled water, and time 5 min) except for the concentration of emulsifier, which varied, as shown in Table 1.

The pomegranate emulsion was also chosen as a stable emulsion at 3% of Tween 20, rpm 20000, oil 1 g w/v, time 5 min). The CMC was taken at very low concentrations from 0.1% to 0.5%. As 0.5% was too high a concentration and formed a viscous emulsion that could not be used for drying purposes and high droplet size was obtained. On the other hand, 0.3% concentration showed a smaller size of 5.3 µm of the droplet as shown in Table 1 with

viscous emulsion. Arancibia *et al*., (2013) also reported the effect of CMC concentrations on the droplet size when 15% of the oil was emulsified with 0.3% CMC and obtained 9.21 µm. In another study, even smaller droplet size (2 µm) of olive oil with a 0.5% concentration of CMC was reported (Arancibia *et al*., 2016).

After optimization of the effect of CMC concentration on droplet size, WP was used as an emulsifier as well as a stabilizer to check the concentration effect (0.5 to 7.0%) on droplet size. It was observed that as the concentration of WP increased, smaller droplet size was obtained. When concentration further increased above 3%, the reverse trend in droplet size was observed with 10.60 µm and 12.26 µm, respectively, for 5 and 7% concentrations. It can be explained that the excess surface-active molecules that may accumulate on the droplet surface resulted in droplet destabilization and the larger droplet size. Furthermore, some structures can form micelles. At 3% emulsion concentration, the smallest droplet size was found to be 9.78 µm as shown in Table 1. The results obtained in this study also matched the work of Akhtar and Dickinson (2007), who reported 10 µm droplet size of WPI emulsion with triglyceride oil. A similar type of result (6.1 µm) was also found by Hebishy *et al*., (2017) when 4% WPI in 30% sunflower/olive oil was homogenized according to the conventional mill method.

A combination of emulsions was also used to measure emulsion droplet size in the continuation of a single emulsifier. The concentrations of WP and MD remained constant (3.5% of total emulsion) while varying the concentrations of both individually to check the effect on droplet size as shown in Table 1. It was observed that lower emulsion droplet size was obtained when the concentrations of WP and MD were kept at 2.5% and 1%, respectively; while other studied combinations produced higher emulsion droplet size (Table 1). In contrast to our results, Akhtar and Dickinson (2007) reported lower emulsion droplet size (1 µm) with a combination of WP/MD in a ratio of 1:2 than using alone WP.

The droplet size of various emulsions was also confirmed with the optical microscope, which supported our claim that smaller droplets were thicker in CMC images than WPC-MD, and larger droplets, but less thick in WP and Tween 20 as shown in Figure 3.

Table 1. Effect of concentration of emulsions on droplet size. The experiment was done in triplicate and the Tukey test was used for the comparison of mean values ($p<0.05$). Letters a–e indicates a significant difference among a different concentration of the same emulsifier.

Emulsions	**Concentration (%)**	$d_{3,2}$ (µm) **Mean±SD**
Tween 20	0.5	15.39±0.17^{a}
	1.0	12.78±0.20^{b}
	3.0	10.27±0.35^{e}
	5.0	11.56±0.28^{c}
	7.0	11.20±0.45^{d}
CMC	0.1	13.03±0.18^{a}
	0.2	9.72±0.65^{c}
	0.3	5.37±0.81^{e}
	0.4	10.63±0.42^{b}
	0.5	7.45±0.33^{d}
WP	0.5	14.33±0.11^{a}
	1.0	10.72±0.50^{d}
	3.0	9.54±0.74^{e}
	5.0	11.60±0.68^{c}
	7.0	12.26±0.85^{b}
WP/MD	1.0/2.5	24.28±0.93^{a}
	1.5/2.0	18.51±0.26^{b}
	2.0/1.5	15.83±0.48^{c}
	2.5/1.0	9.12±0.39^{e}
	1.75/1.75	12.65±0.57^{d}

CMC, WP, WP-MD, $d_{3,2}$, and SD stand for carboxy methyl cellulose, whey protein, whey protein/maltodextrin, sauter mean diameter, and standard deviation, respectively

3.3. Emulsion stability measurement during storage

The stable emulsion must prevent change in the size of droplets during storage with time and it is an immense challenge for the scientific community and industrial production. The results of our stability measurements on emulsions containing CMC, Tween 20, WP, WP-MD were checked on different days with the Lumisizer. As can be seen in Figure 4, the lower instability index value of CMC showed

higher stability and less separation of emulsion than using WP-MD; while higher instability index values were observed for WP and Tween 20, respectively, which led to instability, creaming, and coalescence of emulsions.

Emulsion stability is generally linked to mean droplet diameter and variation in rheological properties. When $d_{3,2}$ is small, viscosity variation is slow and the emulsion is more stable, as described elsewhere (Tadros, 2004; Sánchez *et al*., 1998). Smaller droplets and narrow size distribution of CMC were observed (Figure 4) because of high molecular weight and pseudo plastic behavior compared to WP/MD, and WP; and Tween 20 can have low shear thinning behavior.

Furthermore, we also checked the storage stability of emulsion for 16 days and measured droplet size on the Mastersizer. As days increased from 1 to 16, the size of droplets increased, which led to instability of emulsion and formation of creaming and coalescence. The CMC emulsion has more thickness and lower droplet size on the first day at 5.37 µm. Hence, it showed more stability than other emulsions, but its droplet size increased gradually with the passing of days and finally reached at 52.36 µm on day 16. While other emulsions showed a comparatively higher increase in droplet size as days passed because of less thickness and higher droplet size on initial days. In high viscosity, the negative or positive charge on molecules causes them to repel each other and does not allow movement of droplets easily so fewer chances for coalescence and creaming exist (McClements, 2015). WP-MD emulsion showed the second best stability due to high thickness, lower droplet size, and a combination of two emulsifiers. The decreasing stability order of emulsions was observed as: CMC> WP/MD > WP > Tween 20, as shown in Table 2.

4. CONCLUSIONS

This is the very first time we have reported pomegranate oil-in-water emulsions stabilized by surfactants and emulsifiers to get finer droplets. The experimental conditions allowed us to form very stable emulsions with very small droplet size $d_{3,2}$ (5.37 µm) and narrow distribution with a single surfactant. The Lumisizer results during storage also revealed higher emulsion stability with CMC. The higher stability of emulsion (0.3% CMC) can be attributed to the competing role of negative repulsive versus attractive depletion forces such as the anionic polysaccharide structure of CMC. These stable emulsions would be utilized for related food applications.

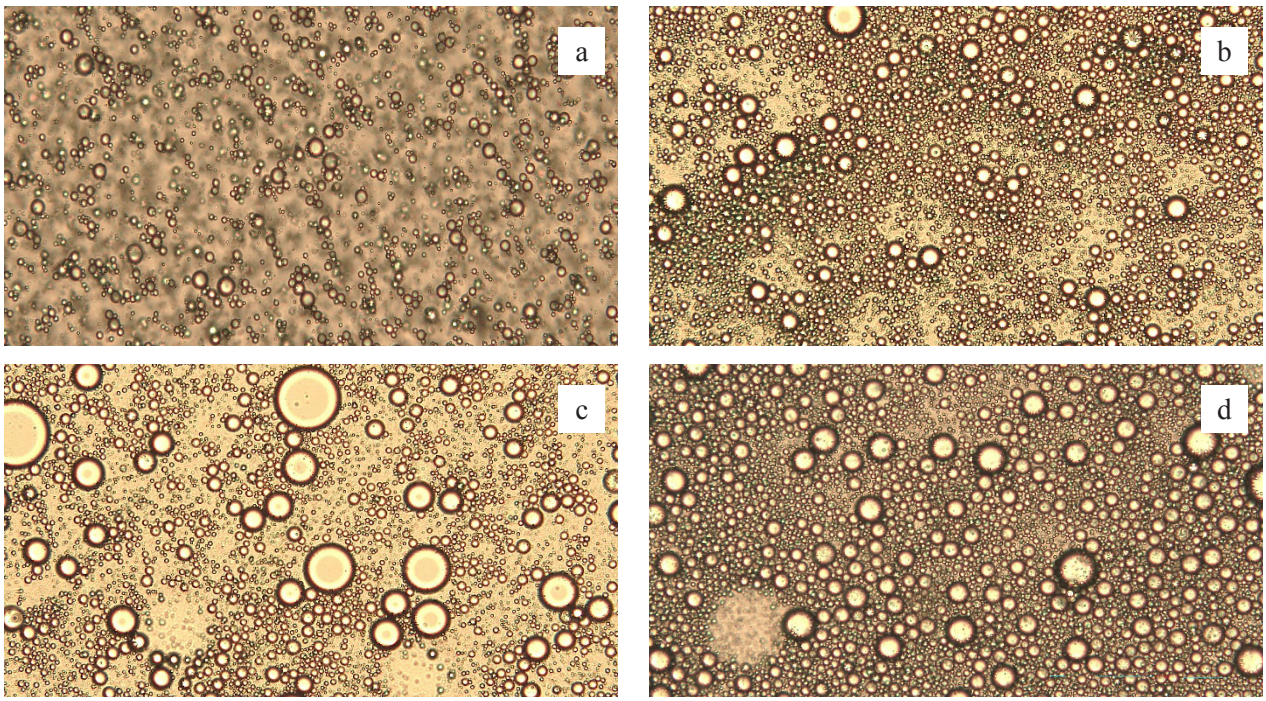


Figure 3. Optical microscope images of emulsion at 20×100 magnification, (a) Tween 20, (b) CMC, (c) WP, (d) WP/MD

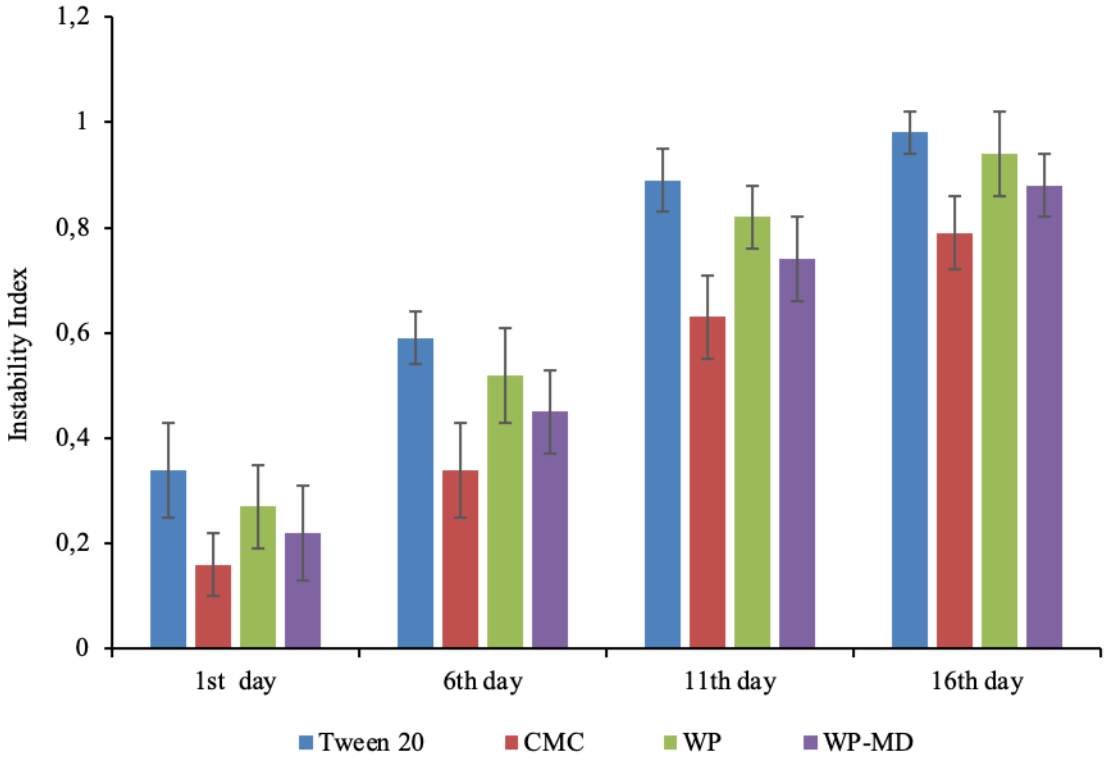


FIGURE 4. Instability index profile of emulsions during storage. The values provided in the figure are the mean values of triplicate analyses with standard deviation

ACKNOWLEDGMENTS

The authors would like to thank Higher Education Commission (HEC), Pakistan for the financial support through grant No. 6716 /Sindh/NRPU/R&D/ HEC, Prof. Dr. David A. York and Dr. Muhammad Soyab Manga, University of Leeds, UK for their help in experimental work and useful discussions.

Conflict of Interest

The authors have declared no conflict of interest.

TABLE 2. Effect of storage on the stability of various emulsions on droplet size. The experiment was done in triplicate and the Tukey test was used for the comparison of mean values ($p < 0.05$).

Emulsion	$d_{3,2}$ (μm) ±SD Days			
	1	6	11	16
Tween 20	10.27±0.35^{d}	32.21±0.14^{c}	54.32±0.33^{b}	106.87±0.72^{a}
CMC	5.37±0.81^{d}	17.56±0.47^{c}	31.21±0.27^{b}	52.36±0.64^{a}
WP	9.54±0.74^{d}	21.43±0.69^{c}	38.65±0.46^{b}	78.29±0.85^{a}
WP-MD	9.12±0.39^{d}	19.21±0.82^{c}	36.32±0.28^{b}	57.87±0.94^{a}

CMC, WP, WP-MD, d32, and SD stand for carboxy methyl cellulose, whey protein, whey protein-maltodextrin, sauter mean diameter, and standard deviation, respectively.

REFERENCES

Akhtar M, Dickinson E. 2007. Whey protein–maltodextrin conjugates as emulsifying agents: an alternative to gum arabic. *Food Hydrocoll.* **21**, 607-616. https://doi.org/10.1016/j.foodhyd.2005.07.014

Arancibia C, Bayarri S, Costell E. 2013. Comparing carboxymethyl cellulose and starch as thickeners in oil/water emulsions. Implications on rheological and structural properties. *Food Biophys.* **8**, 122-136. https://doi.org/10.1007/s11483-013-9287-2

Arancibia C, Navarro-Lisboa R, Zúñiga R, Matiacevich S. 2016. Application of CMC as thickener on nanoemulsions based on olive oil: physical properties and stability. *Int. J. Poly. Sci.* **10** , https://doi.org/10.1155/2016/6280581

Bai L, Huan S, Gu J, Mcclements DJ. 2016. Fabrication of oil-in-water nanoemulsions by dual-channel microfluidization using natural emulsifiers: Saponins, phospholipids, proteins, and polysaccharides. *Food Hydrocoll.* **61**, 703-711. https://doi.org/10.1016/j.foodhyd.2016.06.035

Bendjaballah M, Canselier JP, Oumeddour R. 2010. Optimization of oil-in-water emulsion stability: experimental design, multiple light scattering, and acoustic attenuation spectroscopy. *J. Disper. Sci. Technol.* **31**, 1260-1272. https://doi.org/10.1080/01932690903224888

Charcosset C. 2009. Preparation of emulsions and particles by membrane emulsification for the food processing industry. *J. Food Eng.* **92**, 241-249. https://doi.org/10.1016/j.jfoodeng.2008.11.017

Chen L, Remondetto GE, Subirade M. 2006. Food protein-based materials as nutraceutical delivery systems. *Trends Food Sci. Techol.* **17**, 272-283. https://doi:org/10.1016/j.tifs.2005.12.011

Curt C. 1994. Review : evaluation of emulsion stability : principle, applications, advantages and drawbacks. *Food Sci.* **14**, 699-724. http://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=3366325

Dickinson E. 2003. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocoll.* **17**, 25-39. https://doi.org/10.1016/S0268-005X(01)00120-5

Dickinson E. 2009. Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocoll.* **23**, 1473-1482. https://doi.org/10.1016/j.foodhyd.2008.08.005

Fadavi A, Barzegar M, Azizi MH. 2006. Determination of fatty acids and total lipid content in oilseed of 25 pomegranates varieties grown in Iran. *J. Food Compost. Anal.* **19**, 676-680. https://doi.org/10.1016/j.jfca.2004.09.002

Galooyak SS, Dabir B. 2015. Three-factor response surface optimization of nano-emulsion formation using a microfluidizer. *J. Food Sci. Technol.* **52**, 2558-2571. https://doi.org/10.1007/s13197-014-1363-1

Hashtjin AM, Abbasi S. 2015. Optimization of ultrasonic emulsification conditions for the production of orange peel essential oil nanoemulsions. *J. Food Sci. Technol.* **52**, 2679-2689. https://doi.org/10.1007/s13197-014-1322-x

Hebishy E, Zamora A, Buffa M, Blasco-Moreno A, Trujillo AJ. 2017. Characterization of whey protein oil-in-water emulsions with different oil concentrations stabilized by ultra-high pressure homogenization. *Processes.* **5** (6), 1-18. https://doi.org/10.3390/pr5010006

Horozov T S, Binks BP. 2004. Stability of suspensions, emulsions, and foams studied by a novel automated analyzer. *Langmuir.* 20, 9007-9013. https://doi.org/10.1021/la0489155

Jiao J, Burgess DJ. 2003. Rheology and stability of water-in-oil-in-water multiple emulsions containing Span 83 and Tween 80. *Am. Assoc. Pharm. Sci.* **5**, 62-73. https://doi.org/10.1208/ps050107

Krstonošić V, Dokić L, Dokić P, Dapčević T. 2009. Effects of xanthan gum on physicochemical properties and stability of corn oil-in-water emulsions stabilized by polyoxyethylene (20) sorbitan monooleate. *Food Hydrocoll.* **23**, 2212-2218. https://doi.org/10.1016/j.foodhyd.2009.05.003

Lerche D. 2002. Dispersion stability and particle characterization by sedimentation kinetics in a centrifugal field. *J. Dispers. Sci. Technol.* **23**, 699-709. https://doi.org/10.1081/DIS-120015373

Li Y, Xiang D. 2019. Stability of oil-in-water emulsions performed by ultrasound power or high-pressure homogenization. *PloS One* **14**(3),1-14 https://doi.org/10.1371/journal.pone.0213189

Mackler AM, Heber D, Cooper EL. 2013. Pomegranate: its health and biomedical potential. *Evid. Based Compl. Altern. Med.* 2013, PAGE 2. https://doi.org/10.1155/2013/903457

Matalanis A, Jones OG, Mcclements DJ. 2011. Structured biopolymer-based delivery systems for

encapsulation, protection, and release of lipophilic compounds. *Food Hydrocoll.* **25**, 1865-1880. https://doi.org/10.1016/j.foodhyd.2011.04.014

Mcclements DJ. 2015. *Food emulsions: principles, practices, and techniques*, CRC press.

MinemotoY, Hakamata K, Adachi S, Matsuno R. 2002. Oxidation of linoleic acid encapsulated with gum arabic or maltodextrin by spray-drying. *J. Microencap.* **19**, 181-189. https://doi.org/10.1080/02652040110065468

Özgül-Yücel S. 2005. Determination of conjugated linolenic acid content of selected oil seeds grown in Turkey. *J. Am. Oil Chem. Soc.* ***82,*** 893-897. https://doi.org/10.1007/s11746-005-1161-7

Sánchez MC, Berjano M, Guerrero A, Brito E, Gallegos C. 1998. Evolution of the microstructure and rheology of o/w emulsions during the emulsification process. *Can. J. Chem. Eng.* **76**, 479-485. https://doi.org/10.1002/cjce.5450760318

Silva KA, Rocha-Leão MH, Coelho MAZ 2010. Evaluation of aging mechanisms of olive oil–lemon juice emulsion through digital image analysis. *J. Food Eng.* **97**, 335-340. https://doi.org/10.1016/j.jfoodeng.2009.10.026

Tadros T. 2004. Application of rheology for assessment and prediction of the long-term physical stability of emulsions. *Adv. Colloid Interface Sci.* **108**, 227-258. https://doi.org/10.1016/j.cis.2003.10.025

Tcholakova S, Denkov ND, Ivanov IB, Campbell B. 2006. Coalescence stability of emulsions containing globular milk proteins. *Adv. Colloid Interface Sci.* **123**, 259-293. https://doi.org/10.1016/j.cis.2006.05.021

Traynor M, Burke R, Frias JM, Gaston E, Barry-Ryan C. 2013. Formation and stability of an oil in water emulsion containing lecithin, xanthan gum and sunflower oil. *Inter. Food Res. J.* **20 (**5), 2173.

Yerramilli M, Ghosh S. 2017. Long-term stability of sodium caseinate-stabilized nanoemulsions. *Food Sci. Tech.* **54**, 82-92. https://doi.org/10.1007/s13197-016-2438-y

GRASAS Y ACEITES 72 (2)
April-June 2021, e411
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0222201

Characterization of the chemical and nutritive quality of cold-pressed grape seed oils produced in the Republic of Serbia from different red and white grape varieties

V.B. Vujasinović[a,✉], M.M. Bjelica[b], S.C. Čorbo[c], S.B. Dimić[d] and B.B. Rabrenović[e]

[a]University of Novi Sad, Faculty of Sciences, 21000 Novi Sad, Serbia

[b]University of Novi Sad, Faculty of Technology, 21000 Novi Sad, Serbia

[c]Faculty of Agriculture and Food Sciences, 71000 Sarajevo, Bosna and Herzegovina

[d]Bimal Trading d.o.o. Beograd, 11000 Belgrade, Serbia

[e]University of Belgrade, Faculty of Agriculture Zemun, 11000 Belgrade, Serbia

✉Corresponding author: vesna.vujasinovic@dgt.uns.ac.rs

Submitted: 27 February 2020; Accepted: 28 May 2020; Published online: 03 June 2021

SUMMARY: Six cold-pressed oil samples obtained from the seeds of different grape varieties grown in a Fruška Gora vineyard in the Republic of Serbia were examined for chemical and nutritive quality, as well as antiradical capacity. All the tested samples were of good quality, but the results showed noticeable differences in seed oil properties for red and white grape varieties. The highest content in total tocols, 575.23 ± 4.46 mg/kg, was found in the red grape seed oil of the Merlot variety, but the vitamin E activity of white grape seed oils was significantly higher. Regarding single phenols, the most prevalent fraction in all the oil samples was ursolic acid, up to 336.3 ± 4.8 μg/g in the grape seed oil of the Hamburg variety. The highest content in the total phenolic compounds, 54.92 ± 0.93 μg GAE/g, was detected in the oil of red grape seeds. Although significant differences existed between samples, EC_{50} values were the lowest for the red grape seed oils, varying from 29.84 ± 1.07 to 65.34 ± 0.32 mg oil/mg DPPH, indicating that these oils had the highest antiradical capacity.

KEYWORDS*: Acidity; Antiradical capacity; Grape seed oil; Phenols; Tocopherols; Totox*

RESUMEN: ***Calidad química y nutricional de los aceites de semilla de uva prensados en frío producidos en la República de Serbia a partir de diferentes variedades de uva roja y blanca*****.** Se examinaron seis muestras de aceite prensado en frío obtenidas de semillas de diferentes variedades de uva cultivadas en un viñedo de Fruška Gora en la República de Serbia, para determinar su calidad química y nutricional, así como su capacidad antirradicalaria. Todas las muestras analizadas fueron de buena calidad, pero los resultados mostraron propiedades de los aceites de semillas notablemente diferentes para las variedades de uva roja y blanca. El mayor contenido de tocols totales, 575,23 ± 4,46 mg/kg, se encontró en el aceite de semilla de uva roja de la variedad Merlot, pero la actividad de la vitamina E de los aceites de semilla de uva blanca fue significativamente mayor. Con respecto a los fenoles simples, la fracción prelevante en todas las muestras de aceite contenía ácido ursólico, hasta 336,3 ± 4,8 μg/g en el aceite de semilla de uva de la variedad Hamburg. El mayor contenido de los compuestos fenólicos totales, 54,92 ± 0,93 mg GAE/g, se detectó en el aceite de semillas de uva roja. Aunque existieron diferencias significativas entre las muestras, los valores de EC50 fueron más bajos para los aceites de semilla de uva roja, variando de 29,84 ± 1,07 a 65,34 ± 0,32 mg de aceite/mg de DPPH, lo que indica que estos aceites tenían la mayor capacidad antirradicalaria.

PALABRAS CLAVE*: Aceite de semilla de uva; Acidez; Capacidad antirradicalaria; Fenoles; Tocoferoles; Totox*

Citation/Cómo citar este artículo: Vujasinović VB, Bjelica MM, Čorbo SC, Dimić BS, Rabrenović BB. 2021. Characterization of the chemical and nutritive quality of cold-pressed grape seed oils produced in the Republic of Serbia from different red and white grape varieties. *Grasas Aceites* **72** (2), e411. https://doi.org/10.3989/gya.0222201

1. INTRODUCTION

The use of by-products from various food and beverage technologies, including the processing of grapes, has become a necessity in terms of producing a new product instead of creating waste. The utilization of by-products has also become an integral part of the regular production process, both due to increased processing efficiency and energy consumption. Grapes are one of the most extensively cultivated crops in the world with a global production of around 74 million tonnes produced worldwide from a production area of about 6.9 million ha. About 75% of total grape cultivation is used for wine production. Europe produced 23.7 million tonnes of grapes in 2017, which amounts to 34.9% of the total world production. Grape production is mostly concentrated in moderately warm climatic zones, such as Italy (7.17 million tonnes), France (5.92 million tonnes) and Spain (5.39 million tonnes) in 2017 (FAOSTAT, 2018).

Viniculture is a very important activity in the field of agricultural production in the Republic of Serbia. The total area under grapevine crops is 22,149.97 ha. Natural potential, climate conditions, tradition, as well as the subsidizing of new wine planting will allow further consolidation of viniculture and winemaking. According to the National Statistics Office of Serbia, grape production in 2018 was 149.595 tonnes (Jakšić *et al*., 2019).

Grape seed oil (mainly solvent extracted and subsequently refined), has been produced for decades, and is gaining market prevalence as a gourmet oil (Dwyer *et al*., 2014). Nowadays, however, the cold-pressing process is preferred for the extraction of oil from plant seeds, to a conventional process in which oil extraction is carried out by organic solvents. Since there are no processing steps that would allow for the removal of undesired contaminants from the oil in producing cold-pressed, i.e. edible non-refined oils, high quality of the raw material is required (Mätthaus and Spener, 2008). Also, the cold-pressing process ensures the maximum retention of bioactive compounds, such as essential fatty acids, phenols and flavonoids, tocopherols, and the like (Teh and Birch, 2013; Radočaj and Dimić, 2013).

Grape seed oil has recently become of great interest for two reasons: first, the oil extraction from seeds contributes to reducing the problem of waste from the processing of grapes; second, nutritionists increasingly recommend the consumption of this oil for its unsaturated fatty acids, phenolic compounds, tocopherols, pigments and low cholesterol content. These minor fractions play a very important role in determining the nutritional and health impact of this seed and its oil (Pardo *et al*., 2009; Garavaglia *et al*., 2016, Cicero *et al*., 2018; Unusan, 2020).

The most important compounds, based on which the nutritive value of grape seed oil is examined, are various biologically active components. These ingredients, although present in very small amounts in oil, have a high metabolic value in the body, showing antioxidant, antiradical and vitamin activity –all different health protective effects (Fernandes *et al*., 2013; Fagundes Assumpção *et al*., 2015; Dabetic *et al*., 2020). An important argument for the use of grape seed oil in medical terms is the high contents in the oligomeric phenolic compounds in this oil (Matthäus, 2008). Bioactive components are of great importance for the application of oils to food and pharmaceutical industries due to their anti-lipid, anticancer and antimutagenic effects, as well as for reducing the risk of cardiovascular diseases (Garavaglia *et al.*, 2016; Shinagawa *et al,* 2015).

Moreover, it is worth mentioning that an edible oil's qualitative and quantitative composition widely depends not only on botanical species, but also on soil characteristics, agronomic and climatic conditions, seed quality, ripening stage, oil extraction process and storage procedure (Dwyer *et al*., 2014; Cicero *et al*., 2018).

Since cold-pressed grape seed oil is becoming very popular, compared to refined oil, the aim of this study was to characterize some quality parameters and the nutritive profile of cold-pressed grape seed oils obtained from different red and white grape varieties. Since the oil's quality depends on agronomic and climatic diversity, results obtained by investigations of cold-pressed grape seed oils produced in the Republic of Serbia from Fruška Gora vineyards are presented here. To the best of our knowledge, until now, there was only very limited, published data on the quality of cold-pressed grape seed oils processed in the Republic of Serbia.

2. MATERIALS AND METHODS

2.1. Materials

Oil samples tested in this work were prepared by the cold pressing of seeds obtained from grapes

during the process of wine production. The fresh wine pomace from the red (Merlot and Hamburg) and white grape (Italian Riesling and Sila-Serbian autochthonous) varieties was gathered within a few hours after leaving the largest winery on the slopes of the Fruška Gora vineyard in the Republic of Serbia. Then, for a very short time, the pomace was purified using a machine with a rotating, perforated drum, separating the seeds from the grapes' skins. Immediately after that, the seeds were dried in a stream of warm air at about 30 °C for 24 hours. The content in impurities in cleaned and dried seeds was about 0.2-0.3% (mostly parts of skins and stunted seeds). Two samples of a blend of different varieties of red, as well as white grape seeds were purchased from small wineries. In this case, wine pomace was collected over a longer period of time; the samples were dried naturally at ambient temperature (at about 20 °C for several days) and the seeds were separated. All grape cultivars were grown in the same geographical region with similar climatic conditions and soil characteristics, and were harvested at their best maturity. In grape seed samples prepared for pressing, by standard laboratory methods, the contents of moisture (ISO 665:2000) and oil (ISO 659:2009-Soxhlet method) were determined. The obtained results, as well as label information, are presented in Table 1.

2.2. Production of cold-pressed grape seed oils

The oil samples were obtained by pressing the cleaned and dried grape seeds with a screw press (Koprulu Machine, Type KYP20D, Turkey), run by a 1.8 kW electric motor, capacity of 5-7 kg/h in a small-size facility for cold-pressed oils. Initially, the press head was heated up to 90 °C. Subsequently, the heaters were switched off and the pressing material was released. The speed of the screw was 25 rpm. When the oil started coming out, the speed slowed down to 20 rpm. The temperature of the outgoing oil, due to pressure and friction, was about 50-55 °C. After pressing the oil samples were stored for three days at room temperature for natural sedimentation of insoluble impurities to occur, after which they were decanted. Finally, clear oil samples for further analysis were used as average samples from several series of pressing. The samples (each of 1000 mL) were kept in a refrigerator at 8 ± 1 °C until analysis to avoid oxidation processes (Bjelica *et al*., 2019).

2.3. Methods

Free acidity, given as *acid value* (AV) (ISO 660:2009), expressed in mg KOH/g, was determined by volumetric titration of a solution of oil dissolved in ethanol:diethyl ether (1:1) with an ethanolic solution of 0.1 M KOH.

The *peroxide value* (PV) (ISO 3960:2017), expressed in meqO_2/kg, was determined by the reaction of oil (dissolved in acetic acid and isooctane) with a solution of potassium iodide. The liberated iodine was then titrated with a standard volumetric sodium thiosulfate solution.

The *p-anisidine value* (*p*-AnV) was determined following the ISO standard spectrophotometric method (ISO 6885:2016), measuring the absorbance at 350 nm.

The *Totox value* was calculated as follows: Totox = 2 PV + *p*-AnV

The *content of total tocopherols and tocotrienols* was determined by using the spectrophotometric method according to Paqout *et al*., (1967). The method is based on the reduction properties of tocophenols/tocotrienols, which reduce Fe^{3+} from $FeCl_3$ in the presence of 2-2'dipyridyl and create a red-colored

Table 1. Label information, moisture and oil content of investigated grape seed samples

Sample codes	Grape varieties	Moisture (%)	Oil (% d.m.*)
M	**M**erlot – red grape	10.12 ± 0.05	9.87 ± 0.14
H	**H**amburg – red grape	8.63 ± 0.03	11.92 ± 0.36
R	**Blend** of different varieties of red grape	7.58 ± 0.06	11.06 ± 0.28
IR	**I**talian **R**iesling – white grape	10.02 ± 0.05	8.89 ± 0.68
S	**S**ila – domestic white grape**	8.62 ± 0.04	12.74 ± 0.40
W	**Blend** of different varieties of white grape	7.98 ± 0.07	11.28 ± 0.42

Values are means ± standard deviation (n = 3); *on a dry matter basis; ** Serbian autochthonous grape variety developed at the University of Novi Sad, Faculty of Agriculture, Novi Sad

complex with Fe^{2+} ions. The determination was made from unsaponified matter, so the oil saponification and the extraction of unsaponifiable matter was performed, and then a benzene solution of the unsaponified matter developed a colored reaction, where absorbance was measured at 520 nm wavelength using a spectrophotometer with a blank test.

The *determination of tocopherols* was carried out using detector RF-535 (Shimadzu, Fluorescence HPLC) coupled to HPLC (Waters M600E, USA) on a reversed-phase Nucleosil 50-5 C18 column (Macherey-Nagel, Germany), using a method based on the procedure described by Carpenter (1979) with some modifications. Operating conditions of the HPLC system were the following: mobile phase: 95% CH_3OH; flow rate: 1.0 mL/min.; column temperature: 20 °C; wavelength: λex = 295 nm, λem = 330 nm. The relative retention time and maximum values of absorption at the given relative retention time were used for the identification of tocopherols in the oil samples. Commercial tocopherol standards were suitably diluted and were used for method validation (solution series from 0.01 to 0.5 μg/mL), as well as for quantification purposes (solution series from 1.0 to 20.0 μg/mL).

Vitamin E activity, i.e. vitamin E equivalent of tocopherols (Mag *et al.*, 2002) was calculated following the formula:

Vitamin E = α-toc. + 0.25 β+γ-toc. + 0.01 δ-toc.

The *content in total phenolic compounds* was estimated according to the Folin–Ciocalteu colorimetric method (based on the reaction of the reagent with the functional hydroxyl groups of the phenols) as described by Haiyan *et al*. (2007). The oil was dissolved in hexane and extracted with methanol (three times). The sample was left to stand overnight. After that, the methanolic extract was washed with hexane and an aliquot (1 mL) was transferred to a volumetric flask (10 mL), to which the Folin–Ciocalteu reagent (0.5 mL) was added. The solution was shaken and left to stand for 3 min. prior to the addition of a saturated solution of sodium carbonate, and dilution with water was made. After 1hour, the absorbance at 725 nm was measured against a reagent blank using a spectrophotometer. Calibration was performed using gallic acid. The total phenol content was expressed as the equivalent of gallic acid in mg per kilogram of oil.

Determination of single polyphenols. Phenols from the grape seed oil samples were extracted using a modified procedure by Gouvinhas *et al*., (2014) and determined on Agilent Technologies 1200 HPLC-DAD, coupled with Agilent Technologies 6410A ESI-QqQ-MS/MS, described previously (Bjelica *et al*., 2019). Approximately 800 μL of the sample were transferred to the 4 mL vial; the mass was accurately measured, and the sample was diluted with 400 μL of hexane. The mixture was extracted with 80% methanol (600 μL) with vigorous shaking using the vortex device. After centrifugation (10 minutes at 2500 rpm), the aqueous methanol layer was transferred to a 2 mL vessel, and the oil layer was extracted two more times with 80% methanol. The combined extracts were supplemented with 80% methanol to 2 mL, filtered through a 0.45 μm regenerated cellulose membrane filter and analyzed using LC-DAD-MS/MS. The content of the selected secondary biomolecules was determined by the LC-DAD-MS/MS method. A 5 μL sample was injected. The separation was performed by the Zorbax Eclipse XDB-C18 column (50 mm x 4.6 mm, 1.8 μm) at 50 °C. The components were eluted with a mobile phase based on 0.05% aqueous solution of formic acid (A) and methanol (B) at a flow rate of 1.0 mL/min, in gradient mode: 0 min., 30% B, 6 min. 70% B, 9 min. 100% B, 12 min. 100% B; re-equilibrium time 3 min. For identification purposes and identity validation, the UV/VIS signal was observed in the range of 190-700 nm. Effluents passed the MS/MS detector without flow sharing. The ion source parameters were: the pressure of the nebulizer 50 psi, temperature and flow of drying gas (N_2) 350 °C and 10 L/min.; the voltage on the capillary 4000V; negative polarity. The compounds were monitored in a dynamic SRM (selected reaction monitoring) mode, with optimized parameters. By sequential dilution of 1:1, a series of commercial standard solutions were prepared in a concentration range of 1.53 ng/mL to 25.0 μg/mL. The concentration of the analyte was determined by the external standard method, using calibration curves in a narrower band-adjusted concentration in the sample. For the quantification, points that directly surrounded the readings in the samples were taken. In most cases, the correlation was not linear but square. For all the compounds, peak areas were determined using Agilent MassHunter Workstation Software – Qualitative Analysis (ver. B.03.01). Calibration curves were plotted and sample concentrations calculated by using the OriginLabs Origin Pro (ver. 8.0) software.

The *antiradical capacity of oils* in the current study was assessed by the evaluation of the free radical scavenging effect on 2, 2-diphenyl-1-picrylhydrazyl radicals (DPPH•), according to the method proposed by Martínez and Maestri (2008). The absorbance was measured on spectrophotometer at 515 nm using toluene as the blank. Antiradical action toward DPPH radicals was estimated from the difference in absorbance with or without oil sample (control) and the percent of inhibition was calculated from the following formula:

$$\% \text{ Inhibition} = [A_C - A_S / A_C] \times 100$$

where: A_C = absorbance of the control; A_S = absorbance of the sample.

EC_{50} (mgoil/mgDPPH•) values were expressed as the inhibitory concentration of the oil necessary to decrease the initial DPPH• absorbance by 50%. A lower EC_{50} value indicates a higher antiradical capacity.

All above-mentioned spectrophotometric measurements were conducted using a UV/VIS spectrophotometer model T80+ (PG Instruments Limited, London).

2.4. Chemicals and reagents

The solvents and chemicals used in these investigations were of analytical grade, except chemicals for the extraction procedure and chromatographic analysis, which were of chromatographic purity. Methanol, ethanol, KOH, pyrogallol and n-hexane were purchased from Merck (Darmstadt, Germany); Folin–Ciocalteu reagent and gallic acid were from Institute Mol Ltd.; formic acid was from J.T. Baker (Deventer, Netherlands), as well as other solvents and reagents. Standards were purchased from Sigma-Aldrich Co. (St. Louis, USA).

2.5. Statistical analysis

All results are presented as a mean value ± standard deviation (n = 3). One-way analysis of variance (ANOVA) with a Duncan test was used to determine significant differences among the data at the level of $p < 0.05$. Also, two-way ANOVA with the Tukey test was performed at a statistically significant level ($p < 0.05$). The degree of linear relationship between two variables was measured using the Pearson's correlation coefficient (r). Statistical analysis was performed using the software SPSS Statistica 20 (IBM Corporation, Armonk, New York, U.S.) and OriginPro 8 (OriginLab Corporation, Northampton, MA 01060 USA).

3. RESULTS AND DISCUSSION

3.1. Chemical characteristics of oils

Some quality parameters of grape seed oil samples, like AV, PV, *p*-AnV and Totox, are presented in Table 2.

The acid value measures the content in free fatty acids formed by the hydrolytic degradation of oil molecules. Free fatty acids can contribute to the reduction in the shelf-life of oil, so acidity is a very important quality parameter (Prescha *et al*., 2014). Table 2 shows that all oil samples had low acidity values, below the maximum level (4 mgKOH/g) allowed by Codex Alimentarius (1999). Similar values have been reported by other authors (Pardo *et al*., 2009; Boso *et al*., 2018). The lowest acid value was found in the oil of the red grape Merlot variety (0.62 ± 0.03 mgKOH/g), as well as in the oil of the Serbian autochthonous white grape Sila variety

Table 2. Some chemical quality parameters of red and white cold-pressed grape seed oils

	AV (mgKOH/g)	PV (meqO_2/kg)	*p*-AnV	Totox
Cold-pressed oil of red grape seed				
Merlot	0.62 ± 0.03^{a}	2.48 ± 0.04^{b}	0.64 ± 0.03^{a}	5.60 ± 0.08^{a}
Hamburg	0.94 ± 0.04^{b}	1.90 ± 0.04^{a}	0.56 ± 0.03^{a}	4.36 ± 0.09^{a}
R- blend seeds	1.61 ± 0.05^{c}	8.14 ± 0.07^{e}	1.78 ± 0.05^{b}	18.04 ± 0.53^{d}
Cold-pressed oil of white grape seed				
It. **R**iesling	0.73 ± 0.03^{ab}	5.08 ± 0.21^{c}	2.14 ± 0.06^{d}	12.30 ± 0.38^{b}
Sila	0.66 ± 0.03^{a}	6.84 ± 0.07^{d}	1.83 ± 0.05^{b}	15.51 ± 0.17^{c}
W- blend seeds	2.42 ± 0.28^{d}	10.08 ± 0.18^{f}	1.94 ± 0.04^{c}	22.10 ± 0.38^{e}

AV – acid value; **PV** – peroxide value; ***p*-AnV** – *para*-Anisidine value; **Totox** value = 2PV + *p*-AnV: Values are means ± standard deviation (n = 3); Different letters in the same column indicate significantly different values ($p < 0.05$, one-way ANOVA with Duncan test)

(0.66 ± 0.03 mgKOH/g). The acidity of the oils was slightly higher in the two other grape varieties (Hamburg - 0.94 ± 0.04 and Ital. Riesling - 0.73 ± 0.03 mgKOH/g); however, the oils extracted from a blend of different red (R) and white (W) grape varieties had increased acidity, 1.61 ± 0.05 and 2.42 ± 0.28 mgKOH/g, respectively. An explanation for the low acid values can be the fact that grape cultivars were harvested at their highest level of maturity. Namely, Rubio *et al*., (2009) revealed that the oil from the grape seeds collected at an early stage of growth had a considerably higher free acidity value compared to the oil extracted from grapes collected at later stages of maturity. Furthermore, the pomace from a large winery company was dried quickly, in contrast to collecting and drying pomace from small wineries. Careful and fast drying of the pomace after production of wine is required to achieve high valuable grape seed oils. Otherwise, the quality of the resulting oil is affected by various microorganisms which enhance acidity (Matthäus, 2008).

PV defines the contents in lipid hydroperoxides in oils formed during auto- and photo-oxidation processes. All investigated oil samples were characterized by such values for PV that did not exceed the limit for cold-pressed oils of 15 meqO_2/kg, recommended by Codex standards for vegetable oils (Codex, 1999). The PV for the oil from red grape seeds were lower and ranged from 1.90 ± 0.04 (Hamburg) to 8.14 ± 0.07 meqO_2/kg (R-blend), in contrast to the oil from white grape seeds with a level of 5.08 ± 0.21(Ital. Riesling) to 10.08 ± 0.18 meqO_2/kg (W-blend). Regarding the PV, it can be concluded that there were statistically significant differences among all the oil samples at $p < 0.05$.

The data published for the PV of grape seed oil commercially available in the UK was 1.0 ± 0.0 meqO_2/kg (Madawala *et al*., 2012). In pressed oil obtained from different red grape varieties grown in Spain, Pardo *et al*., (2009) found PV values from 5.99 meqO_2/kg (hot air drying of seed, at 50 °C for 6h) to 13.50 meqO_2/kg (ambient temperature dried seeds, at 20 °C for 7 days). In oils obtained from cold extraction of bagasse-derived seeds from the white grape varieties, PV values were in the range of 11 ± 1.69 to 26 ± 2.89 meqO_2/kg (Boso *et al*., 2018). Kiralan *et al*., (2019) concluded that the PV values for fresh cold-pressed oils were 1.82, 12.2 and 26.5 meqO_2/kg for flaxseed, grape seed and black cumin seed oils, respectively. The PV value of commercial cold-pressed grape seed oil from the European market by Radočaj and Dimić (2013) was 5.07 ± 0.05 meqO_2/kg.

The *p*-AnV reflects the contents in secondary lipid oxidation products (saturated and unsaturated carbonyl compounds), resulting from the decomposition of hydroperoxides, so *p*-AnV along with PV, indicate the progression of rancidity in the oils. The *p*-AnV in all the analyzed oil samples (Table 2) were in the range of 0.56 ± 0.03 to 2.14 ± 0.06; whereby the lowest *p*-AnV was found in the oil of the red grape Hamburg variety. Generally, all three red grape seed oils had somewhat lower *p*-AnV in relation to oils from white grape seeds. The *p*-AnV of the oils had the following values: Merlot (0.64 ± 0.03) and Hamburg (0.56 ± 0.03) (without significant differences between these two samples, $p < 0.05$), < R-blend (1.78 ± 0.05) and Sila (1.83 ± 0.05) (without significant differences, $p < 0.05$), < W-blend (1.94 ± 0.04) and < Ital. Riesling (2.14 ± 0.06).

The food safety of these oils, with respect to the *p*-AnV value, is difficult to evaluate because of the lack of an established limit value for this parameter in cold-pressed oils (recommended *p*-AnV value for fresh fully refined oil is <10). However, cold-pressed seed oils, as a rule, have very low *p*-AnV, indicating low levels of carbonyl compounds because of the absence of heat during the oil extraction process. The mean values of *p*-AnV in a number of fresh cold-pressed oils from the seeds and fruits of different plants (other than grape seed oil) found in the Polish market (Prescha *et al*., 2014) were in the range of 0.25 ± 0.05 to 8.60 ± 3.20. According to their results, the lowest *p*-AnV was found for sesame oil (0.2-0.3), but the largest variability occured in rose hip oil (4.22-11.88) and pumpkin seed oil (1.48-8.17).

The total secondary oxidation products, i.e. Totox, in our samples, measured as a sum of PV and *p*-AnV, ranged from 2.45 ± 0.09 to 12.03 ± 0.38. The Totox value for oils from the red grape Merlot and Hamburg varieties were the smallest; whereas they were 2 to 3 times larger in the oils of white grape Ital. Riesling and Sila varieties. The largest Totox values of 10.24 ± 0.53 (R) and 12.03 ± 0.38 (W) were found in the oils from blended seeds, which may be a reflection of pomace collection and the drying process. In the reviewed literature, data on the Totox values for grape seed oil were very limited.

In grape seed oil, as a specialty oil from a local retail store in Reading (UK), the Totox value was 17.5 ± 0.1 (Madawala *et al*., 2012), but there was a lack of accurate background information on the sample.

3.2. Nutritive value of oils

As cold pressing does not involve chemicals or heat treatment during the oil production process, it implies that cold-pressed seed oils may contain phytochemicals, as well as natural antioxidants, more than refined oils. Some results from our previous work about total tocopherol and phenol contents and their fractionation (Bjelica *et al*., 2019) have been included to support this new investigation.

3.2.1. Tocopherol profile and vitamin E activity

The profile of tocopherols in the investigated grape seed oils is characterized by the presence mainly of the α-tocopherol (Table 3), where the amount ranged from 29.5 ± 1.09 to 65.5 ± 1.63 mg/kg. Other forms of tocopherols were found at considerably lower concentrations, at most up to 7.6 ± 0.2 mg/kg for β+γ-tocopherols and up to 3.9 ± 0.1 mg/kg for δ-tocopherols. All the results were significantly different at $p < 0.05$. Vitamin E is of particular importance for the nutritive value of the oil because of its excellent antioxidant properties (Crews *et al*, 2006). The biologically important vitamin E activity (expressed as α-tocopherol equivalent) of the samples is somewhat higher and ranges from 30.0 to 66.9 mg/kg, since the share of α-tocopherol is the highest in all samples and ranged from about 85 to 92%. It can be concluded that the vitamin E activity of white grape seed oil is significantly higher compared to red grape seed oil. The highest vitamin E activity was determined in the oil of a blend of different unknown white grape seed varieties, 66.9 mg/kg, as well as in the oil of Ital. Riesling white grape seeds, 65.9 mg/kg.

By analyzing the results of the vitamin E activity in Table 3 according to the Canadian Food Inspection Agency (1996), it can be said that only white grape seed oils could be labelled as a "Source of vitamin E", since they contain vitamin E in the amount of at least 0.5 mg per 10 g of oil (table portion), i.e. they provide between 5 and 15% of the recommended daily intake. From this aspect, white grape seed oil can be compared to peanut oil, palm and soybean oil, unlike corn and rapeseed oil that represent "A good source of vitamin E." Sunflower and cotton oils could be claimed as "An outstanding source of vitamin E" (Mag *et al*., 2002). The relevant literature data on the contents in tocopherol and tocotrienol in seed oils are very different. The biologically active vitamin E content relative to that of α-tocopherol ranged from 0.5 to 118 mg/kg of oil for the tannin-removed grape seed sample and the commercial grape seed oil, respectively (Oomah *et al*., 1998). Crews *et al*., (2006) quoted data for α-tocopherol in grape seed oil (extracted with hexane) from less than 10 mg/kg up to 229 mg/kg, indicating significant variations depending on the variety of the grape and the production area (France, Italy, Spain). Also, according to their results, the major

Table 3. Tocopherol content and vitamin E activity of cold-pressed red and white grape seed oils

Tocopherols (mg/kg)	Red grapes			White grapes		
	Merlot	**H**amburg	**R**-blend	**I**tal. **R**iesling	**S**ila	**W**-blend
α-	38.4 ± 1.2^{b}	39.8 ± 1.5^{b}	29.5 ± 1.1^{a}	64.0 ± 1.9^{d}	49.6 ± 1.4^{c}	65.5 ± 1.6^{d}
β+γ-	3.0 ± 0.1^{b}	5.2 ± 0.2^{d}	2.0 ± 0.1^{a}	7.6 ± 0.2^{f}	4.6 ± 0.1^{c}	5.5 ± 0.2^{e}
δ-	1.2 ± 0.0^{b}	1.8 ± 0.0^{c}	0.8 ± 0.0^{a}	2.8 ± 0.1^{d}	3.9 ± 0.1^{f}	3.5 ± 0.4^{e}
Vitamin E activity*	39.1^{b}	41.1^{c}	30.0^{a}	65.9^{e}	49.8^{d}	66.9^{f}
Share of α-toc. (%)	90.1^{e}	85.0^{b}	91.3^{f}	86.0^{c}	84.8^{a}	87.9^{d}
Total tocopherols	42.6 ± 0.6	46.8 ± 0.8	32.3 ± 0.6	74.8 ± 1.0	58.1 ± 0.8	74.5 ± 0.7

Values are means ± standard deviation (n =3); *expressed as α-tocopherol equivalent (mg/kg);
Different letters in the same row indicate significantly different values ($p < 0.05$, one-way ANOVA with Duncan test)

tocopherol was the α-form. Other tocopherol forms were found as follows: β-tocopherol <10 to 133 mg/kg, γ-tocopherol < 10 to 169 mg/kg and δ-tocopherol < 10 to 69 mg/kg. The contents in α-tocopherol in cold-pressed seed oils of seven local grape cultivars from the south-eastern part of Turkey were much higher than in our investigation and ranged from 77.33 to 225.40 mg/kg. However, β- and δ-tocopherol were not detected in any of the oils (Demirtas *et al*., 2013). In the cold-pressed grape seed oil of the Merlot variety, Oomah *et al*., (1998) determined a vitamin E content of 10.6 mg/kg; whereas in the seed oil of the same variety grown in China, When *et al*., (2016) determined a vitamin E (α-tocopherol) content of 90.00 ± 1.28 mg/kg (the seeds were subjected to supercritical carbon dioxide extraction). Fernandes *et al*., (2013) published that the seed oils of ten Portuguese grape varieties were also a very good source of vitamin E (148 to 358 α-tocopherol equivalents-including tocotrienol forms). The tocopherol profile of cold-pressed grape seed oil from a local cold pressing company in Turkey was as follows: 123.0 ± 1.76 mg/kg, 16.7 ± 0.01 mg/kg and 0.56 ± 0.01 mg/kg for α-, γ- and δ-tocopherol, respectively (Kiralan *et al*., 2019).

3.2.2. Profile of phenolic compounds

Belonging to the group of very important biomolecules, the phenolic fraction of vegetable oil contributes not only to its sensory taste, but also to the nutritive quality and shelf-life of the edible oils which exhibit strong antioxidant activity. Matthäus (2008) stated that grape seeds contain significant amounts of various phenolic compounds. However, due to their limited solubility, only small amounts pass into the oil. The composition of the phenolic compounds in our investigated cold-pressed oil samples is presented in Table 4.

As shown in Table 4, the most significant fraction of phenolic compounds in all the oil samples was ursolic acid, and the highest content was found in the oil of red grape varieties. The statistical analysis of the ursolic acid content showed significant differences ($p < 0.05$) among all samples and had the following order: Hamburg (336.3 ± 4.8 μg/g) > R-blend (305 ± 11μg/g) > S (212 ± 17 μg/g) > Ital. Riesling (159 ± 32 μg/g) > Merlot (82.3 ± 12.5 μg/g) and > W-blend (47.1 ± 6.3 μg/g). The presence of ursolic acid in all the oil samples might be due to the fact that the grape cultivars analyzed in this study originated from the same geographical region and

Table 4. Phenolic composition in cold-pressed red and white grape seed oils

Phenols (μg/g)	Red grapes			White grapes		
	Merlot	**H**amburg	**R**-blend	**I**tal. **R**iesling	**S**ila	**W**-blend
p-OH benzoic acid	<0.08	<0.08	<0.08	<0.09	<0.09	0.495 ± 0.031
p-coumaric acid	<0.08	0.398 ± 0.030^{b}	0.475 ± 0.006^{c}	<0.09	0.126 ± 0.007^{a}	0.146 ± 0.026^{a}
Vanillic acid	<0.3	0.878 ± 0.116^{b}	1.70 ± 0.04^{c}	<0.3	<0.3	0.50 ± 0.11^{a}
Ferulic acid	<0.08	0.226 ± 0.012^{c}	0.191 ± 0.024^{b}	<0.09	<0.09	0.090 ± 0.001^{a}
Resveratrol	<0.3	5.16 ± 0.08^{b}	3.99 ± 0.21^{a}	<0.3	<0.3	<0.3
Morin	<0.3	0.979 ± 0.011^{c}	0.842 ± 0.109^{b}	<0.3	<0.3	0.19 ± 0.02^{a}
Naringenin	0.008 ± 0.001^{a}	0.239 ± 0.009^{d}	1.189 ± 0.009^{e}	0.015 ± 0.003^{a}	0.032 ± 0.003^{b}	0.068 ± 0.004^{c}
Ursolic acid	82.3 ± 12.5^{b}	336.3 ± 4.8^{f}	305 ± 11^{e}	159 ± 32^{c}	212 ± 17^{d}	47.1 ± 6.3^{a}
Proto-catechinic acid	<0.04	0.173 ± 0.011^{b}	0.349 ± 0.012^{c}	<0.04	<0.04	0.059 ± 0.011^{a}
Krizoeriol	0.023 ± 0.003^{b}	0.216 ± 0.004^{c}	0.314 ± 0.005^{d}	0.010 ± 0.001^{a}	0.015 ± 0.001^{a}	0.011 ± 0.001^{a}
Amentoflavon	0.216 ± 0.133^{b}	0.399 ± 0.068^{c}	0.555 ± 0.018^{d}	0.016 ± 0.001^{a}	<0.009	<0.009
Kaempherol	<0.3	5.80 ± 0.16^{c}	4.68 ± 0.21^{b}	<0.3	<0.3	1.18 ± 0.36^{a}

Values are means ± standard deviation (n =3); Different letters in the same row indicate significantly different values ($p < 0.05$, one-way Anova with Duncan test)

growing conditions. Namely, Cicero *et al*., (2018) suggested that the polyphenol profile of grape seed oil may be affected by the variety of grape seeds and environmental growing conditions, rather than extraction procedures. The interest in ursolic acid is related to its beneficial effects on human health due to antioxidant, antimicrobial, anti-inflammatory, hepatoprotective, immunomodulatory, anti-tumor, cardioprotective, and antihyperlipidemic activities, among others (López-Hortas *et al*., 2018). When considering other phenolic fractions in a significant amount (Table 4), it was resveratrol (< 0.3 to 5.16 ± 0.08 μg/g) and kaempherol (< 0.3 to 5.80 ± 0.16 μg/g), but they were far less compared to ursolic acid. By applying two-way ANOVA on the obtained results of these phenolic fractions, as well as the results of the Tukey test, it can be concluded that all oil samples were statistically significant. Among the investigated samples, on average, red grape seed oils had higher contents in phenolic compounds, except for p-OH benzoic acid, with respect to the white grape seed oils. Oil from the red grape Hamburg variety was the richest in all phenolic fractions, about 336.3 ± 4.8 μg/g.

Bail *et al*., (2008) determined the total phenolic components of 9 samples of cold-pressed seed oils of different grape varieties and published the contents to be ranging from 59.0 ± 0.02 to 115.5 ± 0.01 μg/g. Lower values were found for refined oils, as well as for seed oil which was heated at 60 °C for 30 min. before pressing. The same authors also found lower contents in phenolic ingredients in white grape seed oil. Recently, Cicero *et al*., (2018) published that cold-pressed grape seed oil from the Brazilian region, Minas Gerais, had the lowest contents in polyphenols. The phenolic profile of the investigated oils from these authors was dominated by the flavonoids apigenin 7-glucoside (0.74 μg/g), and luteolin (0.63 μg/g); whereas phenolic acids, such as *p*-coumaric (0.19 μg/g), and caffeic (0.27 μg/g) acids, were detected in lower amounts. An exception was presented by gallic acid, which was found in the amount of 2.97 μg/g. Kiralan *et al*., (2019) determined 8 bioactive and phenolic compounds including vanillin (0.33 μg/g), vanillic acid (0.39 μg/g), *p*-coumaric acid (0.08 μg/g), dihydroquercetin (2.49 μg/g), luteolin (0.67 μg/g), campherol (0.34 μg/g), apigenin (0.01 μg/g) and acacetin (0.31 μg/g) in cold-pressed grape seed oil.

3.2.3. Total contents in biomolecules and EC_{50} values

Total contents in biomolecules, like tocopherols and tocotrienols and phenolic compounds, as well as antiradical capacity, assessed by the evaluation of the free radical scavenging effect of oils, are presented in Table 5.

Comparing the results of the total tocopherol and tocotrienol contents (Table 5) with the results of total tocopherol contents (Table 3), it can be concluded that the investigated grape seed oils showed a significantly higher content in tocotrienols than tocopherols. This is also evidenced by the literature data. The major compound found in grape seed oil is γ-tocotrienol (Crews *et al*., 2006; Fernandes *et al*., 2013). The contents in all tocols in our tested samples ranged from at least 338.14 ± 3.35 mg/kg (blend of different red grape varieties) up to 575.23 ± 4.46 mg/kg (Merlot variety). Although all samples

Table 5. Total tocol and total phenol contents and EC_{50} values for cold-pressed red and white grape seed oils

	Total tocols* (mg/kg)	Total phenols (mgGAE/kg)	EC_{50}**
Cold-pressed oil of red grape seed			
Merlot	575.23 ± 4.46^{f}	12.66 ± 1.07^{b}	65.34 ± 0.32^{c}
Hamburg	338.14 ± 3.35^{a}	44.69 ± 1.56^{c}	32.66 ± 0.43^{b}
R-blend	373.55 ± 2.88^{b}	54.92 ± 0.93^{d}	29.84 ± 1.07^{a}
Cold-pressed oil of white grape seed			
Ital. **R**iesling	463.99 ± 2.57^{e}	9.29 ± 0.84^{a}	95.63 ± 1.48^{f}
Sila	438.77 ± 5.37^{c}	11.94 ± 1.72^{b}	89.45 ± 1.03^{d}
W-blend	452.41 ± 0.89^{d}	8.27 ± 0.53^{a}	92.50 ± 1.17^{e}

*Content in total tocopherols and tocotrienols; **(mg oil/mg DPPH radicals needed to decrease the initial DPPH• concentration by 50%); Values are means ± standard deviation (n = 3); Different letters in the same row indicate significantly different values (p < 0.05, one-way ANOVA with Duncan test)

were significantly different, it can be said that the total contents in tocols in oils from white grape seeds were relatively homogeneous, from 438.77 ± 5.37 to 463.99 ± 2.57 mg/kg. According to the literature data (Baydar and Akkurt, 2001) in the seed oils from the grape cultivar Hamburg and Riesling from the vineyard of Ankara (Turkey), the concentration of total tocopherols was 401 ± 11.04 and 364 ± 14.25 mg/kg, respectively. The same authors found that, generally, the tocopherol concentration in red grape seed oil was higher (357 ± 9.96 to 578 ± 27.65 mg/kg) in comparison with white grape seed oil (364 ± 14.25 to 486 ± 23.66 mg/kg). The total tocopherol and tocotrienol contents in new monovarietal cold-pressed grape seed oils derived from white grape varieties collected in North-western Spain ranged between 360 ± 2.61 and 445.5 ± 36.5 mg/kg (Boso *et al*., 2018). Demirtas *et al*., (2013) observed variation in total tocol contents (394-755 mg/kg) in cold-pressed oil from grape cultivars originating from the same geographical region in the south-eastern part of Turkey with similar climatic and soil characteristics. Our results are in accordance with the cited literature data.

Regarding the total phenol contents, it can be said that depending on the variety, it was on average higher in the cold-pressed oils obtained from red grape varieties (12.66 ± 1.07 to 54.92 ± 0.93 mgGAE/kg) compared to white grape varieties (8.27 ± 0.53 to 11.94 ± 1.72 mgGAE/kg). Demirtas *et al*., (2013) reported that the total phenol contents in seven different grape seed oils ranged from 21.9 ± 0.14 to 47.0 ± 0.30 mgGAE/kg oil and these values are similar to our results. Bail *et al*., (2008) measured the total phenol contents in virgin cold-pressed unfiltered grape seed oils and the contents ranged from 59 ± 0.02 to 115.5 ± 0.01 mgGAE/kg of oil. According to Lutterodt *et al*., (2011) the total phenolic content was much higher when using a Soxhlet extraction method for grape seed oil (ethanol, 3h) and it ranged from 160 ± 0.00 to 800 ± 0.01 mg GAE/kg of oil. Wen *et al*., (2016) reported the contents in total phenolics of 98.19 ± 0.02 mgGAE/kg for the red grape Cabernet Sauvignon variety, while Chardonnay (white grape variety) represented total phenolic contents of 46.6 ± 3.24 mgGAE/kg. The authors concluded that this may be due to the presence of anthocyanins with the main phenolics, which appeared to be red in color. Recently, Kiralan *et al*., (2019) published that many more total phenolic compounds, 924.2 ± 20.41 mgGAE/kg, were present in grape seed oil obtained from a local cold-pressing company (Istanbul, Turkey). The amounts of phenolics in grape seeds may vary greatly according to location, climate, cultivar, vintage, post-harvest treatment and storage conditions. On the other hand, low molecular weight phenolics have low solubility in oil and some phenolic acids can be bound to the seed material, so the total phenolic content in oil may also vary (Lutterodt *et al*., 2011).

The EC_{50} values were calculated as the inhibitory concentration of the oil necessary to decrease initial DPPH radical absorbance by 50%, whereby a lower EC_{50} value indicates a higher antiradical capacity. The EC_{50} value of grape seed oils in this study ranged from 29.84 ± 1.07 to 95.63 ± 1.48 mg oil/mg DPPH (Table 5). Consequently, the highest EC_{50} was registered for the white grape seed oil of the Ital. Riesling variety; while the lowest EC_{50} was found for the oil of unknown varieties of red grapes, R-blend ($p < 0.05$). In accordance with this, the oil from the red grape seed blend had the highest antiradical power followed by the Hamburg and Merlot varieties; while the lowest antiradical potential was found for grape seed oil It. Riesling ($p < 0.05$). This can be explained by the fact that the red grape oils have far higher contents in total phenolic compounds compared to white grape oils.

It is worth mentioning that a strong negative relationship between EC_{50} and content in total phenols was observed, where (r) was -943 ($p = 0.01$). However, Wen *et al*., (2016) indicated that the antioxidant activities of grape seed oil are not just the function of phenolics, but might be a combined effect of phenolics, vitamin E and sterols. Furthermore, a positive relationship between EC_{50} and total tocopherol/tocotrienol content was determined (r value was 0.543 at $p = 0.05$). On the other hand, the content in total tocols showed a negative moderate correlation with the content in total phenols, where (r) was -0.732 ($p = 0.01$).

Two-way ANOVA and the Tukey test were performed at a significance level of $p < 0.05$, even though factorial analysis was not the focus of this paper. The relevant factors were grape color (red or white) and the origin of the seeds (variety or blend of different varieties). The Tukey test showed that there was no statistically significant difference in total

tocol contents in cold-pressed oils obtained from red or white grape varieties or a blend of different red and white seed varieties.

The opposite effect was found for total phenols. There was a statistically significant difference between the total phenol contents in cold-pressed oils obtained from red or white grape varieties and the total phenol contents in the cold-pressed oils obtained from the seed of the grape varieties or a blend of different varieties.

By applying two-way ANOVA on the obtained results for $EC_{50,}$ as well as the analysis of the Tukey test ($p < 0.05$), we can identify a statistically significant difference in EC_{50} in cold-pressed oils obtained from red or white grape varieties, but there was no statistically significant difference in the EC_{50} in cold-pressed oils obtained from the seeds of the grape varieties, or a blend of different varieties.

4. CONCLUSIONS

The results of the investigation of cold-pressed oil samples obtained from grapes grown in the Fruška Gora vineyard in the Republic of Serbia showed good oil quality, but pronounced seed oil nutritive profile differences for red and white grape varieties were observed. The highest content, 575.23 ± 4.46 mg/kg, of total tocols was found in the red grape seed oil of the Merlot variety. Red grape seed oils also indicated better DPPH radical scavenging capacity, but the vitamin E activity of 49.8 to 66.9 mg/kg was significantly higher in white grape seed oils. The most prevalent phenolic fraction in all oil samples was ursolic acid, at up to 336.3 ± 4.8 μg/g in the grape seed oil from the Hamburg variety.

All the presented quality characteristics of cold-pressed grape seed oils are highly valued and encourage production this kind of oil in larger quantities. However, the chemical quality of oil highly depends on the period of time needed for seed extraction from the pomace, in terms of avoiding hydrolytic and oxidative degradation. According to our results, a longer period of time indicated higher acid, peroxide and Totox values (2 to 4 times more) in oils from blends of different grape seed varieties obtained from pomace collected from small wineries. Overall, the results could be used for the insights of some chemical parameters and the most important bioactive components responsible for the quality of cold-pressed grape seed oil from this region.

REFERENCES

Bail S, Stuebiger G, Krist S, Unterweger H, Buchbauer G. 2008. Characterisation of various grape seed oils by volatile compounds, triacylglycerol composition, total phenols and antioxidant capacity. *Food Chem.* **108**, 1122-1132. https://doi.org/10.1016/j.foodchem.2007.11.063

Baydar NG, Akkurt M. 2001. Oil content and oil quality properties of some grape seeds. *Turk. J. Agric. For.* **25**, 163-168.

Bjelica M, Vujasinović V, Rabrenović B, Dimić S. 2019. Some chemical characteristics and oxidative stability of cold-pressed grape seed oils obtained from different winery waste. *Eur. J. Lipid Sci. Technol.* **121**, 1-10. https://doi.org/10.1002/ejlt.201800416

Boso S, Gago P, Santiago JL, Rodriguez-Canas E, Martinez MC. 2018. New monovarietal grape seed oils derived from white grape bagasse generated on an industrial scale at a winemaking plant. *LWT-Food Sci. Technol.* **92**, 388-394. https://doi.org/10.1016/j.lwt.2018.02.055

Canadian Food Inspection Agency. 1996. Guide to Food Labeling and Advertising, Nutrient Claims, Section VI-53, Table 6.2.6.3.1.

Carpenter AP. 1979. Determination of tocopherols in vegetable oils. *J. Am. Oil Chem. Soc.* **56,** 668-671. https://doi.org/10.1007/bf02660070

Cicero N, Albergamo A, Salvo A, Bua GD, Bartolomeo G, Mangano V, Rotondo A, Di Stefano V, Di Bella G, Dugo G. 2018. Chemical characterization of a variety of cold-pressed gourmet oils available on the Brazilian market. *Food Res. Inter.* **109**, 517-525. https://doi.org/10.1016/j.foodres.2018.04.064

Codex - JOINT FAO/WHO 1999. Food standards programme. In: Codex Alimentarius Commission. Codex standards for named vegetable oils. CX-STAN 210-1999.

Crews C, Hough P, Godward J, Brereton P, Lees M, Guiet S, Winkelmann W. 2006. Quantitation of the main constituents of some authentic grape-seed oils of different origin. *J. Agric. Food Chem.* **54**, 6261-6265. https://doi.org/10.1021/jf060338y

Dabetic MN, Todorovic MV, Djuricic DI, Antic Stankovic JA, Basic NZ, Vujovic SD, Sobajic SS. 2020. Grape seed oil characterization: A novel

approach for oil quality assessment. Eur. *J. Lipid Sci. Technol.* 1900447. https://doi.org/10.1002/ejlt.201900447

Demirtas I, Pelvan E, Özdemir IS, Alasalvar C, Ertas E. 2013. Lipid characteristics and phenolics of native grape seed oils grown in Turkey. *Eur. J. Lipid Sci. Technol.* **115**, 641-647. https://doi.org/10.1002/ejlt.20120159

Dwyer K, Hosseinian F, Rod M. 2014. The market potential of grape waste alternatives. *J. Food Res.* **3**, 91-106. https://doi.org/10.5539/jfr.v3n2p91

Fagundes Assumpção C, Larroza Nunes I, Alcântara Medonça T, Calixto Bortolin R, Jablonski A, Hickmann Flôres S, Oliviera Rios A. 2016. Bioactive compounds and stability of organic and conventional *Vitislabrusca* grape seed oils. *J. Am. Oil Chem. Soc.* **93**, 115-124. https://doi.org/10.1007/s11746-015-2742-0

FAOSTAT 2018. FAO Statistical Database, http://www.fao.org.

Fernandes L, Casal S, Cruz R, Pereira JA, Ramalhosa E. 2013. Seed oils of ten traditional Portuguese grape varieties with interesting chemical and antioxidant properties. *Food Res. Inter.* **50**, 161-166. https://doi.org/10.1016/j.foodres.2012.09.039

Garavaglia J, Markoski MM, Oliveria A, Marcadenti A. 2016. Grape seed oil compounds: Biological and chemical actions for health. *Nutr. Metab. Insight*. **9**, 59-64. https://doi.org/10.4137/NMI.S32910

Gouvinhas I, Machado J, Gomes S, Lopes J, Martins-Lopes P, Barros A. 2014. Phenolic composition and antioxidant activity of monovarietal and commercial portuguese olive oils. *J. Am. Oil Chem. Soc.* **91**, 1197–1203. https://doi.org/10.1007/s11746-014-2462-x

Haiyan Z, Bedgood Jr. DR, Bishop AG, Prenzler PD, Robards K. 2007. Endogenous biophenol, fatty acid and volatile profiles of selected oils. *Food Chem.* **100**, 1544-1551.

Jakšić D, Bradić I, Beader M, Ristić M, Popović D, Mošić I, Dodok I. 2019. *Vinogradarstvo i vinarstvo Srbije*, Studija, Analiza sektora proizvodnje i prerade grožđa i proizvodnje vina. Centar za vinogradarstvo i vinarstvo Niš, Niš, Serbia, pp 9 and 147.

Kiralan M, Çali G, Kiralan S, Özaydin A, Özkan G, Ramada MF. 2019. Stability and volatile oxidation compounds of grape seed, flax seed and black cumin seed cold-pressed oils affected by thermal oxidation. *Grasas Aceites* **70** (1), e295. https://doi.org/103989/gya.0570181

López-Hortas L, Pérez-Larrán P, González-Muñoz MJ, Falqué E, Domínguez H. 2018. Recent developments on the extraction and application of ursolic acid. A review. *Food Res. Int.* **103**, 130-149. https://doi.org/10.1016/j.foodres.2017.10.028

Lutterodt H, Slavin M, Whent M, Turner E, Yu L. 2011. Fatty acid composition, oxidative stability, antioxidant and antiproliferative properties of selected cold-pressed grape seed oils and flours. *Food Chem.* **128**, 391-399. https://doi.org/10.1016/j.foodchem.2011.03.040

Madawala SRP, Kochar SP, Dutta PC. 2012. Lipid components and oxidative status of selected specialty oils. *Grasas Aceites* **63** (2), 143-151. https://doi.org/10.3989/gya.083811

Mag TK, Mag T, Reichert RD. 2002. A new recommended calculation of vitamin E activity: Implications for the vegetable oil industry. *INFORM* **13**, 836-839.

Martinez M, Maestri D. 2008. Oil chemical variation in walnut (*Juglans regia* L.) genotypes grown in Argentina. *Eur. J. Lipid Sci. Technol.* **110**, 1183-1189. https://doi.org/10.1002/ejlt.200800121

Matthäus B, Spener F. 2008. What we know and what we should know about virgin oils – a general introduction. *Eur. J. Lipid Sci. Technol.* **110**, 597-601. https://doi.org/10.1002/ejlt.200800118

Matthäus B. 2008. Virgin grape seed oil: Is it really a nutritional highlight? *Eur. J. Lipid Sci. Technol.* **110**, 645-650. https://doi.org/10.1002/ejlt.200700276

Oomah BD, Liang J, Godfrey D, Mazza G. 1998. Microwave heating of grapeseed: Effect on oil quality. *J. Agric. Food Chem.* **46,** 4017-4021. https://doi.org/10.1021/jf980412f

Paqout C, Mercier J, Lefort D, Mathieu A, Perron R. 1967. *Les Methodes Analitiques des Lipides Simples* (in Serbian), Poslovno udruženje proizvođača biljnih ulja, Belgrade, pp. 175–179.

Pardo JE, Fernández E, Rubio M, Alvarruiz A, Alonso GL. 2009. Characterization of grape seed oil from different grape varieties (*Vitis vinifera*). *Eur. J. Lipid Sci. Technol*. **111**, 188–193. https://doi.org/10.1002/ejlt.200800052

Prescha AM, Grajzer M, Dedyk M, Grajeta H. 2014. The antioxidant activity and oxidative stability of cold-pressed oils. *J. Am. Oil Chem. Soc.* **91**, 1291-1301. https://doi.org/10.1007/s11746-014-2479-1

Radočaj O, Dimić E. 2013. Physico-chemical and nutritive characteristics of selected cold-pressed oils found in the European market. *Rivista Ital. Sost. Grasse* **90** (4), 219-228.

Rubio M, Alvarez-Orti M, Alvarruiz A, Fernández E, Pardo JE. 2009. Characterization of oil obtained from grape seeds collected during berry development. *J. Agric. Food Chem.* **57**, 2812-2815. https://doi.org/10.1021/jf803627t

Shinagawa FB, de Santana FC, Torres LRO, Mancini-Filho J. 2015. Grape seed oil: a potential functional food? *Food Sci.* **35** (3), 399-406. https://doi.org/10.1590/1678-457X.6826

Teh SS, Birch J. 2013. Physicochemical and quality characteristics of cold-pressed hemp, flax and canola seed oils. *J. Food Compos. Anal.* **30**, 26–31. https://doi.org/10.1016/j.jfca.2013.01.004

Unusan N. 2020. Proanthocyanidins in grape seeds: An updated review of their health benefits and potential uses in the food industry. *J. Funct. Food.* 67, 103861. https://doi.org/10.1016/j.jff.2020.103861

Wen X, Zhu M, Hu R, Zhan J, Chen Z, Li J, Ni Y. 2016. Characterisation of seed oils from different grape cultivars grown in China. *J. Food Sci. Technol.* **53** (7), 3129-3136. https://doi.org/10.1007/s13197-016-2286-9

GRASAS Y ACEITES 72 (2)
April-June 2021, e412
ISSN-L: 0017-3495
https://doi.org/10.3989/gya.0214201

Physicochemical properties of red palm oil extruded potato and sweet potato snacks

Y.Y. Liu[a], T.M. Olajide[a], M. Sun[a], M. Ji[b], J.H. Yoong[b] and X.C. Weng[a, ✉]

[a]School of Life Sciences, Shanghai University, 333 Nanchen Road, Shanghai, 200444, China.

[b]Palm Oil Research and Technical Service Institute of Malaysian Palm Oil Board (PORTSIM), Shanghai, 201108, China.

✉Corresponding author: wxch@staff.shu.edu.cn

Submitted: 07 February 2020; Accepted: 02 June 2020; Published online: 03 June 2021

SUMMARY: Extruded potato (P) and sweet potato (SP) products with red palm oil (RPO) were prepared under different conditions. Superior product characteristics such as sensory score, expansion ratio, and water solubility index, among others, were obtained at high extrusion temperature (150-155 °C) and low water feed rate to the extruder (50.4-50.8 mL/min). The optimal products, P_1 and SP_1, had high micronutrients as their total contents of β-carotene, squalene, tocopherols, and tocotrienols were 883.2, 304.4, 262.4, and 397.0 mg/kg of oil, respectively. The average peroxide value was 4.3 meq O_2/kg oil, *p*-anisidine value 3.3, and induction period (100 °C) 11.4 h. Moreover, RPO extruded with P showed a better extrusion behavior but lower micronutrient retention and oxidative stability than that extruded with SP. Thus, the finding herein is important for investigating extrusion conditions, increasing variety, improving nutritional quality, assessing applicability and predicting the shelf-life of RPO-P/SP-extruded food.

KEYWORDS*: Extrusion; Micronutrients; Oxidation stability; Potato; Red palm oil; Sweet potato*

RESUMEN: ***Propiedades fisicoquímicas de snacks extrusionados de patatas y batatas con aceite de palma roja.*** Se prepararon snacks extrusionados de patatas (P) y batatas (B) con aceite de palma roja (APR) en diferentes condiciones. Se obtuvieron unas características superiores de los productos, como puntuación sensorial, relación de expansión, índice soluble en agua, entre otros, a alta temperatura de extrusión (150-155 °C) y baja velocidad de alimentación de agua al extrusionador (50,4-50,8 mL/min). Los productos óptimos, P1 y SP1, contenían altos micronutrientes ya que su contenido total de β-caroteno, escualeno, tocoferoles y tocotrienoles fue de 883,2; 304,4; 262,4 y 397,0 mg/kg de aceite, respectivamente. El índice de peróxido promedio fue de 4,3 meq O_2/kg de aceite, el valor de *p*-anisidina de 3,3 y el período de inducción a 100 °C de 11,4 h. Además, el APR utilizado para la extrusión de P mostró un mejor comportamiento de extrusión, pero menor retención de micronutrientes y estabilidad oxidativa que el extrusionado con SP. Por lo tanto, los datos aquí obtenidos son importantes para profundizar en las condiciones de extrusión, aumentar la variedad, mejorar la calidad nutricional, evaluar la aplicabilidad y predecir la vida útil de los alimentos extrusionados con APR-P / B.

PALABRAS CLAVE*: Aceite de palma roja; Batata; Estabilidad oxidativa; Extrusión; Micronutrientes; Patata.*

Citation/Cómo citar este artículo: Liu YY, Olajide TM, Sun M, Ji M, Yoong JH, Weng XC. 2021. Physicochemical properties of red palm oil extruded potato and sweet potato snacks. *Grasas Aceites* **72** (2), e412. https://doi.org/10.3989/gya.0214201

1. INTRODUCTION

Extruded snacks are foods enjoyed by many for their crispy mouthfeel as well as desirable flavor. They are often made from dry cereals, sugar, oil and other edible materials, extruded at a high temperature within a short period of time. The extruded food retains large amounts of vitamins and other nutrients because of the short processing time involved. Moreover, high temperature and shear force in the barrel enable the degradation of macromolecules which makes the products easy to digest (Ding *et al.*, 2006).

Potatoes (P) and sweet potatoes (SP) are native to South America and are staple foods enjoyed in different cultures around the world. They are high-yielding and adaptable, since their introduction to China during the Ming and Qing dynasties, P and SP have had a tremendous impact on the Chinese society. Nowadays, the production of P and SP in China ranks first in the world (Zhang *et al.,* 2017; Bai *et al.*, 2018). Since the launch of the potato development strategy by the Chinese Ministry of Agriculture in 2015, the value of the P industry has gradually increased. The application of P and SP to the processing of extruded foods does not only broaden the industrial chain of the two crops, but also promotes their variety and enhances the competitiveness of extruded products.

The production of palm oil reached approximately 64.6 Mt in 2017, accounting for 34% of the global oil market. Malaysia alone produced 19.92 Mt and exported 16.56 Mt in that same year. In addition to high yields and low prices, palm oil is also widely known for its oxidative stability and nutritional properties. Palm oil, containing approximately 48% saturated fatty acid and 38% monounsaturated fatty acid is expected to replace hydrogenated oil, which is rich in *trans*-fatty acids in the food industry (Mba *et al.*, 2015). Red palm oil (RPO) is deep red and processed by molecular distillation to retain abundant β-carotene, tocopherols, tocotrienols and squalene. Carotenoids, tocopherols, tocotrienols and squalene have various health-promoting properties, such as the prevention of eye and skin conditions, protection of the biological systems against oxidation, preventive effect on various types of cancers and cardioprotective effect on serum cholesterol. Furthermore, tocopherols and tocotrienols are strong natural antioxidants, which can greatly retard the autoxidation of oils and fats and protect fatty foods from off-flavors (Mba *et al.*, 2015). The development of functional extruded food with low-cost, nutrient-rich, and oxidatively stable red palm oil helps reduce the intake of *trans*-fatty acids, increases the effective intake of carotenoids, tocopherols, squalene and other nutrients, as well as delaying the oxidation of food. This may be useful for expanding the application of RPO by improving malnutrition in poor areas and extending the shelf-life of food.

In the few studies that have evaluated the application of RPO in extruded snacks, Sidhu *et al.,* (2004) used palm oil and palm olein in the production of extrudates, biscuits and bread. The results showed that the content in β-carotene in the extruded product ranged from 136.0 to 495.9 mg/kg, which was higher than that in baked food. Wan *et al.,* (2018), on the other hand, observed that palm oil extruded at a low temperature and high moisture content showed lower oxidative stability than when extruded at a high temperature and low water content and P starch offered a better protection to palm oil against oxidative stability than corn starch. However, based on existing literature, the interactions between co-factors such as composition and extrusion parameters, both of which influence the quality of RPO-extruded food has not been adequately investigated. Therefore, this study optimized products with different expansion conditions (from micro-expansion to complete but not excessive expansion) by adjusting extrusion parameters like temperature (60-100 ºC) and water velocity (50-100 mL/min), and systematically evaluating the effect of the extrusion conditions on product characteristics, nutritional quality and the oxidative stability of RPO-P/SP extrudates.

2. MATERIALS AND METHODS

2.1. Materials

P flour, SP flour (homemade), corn flour, and sugar were purchased from Wal-Mart supermarket (Shanghai, China). Food grade calcium carbonate was purchased from Mingyuan Chemical Material Co., Ltd (Dexing, China) and RPO was donated by Palm Oil Research and Technical Service Institute of Malaysian Palm Oil Board (PORTSIM) (Shanghai, China).

2.2. Sample preparation

The ingredients in extruded P snacks are: corn flour 2400 g, P flour 600 g, RPO 150 g (4.33% of total materials), sugar 300 g, calcium carbonate 17.25 g

(moisture content 10.22%, pH 6.60). The ingredients in extruded SP snacks are: corn flour 2700 g, SP flour 300 g, RPO 150 g (4.33% of total materials), sugar 300 g, calcium carbonate 17.25 g (moisture content 10.54%, pH 6.48). The formulations were derived from a preliminary optimized single factor test and orthogonal experiment similar to Yao *et al.,* (2017).

Extrusion was carried out with the mixed materials after starting up the DTE-35 twin-screw extruder with a water pump (Coperion Machinery Co., Nanjing, China) for 20 minutes. The extrusion conditions, including water velocity, temperature (± 3 ºC), screw speed and cutting frequency were adjusted in real time according to the product characteristics. Samples were collected under specific conditions when the products were in a better and more stable state (i.e. products with proper expansion, smooth surface and crisp texture). The extrudates were cooled in a stainless-steel pallet at room temperature for 15 minutes, dried at 130 °C for 30 minutes for a moisture content less than 3%, and then finally stored in an air-tight condition at -20 °C. Five samples of P and SP with different expansion ratios were collected, respectively. The extrusion conditions of the samples are shown in Table 1.

2.3. Chemical composition of raw materials

Starch, fiber, and protein analyses of the raw materials were determined according to AOAC Official Method (AOAC, 2000). Lipids and moisture analyses were determined according to AACC International Approved Method (AACC, 2002).

2.4. Product characteristics analysis

100 g extruded products were crushed and passed through 60 mesh sieves for the determination of color, water solubility index, and water absorption index. Similarly, another 100 g extruded products were uncrushed for determination of expansion ratio, and specific volume.

2.4.1. Expansion ratio (ER)

The cross-sectional diameter of the samples was measured randomly by a Vernier caliper about 20 times. The ratio of the average sample diameter to die diameter, ER, was then calculated (diameter of the circular die was 3.5 mm) (Ding *et al.*, 2006).

Table 1. The extrusion conditions of P_1-P_5, SP_1-SP_5

Sample	Temperature /ºC	Water velocity /(mL/min)	Screw speed /rpm	Feed frequency /Hz	Slicer frequency /Hz	Melting temperature/ºC
P_1	151	51	251	12	8	141
P_2	126	51	251	12	6	132
P_3	102	62	251	12	6	126
P_4	89	100	251	12	8	112
P_5	67	100	251	12	12	106
SP_1	155	50	251	12	10	121
SP_2	117	50	251	12	10	126
SP_3	107	72	251	12	8	120
SP_4	82	82	251	12	10	113
SP_5	79	100	251	12	12	103

P, potato; SP, sweet potato. The composition of $P_{1\text{-}5}$ ($SP_{1\text{-}5}$): corn flour 2400 g (2700 g), P flour 600 g (SP flour 300 g), red palm oil 150 g, sugar 300 g, calcium carbonate 17 g.

2.4.2. Specific volume (SV)

SV was determined by the millet volume displacement method using a graduated cylinder (Spinello *et al.*, 2014).

2.4.3. Color

A CR-400 chroma meter (Konica Minolta, Japan) was used for color determination, and color calibration was performed on the standard whiteboard (L*, 97.13; a*, 0.21; b*, 1.87). The results are shown as ΔL, Δa, Δb (the differences in brightness, redness, and yellowness between samples and the whiteboard, respectively) (Spinello *et al.*, 2014).

2.4.4. Sensory analysis

The products were poured into randomly numbered containers and the overall acceptability of products on a 9-point hedonic scale was evaluated by a ten-member panel (all from the Food Science Department, Shanghai University, Shanghai, China). The results were expressed as sensory scores (SS), from 9 (extremely like) to 1 (extremely dislike) (Wichchukit and O'Mahony, 2015).

2.4.5. Water solubility index (WSI) and water absorption index (WAI)

WSI and WAI of samples were analyzed by a modification of the method of Spinello *et al.*, (2014) according to Equation (1) and Equation (2). 2 g of sample (m_0) were placed in a centrifuge tube (m_1), and dispersed into 25 mL distilled water. After being kept in the water bath for 30 min (with intermittent shaking every 5 min), the sample was centrifuged at 4,000 rpm for 10 min. The supernatant was decanted into a glass pan (m_2) and dried at 105 ºC to constant weight (m_3). Lastly, the weight of the centrifuge tube and gel of the precipitate (m_4) were measured.

$$\mathrm{WSI}/\% = \frac{m_3/g - m_2/g}{m_0/g} * 100 \quad (1)$$

$$\mathrm{WAI}/(g/g) = \frac{m_4/g - m_1/g}{m_0/g} \quad (2)$$

2.5. Oil extraction

100 g of finely ground extruded snacks were extracted with 500 mL petroleum ether in SK2200H ultrasonic machine (Kedao Ultrasound Instrument Co., Shanghai, China) at room temperature for 10 min. The extraction process was repeated four times. After the rotary evaporation of solvent at 35 °C, the extracted oil was stored at -20 °C for further analysis.

2.6. Oil property analysis

2.6.1. β-Carotene

β-Carotene was analyzed by UV-1800PC ultraviolet spectrophotometer (Mapada Instrument Co., Shanghai, China) (Pan *et al.*, 2016).

2.6.2. Squalene

Squalene was determined by LC-20A High Performance Liquid Chromatography (HPLC) with SPD-M20A UV detector (Shimadzu Co., Japan) (Pan *et al.*, 2016). The unsaponifiable matter of a 3-g samples were extracted, and then dissolved and diluted with 25 mL 0.22 μm membrane filtered n-hexane. The injection volume was 25 μL. An Inertsustain C18 column (250 mm × 4 mm, 5 μm; Japan, Shimadzu Corporation) thermoset at 30 °C was used. The mobile phase was acetonitrile-methanol (4:6 v/v) at a flow rate of 1 mL/min. Peaks were detected at 204 nm. Squalene was identified and quantified using standard squalene (Aladdin Industrial Co., Shanghai, China) as external standard.

2.6.3. Tocotrienols and tocopherols

Tocotrienols and tocopherols were measured by a LC-20A HPLC with RF-10AXL fluorescence detector (Shimadzu Co., Japan) programmed for excitation at 290 nm and emission at 330 nm. 2 g of sample were dissolved in methanol and sonicated for 20 min, centrifuged at 3000 rpm for 10 min, after which 5 μL of the dissolved sample were injected into the HPLC. The separation was achieved on an Inertsustain C18 column (250 mm × 4 mm, 5 μm; Shimadzu Co., Japan) thermo-set at 30 °C. The mobile phase was methanol-water (98:2 v/v) at a flow rate of 1 mL/min. The identification and quantification were based on external standards (α-, β-, γ-, and δ-isoforms, Solarbio Co., Beijing, China).

2.6.4. Peroxide value (PV) and p-anisidine value (p-AV)

The PV and *p*-AV were determined according to methods Cd 8-53 and p 2.4 (AOCS, 1997), respectively.

2.6.5. Induction period (IP)

A Rancimat 743 model (Metrohm Co., Switzerland) was used to determine the IP of the samples according to a previous method with slight modifications (Olajide *et al.,* 2020). 2.5 g of sample were carefully weighed into rancimat tubes and subjected to accelerated oxidation at 100 °C under an air flow of 20 L/h. The IP were printed automatically by the apparatus.

2.7. Statistical analysis

All analyses were performed in triplicate, and the data are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was carried out using Microsoft Excel version 2013, followed by Duncan's multiple range test ($P < 0.05$) and Principle component analysis (PCA).

3. RESULTS AND DISCUSSION

The composition of potato, sweet potato and corn flours, which exerts a considerable influence on the product characteristics is shown in Table 2. P and SP showed significant difference in starch, protein, lipids and fiber contents. Figure 1 shows the appearance of the extruded products.

3.1. Product characteristics

3.1.1. Expansion ratio (ER)

ER is an important index related to the consumer perception of extruded products. The expansion concludes the longitudinal expansion, which is difficult to measure, and the radial expansion determined in Figure 2a as ER. Looking at Figure 2a through Table 1, ER was significantly affected by extrusion conditions. Better expansion can be found at a high temperature and low moisture, for example, P_1 and SP_1 (2.584 mm/mm and 2.35 mm/mm, respectively). Furthermore, similar to the results of Ding *et al.*, (2006), Spinello *et al.*, (2014) and Thymi *et al.*, (2005), a decrease in temperature and an increase in moisture could reduce the ER — a phenomenon which is unsatisfactory in the extrusion industry. During the extrusion process, a high temperature and shear force in the barrel induced order-disorder transformations of starch and bubble nucleation, which meant viscoelastic starch mass was caught and entrapped in the small air bubbles. Then, the pressure difference between the pressure inside the die and the atmospheric pressure led to water evaporation and bubble growth. At a high temperature, starch molecules gelatinized to form a

Table 2. Chemical composition of raw materials

	Starch/%	Protein/%	Lipids/%	Fiber/%	Moisture/%
Potato flour	79.13±1.47^a	8.00±0.04^b	1.36±0.02^c	1.58±0.13^b	6.45±0.67^b
Sweet potato flour	62.50±1.02^c	3.46±0.03^c	3.76±0.03^b	1.93±0.08^a	7.03±0.57^b
Corn flour	77.10±0.55^b	8.28±0.04^a	3.85±0.03^a	1.03±0.07^c	12.10±0.91^a

Values are means ± standard deviations (n = 3).

Means in the same row with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

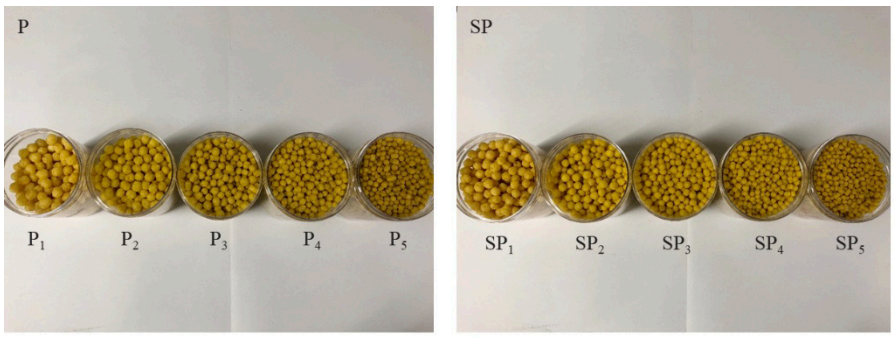


Figure 1. P products (a) and SP products (b) extruded under different conditions. P, potato; SP, sweet potato. The composition of P_{1-5} (SP_{1-5}): corn flour 2400 g (2700 g), P flour 600 g (SP flour 300 g), red palm oil 150 g, sugar 300 g, calcium carbonate 17 g.

stable expanded structure. The thermal degradation of starch led to a lower melting viscosity, accompanied by less resistance to bubble growth, and resulting in larger ER. Moreover, because of the change in amylopectin molecular structure under high moisture content conditions, the melting elasticity reduced, and thus decreased the porosity values and expansion ratio (Thymi *et al.*, 2005).

ER depends not only on extrusion parameters and equipment parameters, but also on the performance of raw materials during extrusion. The ER of SP products was lower than that of P products. On the one hand, the lower starch and higher fiber contents in SP (Table 2) could decrease the ER (Wan *et al.*, 2018); on the other hand, SP contains amylase, which hydrolyzes starch into sugar. Consequently, the existence of sugar may yield poor starch conversion, encourage shrinkage of the bubble walls and cause a reduction in expansion. It has also been reported that extrudability is slightly different among different food raw materials (Corn starch > P starch > SP starch) (Bai *et al.*, 2018; Wan *et al.*, 2018).

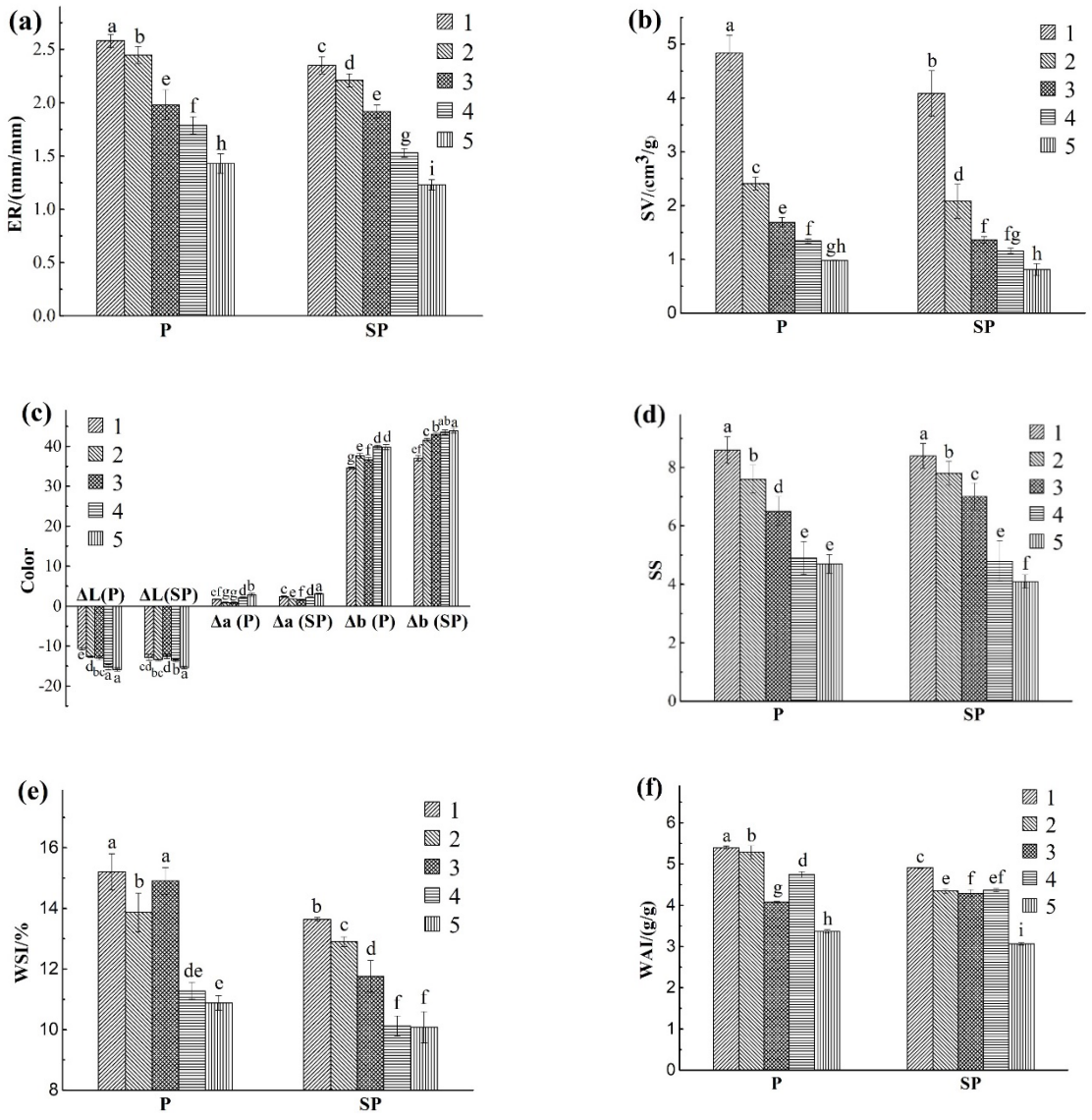


FIGURE 2. Product characteristics of P and SP samples extruded under different conditions. (a) ER, Expansion Ratio (b) SV, Specific Volume (c) Color: ΔL, brightness; Δa, redness; Δb, yellowness (d) SS, Sensory Scores from 10 responses (e) WSI, Water Soluble Index (f) WAI, Water Absorption Index. Values are expressed as Mean ± SD (n = 3). Different letters are significantly different at $P < 0.05$ according to Duncan's test.

3.1.2. Specific volume (SV)

SV, the volume of sample per unit mass, is a measurement of overall expansion. In general, a higher SV results in higher porosity and better texture of the products. Significant differences were observed among the extrusion parameters and starch types. As shown in Figure 2b, the SV of samples ranged from 0.81 to 4.84cm^3/g and products with elevated SV were generated from high temperatures and low moisture. The extrusion of cassava and potato flours showed comparable results (Spinello *et al.*, 2014).

3.1.3. Color

The influence of extrusion variables on the color of the samples was determined (Figure 2c). The variables (temperature and moisture content) displayed a significant effect on the ΔL (lightness), Δa (redness), and Δb (yellowness) of the samples. The ΔL and Δa increased, while Δb decreased as barrel temperature increased (P_1-P_3, SP_1-SP_3), indicating that the product was redder and yellower. However, Δa also increased with the temperature decrease and moisture content increase in P_3-P_5 and SP_3-SP_5. It is known that high temperature and low humidity extrusion processes are conducive to Maillard reaction between free amino groups of protein and carbonyl groups of reducing sugars. The more starch degraded, the more reactive small molecule reducing sugar generated. In a previous study with corn rich in lysine as the main raw material, the two amino groups of lysine enhanced the Maillard reaction (Singh *et al.*, 2007). Owing to the melanoids produced by the Maillard reaction and the tocoquinones formed by the thermal oxidation of tocopherols at a high temperature, the redness and yellowness of P_1 and SP_1 were higher. Besides, the β-carotene in corn, SP and RPO gathered in smaller products prepared by high moisture extrusion. More β-carotene in SP also contributed to higher Δa of products. This color phenomenon was also observed in SP raw materials. In brief, P_1 and SP_1 with preferred expansion and proper color showed better product characteristics.

3.1.4. Sensory characteristics

The maximum and minimum sensory scores corresponded to P_1, SP_1 and P_5, SP_5, respectively (Figure 2d). The sensory properties of extruded snacks are associated with appearance, texture and flavor characteristics. Apart from similar shapes, the main difference in appearance between the samples was color (Figure 1). The texture, which is one of the most important qualities consumers look for, is closely connected to expansion (ER in Figure 2a). Looking at product ER with color in Figure 2 and product appearance in Figure 1, the sensory score gradually decreased with the shrinkage and deepened the color of the products. Furthermore, in Figure 2, SP showed poorer extrusion behaviors and darker color than P, thus making their sensory scores slightly lower. Generally, the results for ER, SV, color and product appearance affirm the changes in the sensory scores in Figure 2.

3.1.5. Water solubility index (WSI) and water absorption index (WAI)

WSI and WAI are the interaction between samples and water. WSI is the solubility of extrudates in water, characterizing food digestibility; while WAI is the volume of swelled starch in water. Higher WAI improves handling characteristics and prevents the drying of products during storage (Ding *et al.*, 2006). It was observed that WSI and WAI decreased obviously with a decrease in expansion (Figure 2e, Figure 2f), which was mainly attributed to the less conversion of starch at a low temperature. A similar observation was reported by Ding *et al.,* (2006) and Ali *et al.,* (2017). High temperature extrusion aggravated starch degradation. Soluble polysaccharides and small molecule sugars were released, causing a positive effect on WSI. In addition, crystalline starch is transformed into amorphous starch at high temperatures, inducing the binding of hydroxyl groups with water, thereby resulting in high WAI (Spinello *et al.,* 2014). Expectedly, the process performance of SP was lower than P in terms of WSI and WAI based on a lower starch content. The changes in ER, SV, WSI and WAI are mainly related to starch conversion in materials as evidenced in this study.

3.2. Oil properties

3.2.1. β-Carotene

A considerable amount of β-carotene remained in the samples after extrusion. Presented in Table 3, the β-carotene content in RPO before extrusion

TABLE 3. β-Carotene, squalene, tocotrienols and tocopherol contents **(mg/kg oil), PV (meq O_2/kg oil), and *p*-AV, IP (h) in oils extracted from samples**

Samples	β-Carotene	Squalene	Tocotrienols				Tocopherols				PV	p-AV	IP
			δ	β/γ	α	Total	δ	β/γ	α	Total			
RPO	524.3±1.0^{a}	185.8±5.3^{a}	56.3±3.3^{a}	218.9±4.9^{a}	73.0±2.2^{a}	348.1±10.4^{a}	33.3±1.1^{b}	42.1±2.2^{a}	224.5±8.8^{a}	299.8±8.2^{a}	9.3±0.1^{a}	0.9±0.0^{k}	41.4±1.3^{a}
P_1	414.7±2.5^{h}	156.9±4.1^{d}	33.3±2.8^{c}	123.2±2.5^{f}	56.1±1.5^{e}	212.6±6.7^{de}	21.2±0.5^{f}	20.4±0.9^{e}	93.3±4.4^{f}	134.0±5.8^{f}	3.9±0.1^{h}	3.4±0.0^{a}	11.4±0.9^{g}
P_2	469.2±2.1^{g}	152.3±2.2^{e}	27.2±3.3^{d}	129.4±3.8^{d}	52.0±3.3^{f}	208.6±10.4^{ef}	21.5±0.1^{f}	20.7±1.8^{e}	110.0±8.2^{d}	152.3±10.1^{e}	3.9±0.1^{h}	3.1±0.0^{c}	12.6±0.4^{g}
P_3	498.5±4.4^{b}	150.9±1.7^{ef}	27.0±1.7^{de}	123.2±5.2^{ef}	52.8±1.0^{f}	202.9±7.8^{f}	24.7±1.2^{e}	23.8±1.5^{d}	110.3±2.6^{d}	158.8±5.2^{e}	4.3±0.1^{g}	2.7±0.0^{d}	23.1±3.8^{d}
P_4	482.2±2.5^{de}	142.2±3.9^{h}	26.3±1.0^{de}	115.2±3.1^{g}	48.7±0.7^{g}	190.2±4.9^{g}	25.1±1.3^{e}	24.1±1.9^{d}	106.4±5.8^{de}	155.7±8.9^{e}	4.5±0.2^{de}	2.4±0.0^{g}	16.5±0.5^{ef}
P_5	468.0±4.9^{g}	144.4±2.4^{gh}	24.0±0.6^{f}	102.3±1.4^{h}	43.9±0.1^{h}	170.2±2.1^{h}	24.9±1.3^{e}	23.9±1.0^{d}	101.1±4.6^{e}	149.9±7.0^{e}	4.7±0.1^{c}	2.4±0.0^{f}	29.0±0.5^{b}
SP_1	468.5±1.6^{g}	147.5±2.4^{fg}	24.9±3.6^{ef}	112.9±4.1^{g}	46.6±2.2^{g}	184.4±9.9^{g}	21.3±0.1^{f}	20.6±1.3^{e}	86.5±6.1^{f}	128.4±7.4^{f}	4.6±0.1^{cd}	3.2±0.0^{b}	11.4±0.7^{g}
SP_2	493.3±0.3^{c}	180.5±5.5^{b}	32.5±1.6^{c}	147.8±5.3^{b}	65.4±1.7^{b}	245.7±8.7^{b}	38.6±4.8^{a}	36.9±3.8^{b}	200.1±10.4^{b}	275.6±18.9^{b}	4.3±0.1^{g}	2.0±0.0^{h}	15.1±0.2^{f}
SP_3	484.0±7.9^{d}	160.3±1.4^{cd}	34.6±2.6^{c}	140.0±6.4^{c}	61.9±3.5^{c}	236.4±12.5^{c}	31.3±1.7^{c}	30.0±3.8^{c}	195.1±8.9^{b}	256.3±14.5^{c}	4.3±0.2^{fg}	1.1±0.0^{i}	26.2±4.1^{c}
SP_4	478.5±2.5^{f}	162.6±2.2^{c}	33.0±4.3^{c}	129.5±5.7^{d}	58.9±3.9^{d}	221.4±12.9^{d}	30.0±1.2^{c}	28.8±3.0^{c}	218.9±8.7^{a}	277.7±12.8^{b}	4.4±0.1^{ef}	1.0±0.0^{j}	25.9±0.4^{c}
SP_5	479.3±0.8^{ef}	177.9±0.9^{b}	39.2±1.6^{b}	127.4±2.7^{de}	53.3±3.1^{f}	220.0±7.4^{d}	27.3±2.2^{d}	26.2±3.2^{d}	179.9±2.4^{c}	233.5±7.7^{d}	4.9±0.4^{b}	2.5±0.0^{e}	18.0±0.1^{e}

Results are expressed as mean ± standard deviation (n = 3).

Means in the same row with different letters are significantly different according to Duncan's multiple range test ($P < 0.05$).

The isomers of tocotrienols and tocopherols are shown as δ, β/γ, α. PV, Peroxide Value; *p*-AV, *p*-Anisidine Value; IP, Induction Period. RPO, red palm oil, is the oil before extrusion; $P_{1\text{-}5}$, potato and $SP_{1\text{-}5}$, sweet potato, represent oils extracted after extrusion. The composition of $P_{1\text{-}5}$($SP_{1\text{-}5}$): corn flour 2400 g (2700 g), P flour 600 g (SP flour 300 g), red palm oil 150 g, sugar 300 g, calcium carbonate 17 g.

was 524.3 mg/kg, after extrusion it was 414.7-498.5 mg/kg, and the retention rate was 79.1-95.1%. The concentration of β-carotene in the extruded food was higher than that of baked food. RPO has also been reported in other functional foods to improve malnutrition in children (Sidhu *et al.*, 2004). Therefore, it is an indisputable fact that red palm oil helps to improve the nutritional quality of foods.

However, the β-carotene retention properties of the samples were quite different from product properties. For instance, P_1 and SP_1 had better product properties; while their β-carotene contents were lower than those of P_3 and SP_2 (Table 3). The phenomenon can be explained such that the extrusion temperature affected the degradation and oxidation of the highly unsaturated β-carotene. A lower retention of carotene at high temperatures was also suggested by Qian *et al.*, (2012). Moreover, the actual dissolved oxygen content in the materials would increase with the increase in water. According to Buton *et al.*, (1984), the oxidation of carotene accelerates under high oxygen partial pressure. Hence, water was the main factor that affected the loss in β-carotene when the temperature was below 100 ºC and the water flow rate was above 70 mL/min (P_3-P_5, SP_3-SP_5) (Table 3). Furthermore, P samples showed better product characteristics, but lower β-carotene content because of the inherent β-carotene in SP.

Extruded products with RPO have potential as a functional food alternative for the intake of β-carotene, since it is a precursor of vitamin A, which cannot be synthesized in the human body. Due to the rupture of the links with other macromolecules, thermal treatment is beneficial for the absorption of carotenoids. Oleic acid, which is abundant in RPO also increases the accessibility and bioavailability of β-carotene (Victoria-Campos *et al.*, 2013). For provitamin A activity, β-carotene plays an important role in cell differentiation, embryonic development, vision and prevention of certain types of cancer (Qian *et al.*, 2012).

3.2.2. Squalene

Similar to β-carotene, the squalene content in the SP samples was also higher than in the P samples, which was in the range of 147.5-180.5 mg/kg, and the retention rate was 76.5-97.2% (Table 3). By contrast, the effect of extrusion conditions on squalene was not exactly the same as the effect on β-carotene. Squalene in extruded products showed a general trend of descending first and then ascending. According to Ogrodowska *et al.*, (2014), high temperature and high-pressure treatments contributed to squalene losses, which is consistent with the results obtained in this research. The extrusion processing system is complex and changeable. Therefore, the mechanism is far from clear.

Using RPO as the source of squalene may increase the content in squalene in the human body because squalene in food can be well absorbed, with the absorption rate reaching 60-85% (Reddy and Couvreur, 2009). In addition, squalene as a nutritional substance is attributed to the absence of cancer in sharks and the low incidence of human cancer among the population with high squalene intake. The anti-cancer effects of squalene include the protective role against carcinogens of hair-cell leukemia and skin cancer, and synergistic effect of antineoplastic drugs. Moreover, squalene has been shown to have powerful antibacterial and antifungal activity, and to protect against coronary heart disease (Reddy and Couvreur, 2009).

3.2.3. Tocotrienols and tocopherols

Tocotrienols and tocopherols in RPO before extrusion and in different oil samples extracted from extruded snacks were identified (Table 3). The contents in tocotrienols and tocopherols in RPO without extrusion were 348.1 mg/kg and 299.8 mg/kg, respectively, and their sum was 647.9mg/kg. Extrusion caused a loss in these substances as evidenced in the samples. Among the several active isomers, the highest contents and the most serious losses were observed in β/γ-tocotrienol and α-tocopherol. Such phenomenon was in agreement with those reported for baking, deep-frying, and other thermal processing. Hamid *et al.*, (2014), and Schroeder *et al.*, (2006) reported the loss sequence in vitamin E as: γ-tocotrienol > α-tocotrienol ≈ α-tocopherol > δ-tocotrienol. However, the retention of the isomers was not identical in previous reports. The stability of the different isomers is related to concentration, temperature, oil system and other factors (Rossi *et al.*, 2007).

The retention of tocotrienols and tocopherols was under the influence of process parameters. It is obvious that the tendencies of β/γ-tocotrienol, α-tocopherol, and total tocotrienols and tocopherols

resembled β-carotene. The loss in vitamin E was at its maximum at high temperatures and low moisture contents (P_1, SP_1 in Table 3, Table 1). The thermal degradation of vitamin E in oils may be aggravated by the increase in temperature (Hamid *et al.*, 2014). Moreover, the influence of water on the degradation of vitamin E was greater than temperature. High water content caused the loss in vitamin E easily by increasing the fluidity of metal catalysts (Klibanov, 1986). The contents in tocotrienols and tocopherols in products was also related to the formula of raw materials. Tocotrienols and tocopherols in SP products were higher than those in extruded P products (Table 3), which were also consistent with β-carotene. It is easy to conclude that SP samples were more nutritious.

The tocotrienols and tocopherols are efficient antioxidants in edible oil and are major bioactive compounds in vivo. Their presence retards the autoxidation of oils and fats greatly and protects fatty foods from off-flavors. In addition, they possess gene regulatory functions (α-tocopherol), natriuretic, anti-inflammatory, antitumor activities (γ-tocopherol), neuroprotective properties (especially α-tocotrienol), preventive effects on cholesterol biosynthesis, and anticancer effects (δ- and γ-tocotrienol) (Kamaleldin and Appelqvist, 1996). In general, considerable tocotrienol and tocopherol retention in food is beneficial to both food storage and human health.

3.2.4. Peroxide value (PV) and p-anisidine value (p-AV)

PV and *p*-AV were used to evaluate the deterioration of oil in the extrusion process. PV represents the main primary products of oil oxidation, like hydrogen peroxide; while *p*-AV represents the small molecular compounds such as aldehydes and ketones produced by further decomposition of hydrogen peroxide. High PV and *p*-AV indicate high oxidation of oil samples. However, as shown in Table 3, the PV and *p*-AV of RPO after extrusion (4.7 meq O2/kg oil and 3.4, respectively) were quite low, thus implying less deterioration and good applicability of RPO in extrusion.

There were minor differences in PV and *p*-AV among the different samples (Table 3). It was expected that the PV and *p*-AV of oil would increase at high temperatures and low moisture. PV and *p*-AV generally elevate with increases in temperature and processing time (Wan *et al.*, 2018). In addition, as early as 1987, studies have shown that a very small content in water (0.2%) can reduce the catalytic activity of metal ions and benefit the stability of lipids; whereas an increased amount of water may promote the hydrolysis of lipids and accelerate oxidation (Chan, 1987). This result was further supported by the retention of β-carotene, tocotrienols and tocopherols. These substances are considered to be antioxidants in food systems which can inhibit the oxidation chain reaction by reacting with peroxyl radicals. Nevertheless, the reduction in the PV value of RPO-mixed products after extrusion as seen in this study could be attributed to the degradation of peroxides in RPO by heat, which increased the amount of secondary product like *p*-AV (Ekwenye, 2006).

Under the same extrusion conditions, the PV of SP and P products showed no significant difference, yet the *p*-AV of P products was slightly higher than that of SP (Table 3). This difference could be attributed to the different composition of P and SP. P starch or SP starch were about 79 or 62%, respectively; different types and amounts of starch may respond differently to thermal and mechanical energies during extrusion (Wan *et al.*, 2018). RPO extruded with SP might reduce the heat transfer between barrel-oil and the contact of oil with oxygen, leading to the decreased oxidation of SP samples. The oxidation of oil can be influenced by many factors, such as unsaturated fatty acid content, antioxidants, metals and photosensitive substances, as well as the moisture, temperature, and oxygen contents in storage containers (Chan, 1987).

3.2.5. Induction period (IP)

Rancimat assay characterizes the oxidation stability of oils. Higher IP reflects a longer shelf-life of products. At 100 ºC, the IP of RPO was 41.4 h (Table 3). The oxidative stability of RPO was higher than that of many oils as reported by Kowalski *et al.*, (2004). Even though extrusion accelerates the oxidation reaction, resulting in the decline of IP, in this study (Table 3), the IPs of oil after extrusion processing were substantial (11.4-29.0 h).

The RPO extruded with P showed no obvious regularity in IP, but the opposite was seen in the RPO extruded with SP. The IP of SP samples decreased with the increase in temperature and water contents. And water can be the dominant factor when water and temperature change simultaneously. These phenomena are well supported by the changes in

β-carotene, tocotrienol and tocopherol contents, as well as PV and *p*-AV. The samples with high micronutrient retention showed less oxidation and greater stability. Endogenous antioxidant components are considerable factors affecting oil stability (Mba *et al.*, 2015). The existence of double bonds in β-carotene and hydroxyl groups in tocopherols can inhibit lipid oxidation by capturing free radicals. Previous studies have also shown that carotenoids have a synergistic effect with tocotrienols and tocopherols (Schroeder *et al.*, 2006). Thus, RPO is a high-quality oil for thermal extrusion processing.

3.3. Principal component analysis (PCA)

To explore the correlation among the measured properties and samples, PCA was performed (Figure 3). Two components were utilized to explain 78.2% of the total variation. The first PC (PC_1) accounted for 56.55% of the total variation and established positive correlations with SV, WSI, ER, WAI, SS, but negative correlations with Δb. Meanwhile, tocotrienols mainly contributed to the PC_2, with a total variance of 21.7%. Figure 3a shows a score plot of products classified into three groups according to their raw materials and extrusion conditions. Group I, constituted by four P samples (P_2-P_5) showed similar product characteristics and oil properties as compared to group II, constituted by four SP samples (SP_2-SP_5). On the other hand, the third group, constituted by P_1 and SP_1, which are samples extruded at temperatures above 150 °C (Table 1), showed different behavior as compared to other samples extruded at temperatures lower than 150 °C. Moreover, from the loading plot shown in Figure 3b, group II was characterized by higher contents in tocotrienols, tocopherols and squalene but lower SV, WSI, ER, WAI and SS. Group III was identified as having higher levels of ER, SV, WSI, WAI, and SS. P_5 and SP_5 were both characterized by lower oxidative stability indices (PV, *p*-AV) and deeper color (Δa and Δb). The above characteristics are in good agreement with the results in Figure 2 and Table 3. In addition, it is evident that SV was closely related to ER, WSI, WAI, and SS, indicating their contribution to the product characteristics of the samples. Tocotrienols, tocopherols and squalene negatively correlated with PV and *p*-AV, which indicate a decrease in the oxidative stability of samples (Figure 3b). According to the results and the above observations, the extruded products were successfully distinguished from each other based on their product characteristics, micronutrient contents and oxidative stability.

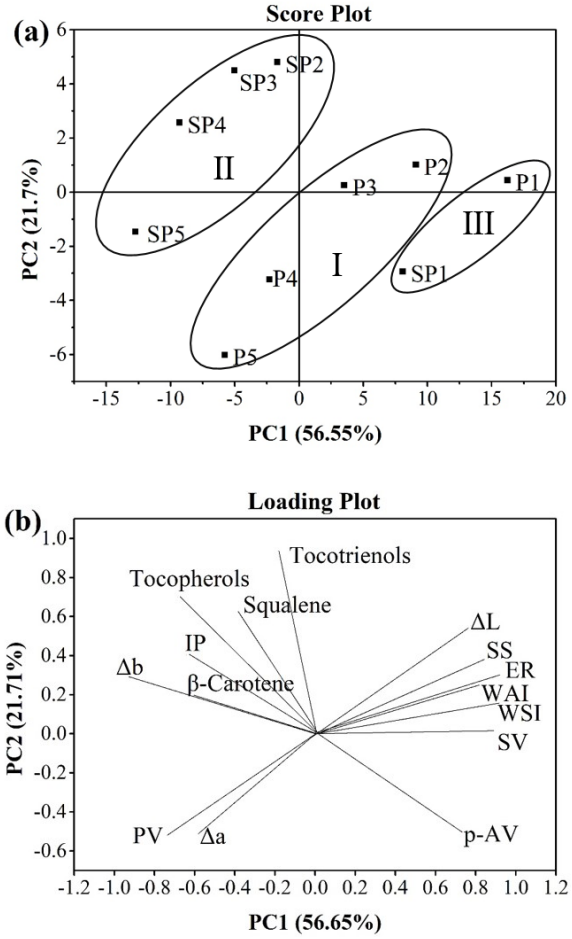


FIGURE 3. Principal component analysis (PCA) of products' score plot (a) and loading plot (b) of P and SP products. PC1, PC2 are principal components 1 and 2. I, II, III are groups of samples classified according to PCA. ER, Expansion Ratio; SV, Specific Volume; SS, Sensory Scores; WSI, Water Soluble Index; WAI, Water Absorption Index; ΔL, brightness; Δa, redness; Δb, yellowness.

4. CONCLUSIONS

This study revealed the product characteristics of extruded snacks as well as micronutrients and oxidative stability changes in extruded RPO produced under different extrusion conditions and types of raw materials, P and SP. The results indicated that superior products can be obtained with high temperature and low moisture extrusion. The optimum extrusion parameters were attributed to P_1 (temperature 151 °C, water velocity 50.8 mL/

min, screw speed 251 rpm, feed frequency 12 Hz). Among the operational variables, water played a decisive role in extrusion. In addition, regardless of the changes in extrusion conditions, P products showed better product characteristics, including ER, WSI, WAI, sensory properties, etc., but lower β-carotene, tocotrienols, tocopherols and oxidative stability than SP products. In this study, extruded P and SP products with RPO showed great expansion behavior, appearance, sensory characteristics, high nutritional value and oxidative stability under the optimized extrusion condition. In general, the findings herein are important for investigating extrusion conditions, increasing variety, improving nutritional quality, assessing applicability and predicting the shelf-life of RPO-P/SP-extruded food.

ACKNOWLEDGEMENTS

The authors thank the Palm Oil Research and Technical Service Institute of Malaysian Palm Oil Board (PORTSIM) for their financial support (PORTSIM 054/2017) and provision of red palm oil, and also Wilmar (Shanghai) Biotechnology R&D Center Co. Ltd. for their technical assistance.

REFERENCES

Ali S, Singh B, Sharma S. 2017. Development of high-quality weaning food based on maize and chickpea by twin-screw extrusion process for low-income populations. *J. Food Process. Eng.* **40**, UNSP e12500. https://doi.org/10.1111/jfpe.12500

AACC. 2002. *Official methods of American Association of Cereal Chemists.* 10th edition, Minnesota, USA.

AOAC. 2000. *Official methods of analysis of Association of Official Analytical Chemists*. 15th edition, Washington DC, USA.

AOCS. 1997. *Official methods and recommended practices of the American Oil Chemists' Society*. 5th edition, Champaign, USA.

Bai J, Peng YJ, Liu L, Li YM, Zhang XF, Jin Y, Zhang Q, Tian X, Guo H. 2018. Extrusion properties and quality properties of different potato materials. *Chin. Food Sci.* **39**, 48-53. https://doi.org/10.7506/spkx1002-6630-201815007

Buton GW, Ingold KU. 1984. beta-Carotene: An unusual type of lipid antioxidant. *Sci.* **224**, 569. https://doi.org/10.1126/science.6710156

Chan H. 1987. *Autoxidation of unsaturated lipids*. Academic Press, London.

Ding QB, Ainsworth P, Plunkett A, Tucker G, Marson H. 2006. The effect of extrusion conditions on the functional and physical properties of wheat-based expanded snacks. *J. Food. Eng.* **73**, 142-148. https://doi.org/10.1016/j.jfoodeng.2005.01.013

Ekwenye UN. 2006. Chemical characteristics of palm biodeterioration. *Biokemistri* **18**, 141-149. https://doi.org/10.4314/biokem.v18i2.56415

Hamid AA, Dek MSP, Tan CP. 2014. Changes of major antioxidant compounds and radical scavenging activity of palm oil and rice bran oil during deep-frying. *Antioxidants* **3**, 502-515. https://doi.org/10.3390/antiox3030502

Kamaleldin A, Appelqvist LA. 1996. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids* **31**, 671-701. https://doi.org/10.1007/bf02522884

Klibanov A. 1986. Enzymes that work in organic solvents. *Chem. Tech.* **6**, 354-359.

Kowalski B, Ratusz K, Kowalska D. 2004. Determination of the oxidative stability of vegetable oils by Differential Scanning Calorimetry and Rancimat measurements. *Eur. J. Lipid Sci. Tech.* **106**, 165-169. https://doi.org/10.1002/ejlt.200300915

Mba OI, Dumont MJ, Ngadi M. 2015. Palm oil: Processing, characterization and utilization in the food industry-A review. *Food Biosci.* **10**, 26-41. https://doi.org/10.1016/j.fbio.2015.01.003

Ogrodowska D, Zadernowski R, Czaplicki S, Derewiaka D, Wronowska B. 2014. Amaranth seeds and products—the source of bioactive compounds. *Pol. J. Food. Nutr. Sci.* **64**, 165-170. https://doi.org/10.2478/v10222-012-0095-z

Olajide TM, Liu T, Liu HA, Weng XC. 2020. Antioxidant properties of two novel lipophilic derivatives of hydroxytyrosol. *Food Chem.* **315**, 126197. https://doi.org/10.1016/j.foodchem.2020.126197

Pan KL, Ji M, Hu MM, OOI C. 2016. Analysis of nutritional components for red palm oil. *Chin. Cereals and Oils* **29**, 79-81.

Qian C, Decker EA, Xiao H. 2012. Physical and chemical stability of beta-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chem.*

132, 1221-1229. https://doi.org/10.1016/j.foodchem.2011.11.091

Reddy LH, Couvreur P. 2009. Squalene: A natural triterpene for use in disease management and therapy. *Adv. Drug Deliver Rev.* **61**, 1412-1426. https://doi.org/10.1016/j.addr.2009.09.005

Rossi M, Alamprese C, Ratti S. 2007. Tocopherols and tocotrienols as free radical-scavengers in refined vegetable oils and their stability during deep-fat frying. *Food Chem.* **102**, 812-817. https://doi.org/10.1016/j.foodchem.2006.06.016

Schroeder MT, Becker EM, Skibsted LH. 2006. Molecular mechanism of antioxidant synergism of tocotrienols and carotenoids in palm oil. *J. Agric. Food Chem*. **54** (9), 3445-3453. https://doi.org/10.1021/jf053141z

Sidhu JS, Al-Hooti SN, Al-Saqer JM, Al-Amiri HA, Al-Foudari M, Al-Othman A, Ahmad A, Al-Haji L, Ahmed N, Mansor IB, Minal J. 2004. Developing functional foods using red palm olein: Pilot-scale studies. *Int. J. Food Prop.* **7**, 1-13. https://doi.org/10.1081/JFP-120022491

Singh S, Gamlath S, Wakeling L. 2007. Nutritional aspects of food extrusion: a review. *Int. J. Food Sci. Tech.* **42**, 916-929. https://doi.org/10.1111/j.1365-2621.2006.01309.x

Spinello AM, Leonel M, Mischan MM, Carmo EL. 2014. Cassava and turmeric flour blends as new raw materials to extruded snacks. *Cienc. Agrotec.* **38**, 68-75. https://doi.org/10.1590/S1413-70542014000100008

Thymi S, Krokida MK, Pappa A. 2005. Structural properties of extruded corn starch. *J. Food Eng.* **68**, 519-526. https://doi.org/10.1016/j.jfoodeng.2004.07.002

Victoria-Campos CI, Ornelas-Paz J, De J, Yahia EM, Failla ML. 2013. Effect of the interaction of heat-processing style and fat type on the micellarization of lipid-soluble pigments from green and red pungent peppers (*Capsicum annuum*). *J. Agr. Food Chem.* **61**, 3642-3653. https://doi.org/10.1021/jf3054559

Wan LT, Li L, Jiao WJ, Mao LL, Li B, Zhang X. 2018. Effect of barrel temperature and moisture content on the composition and oxidative stability of extruded palm oil in an oil-starch model system. *Lwt-Food Sci. Technol.* **98**, 398-405. https://doi.org/10.1016/j.lwt.2018.08.019

Wichchukit S, O'Mahony M. 2015. The 9-point hedonic scale and hedonic ranking in food science: some reappraisals and alternatives. *J. Sci. Food Agric.* **95**, 2167-2178. https://doi.org/10.1002/jsfa.6993

Yao SW, Xie SY, Jiang LZ, Li L. 2017. Effect of dandelion extract, sucrose and starter culture on the viscosity, water-holding capacity and pH of plain yogurt. *Mljekarstvo* **67**, 305-311. https://doi.org/10.15567/mljekarstvo.2017.0408

Zhang H, Xu F, Wu Y, Hu HH, Dai XF. 2017. Progress of potato staple food research and industry development in China. *J. Integr. Agric.* **16**, 2924-2932.

GRASAS Y ACEITES 72 (2)
April-June 2021, e413
ISSN-L: 0017-3495
http://grasasyaceites.revistas.csic.es

INFORMATIVE NOTE

Captive fatty acids of fresh olive oils?

J.A. Cayuela-Sánchez ✉

Department of Biochemistry and Molecular Biology of Plant Products.

Instituto de la Grasa, CSIC, Universidad Pablo de Olavide, 46, Crtra. Utrera, Km. 1, 41013
✉Corresponding author: jacayuela@ig.csic.es

Submitted: 04 May 2020; Accepted: 06 October 2020; Published online: 10 June 2021

SUMMARY: Olive oil is mainly made up of triglycerides. It is well known that olive oil contains free fatty acids, the proportion of which is variable, depending on the extent of the hydrolysis of triglycerides. Besides, globular structures have been reported in cloudy virgin olive oils. The pseudo-walls of these globules would be constituted by amphiphilic molecules, while fatty acids are amphiphilic. This brief review aims to inform on the importance of the possible interaction of 'free' fatty acids of veiled virgin olive oils, as structural units in the pseudo-wall of the colloidal globules, already reported. The binding of fatty acids to the colloidal globules can mean they are not free in the olive oil. They could be 'captive' in said pseudo-walls, thus exerting less influence on the perception of acidity by the consumer or taster of the olive oils. The official method of analysis of olive oil acidity cannot detect this effect. This may suppose that functional acidity is lower than the acidity values determined by chemical analysis in cloudy virgin olive oils.

KEYWORDS*: Captive fatty acids; Colloid; Colloidal globules; Free fatty acids; Veiled virgin olive oil.*

RESUMEN: *¿Ácidos grasos "cautivos" de los aceites de oliva frescos?* El aceite de oliva está compuesto principalmente de triglicéridos. Es bien sabido que contiene ácidos grasos libres, cuya proporción es variable, dependiendo del grado de hidrólisis de los triglicéridos. Se han informado la existencia de estructuras globulares en el aceite de oliva virgen coloidal. Las pseudo-paredes de estos glóbulos estarían constituidas por moléculas anfifílicas, siendo anfifílicos los ácidos grasos. El objetivo de esta breve revisión es informar de la importancia de la posible interacción de los ácidos grasos 'libres' de los aceites de oliva virgen coloidales, como unidades estructurales en la pseudo-pared de los glóbulos coloidales referidos. La unión de los ácidos grasos a los glóbulos coloidales puede significar que no están libres en el aceite de oliva. Podrían estar "cautivos" en dichas pseudo-paredes ejerciendo menos influencia en la percepción de acidez por parte del consumidor o catador de los aceites de oliva. El método oficial de análisis de acidez del aceite de oliva no puede detectar este efecto. Esto puede suponer que la acidez funcional es inferior a los valores de acidez determinados por análisis químico en aceites de oliva virgen coloidales.

PALABRAS CLAVE*: Aceite de oliva sin filtar; Ácidos grasos cautivos; Ácidos grasos libres; Coloide en rama; Glóbulos coloidales.*

Citation/Cómo citar este artículo: Cayuela-Sánchez JA. 2021. Captive fatty acids of fresh olive oils?. *Grasas Aceites* **72** (2), e413. http://grasasyaceites.revistas.csic.es

1. INTRODUCTION

Olive oils (OO), as other vegetable oils, are mainly made up of triglycerides (between 98% and 99%). Their content in free fatty acids is well known (Barjol, 2013). The proportion of free fatty acids of OO is variable, depending on the extent of the hydrolysis of triglycerides. Thus, the free fatty acids in vegetable oils are generally considered as those that do not form part of the triglycerides. Olive oil free acidity is one of the major quality features of virgin olive oils. In fact, according to the standards in force of the European Commission (1991), the classification into Extra Virgin Olive Oil class (EVOO) requires the free acidity to be lower than 0.8%. The class EVOO also requires the fruity sensory attribute to be perceptible, and for it to be free of organoleptic defects. The product without these features but with free acidity up to 2%, belongs to the class VOO. An acidity value higher than 2% implies that the product is to be classified as lampante olive oil, which should be refined. The official method of analysis of olive oil's free acidity (European Commission, 1991) is a volumetric titration. Briefly, 4 to 6 g of olive oil are weighed into 250 mL wide-mouth Erlenmeyer flasks, and 50-mL ethyl alcohol: ethyl ether at 1:1 and a few drops phenolphthalein are added, and it is then neutralized with NaOH 0.1 N until it turns a pink color, and expressed as a percentage of oleic acid. Thus, olive oil acidity is considered to be caused by the fatty acids liberated from triglycerides by hydrolysis processes, and expressed as the analyzed value according to the method explained above.

It is interesting to highlight that the physical state of cloudy virgin olive oil (VOO) is due to a phase formed by microscopic droplets of water in meta-stable dispersion (Lercker *et al*., 1994). Moisture spreads homogeneously in the major mass of triglyceride liquid, making it an emulsion colloid. Besides, VOO has another phase formed by microscopic solid particles, thus it has been described as a sol-emulsion colloid (†Gómez-Herrera, 2007). In this article, we explain how the fatty acids in veiled olive oils could not be free in the triglyceride mass, and that the functional free acidity of these olive oils could be lower than the acidity determined through official analysis.

2. CAPTIVE FATTY ACIDS IN VEILED OLIVE OILS?

A study on the influence of the scattered matter in veiled olive oils on their shelf-life reported that the free fatty acids precipitated with the residue or sediment in the oil (Lercker *et al*., 1994). The cited study related the precipitate of the solid particles contained in the olive oils to the attraction of opposite electrical charges. The same study pointed out that the scattered matter dampened the acidity, at the same time exerting an antioxidant effect.

Together with this, there are several studies on the conservation of alternatively cloudy or filtered olive oils. The literature agrees that changes in the acidity during the period of olive oil storage are greater in veiled olive oils than in the filtered ones (Tsdimidou *et al*., 2005; Fregapane *et al*., 2006; Stefanoudaki *et al*., 2010). The literature establishes that free fatty acids have a prooxidant effect (Frega *et al*., 1999), and that high acidity exerts a negative influence on the stability of olive oil (Ciafardini, *et al.,* 2006; Frega *et al*., 1999). The referred studies concur that a slightly better oxidative stability index (OSI) is determined for cloudy olive oils. Frega *et al*. (1999) reported that the suspended-dispersed material of cloudy virgin oil exerted a positive effect on oxidative stability. Moreover, the same authors found a higher antioxidant effect of the dispersed particles compared to the suspended particles. This last study proved the olive oil quality decay was significantly delayed by removing the scattered matter. The authors pointed out the possibility of an effect from the higher surface to dimension ratio of the dispersed particles compared to the suspended particles. It is possible to think the authors referred to the 'suspended' particles as those with little stability among all participating in the dispersion. Nevertheless, the study cited lacks a clear description of what particles are referred to by the concepts 'suspended' and 'dispersed'. It is also interesting to consider that, according to Chaiyasit *et al*., (2007), the interface of association colloids is a likely site of oxidation reactions since many prooxidants and antioxidants are also surface active and thus would concentrate at the water-lipid interface.

A recent microscopy study (Cayuela-Sánchez and Caballero-Guerrero, 2019) revealed that the major

part of the spread matter in cloudy virgin olive oil is formed by microscopic droplets of water, while solid particles seem to be contained within an aqueous medium inside colloidal globules (Figure 1). The name of reverse micelles does not seem appropriate for these globules, because their size, even larger than 15 μm, is too large for it. The diameter of reverse micelles ranges from 7 10^{-4} μm to 25 10^{-4} μm (Amararene *et al*., 1997), thus quite smaller. The referred solid particles were reported to show a wide diversity both in shape and size. Besides, they were observed in motion. The peculiar movement of said solid particles inside the colloidal globules was recorded in various video sequences (Cayuela-Sánchez and Caballero-Guerrero, 2019). The authors first related the observed movement to the possible physical influence of electrostatic charges. However, the observed particles' agitation clearly matches the Brownian movement according to the literature (Einstein, 1905; Smoluchowski, 1906; Einstein, 1956). This physiochemical effect is observed particularly in emulsions. The major interest of this movement of the solid particles in olive oil colloidal globules is that it confirms the hypothesis the internal content of the colloidal globules is aqueous. This movement of particles is not observed in the mass of veiled olive oil, formed largely by triglycerides, outside the globules. Albert Einstein explained in detail (Einstein, 1905) how the movement Brown had observed in pollen was the result of the particles being pushed by individual water molecules. Briefly explained, the notorious scientist attributed the movement to the atomic bombing force, whose direction is constantly changing. Therefore, the observed movement could exist independently from the microscopy illumination. However, artificial light should provide additional excitation because this physical phenomenon is temperature-dependent (Seddig, 1908). Thus, the heat provided by the light used for microscopy observation could be responsible for most of the observed movement.

The suspended matter identified by Frega *et al*., (1999) probably relates to these colloidal globules, despite such globular structures containing water was not described. Moreover, Frega *et al*., (1999) related the amino groups of lignin, derived from the olive nut, as primarily accountable for the cloudy material. However, the study did not consider the possibility that microscopic droplets of water are directly responsible for the opalescence of veiled olive oils. The referred authors hypothesized that after reacting with FFA, the amino groups may liberate the phenolic groups that were previously bonded to hydrogen, a state in which they were not able to display any antioxidant activity. Frega *et al*., (1999) related this to the observation that FFA bonded to the disperse particles and precipitated with them as a brown colored residue, as reported in a previous study (Lercker *et al*., 1994). However, these studies did not consider that FFA may participate in the colloidal globules reported (Cayuela-Sánchez and Caballero-Guerrero, 2019), among the structural units forming their pseudo-wall (perceptible in Figure 1),

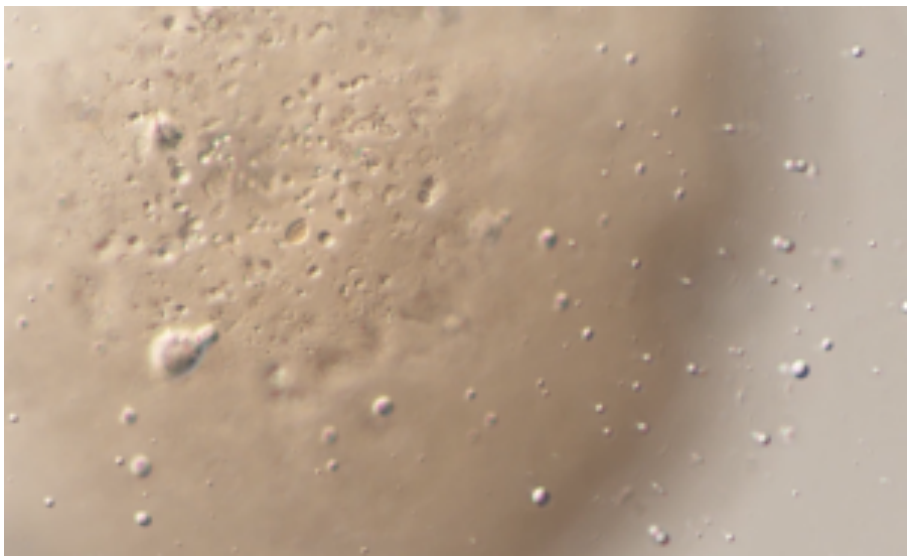


Figure 1. Microscopy focus inside a colloidal globule of fresh virgin olive oil.

as the present study proposes. This last hypothesis agrees that FFA precipitate with the solid residue noted by Lerker *et al*., (1994); although a different scenario for the modulation of the acidity of veiled virgin olive oils can be shown. Frega *et al*., (1999) inferred that the effects from spread matter are so positive that avoidance of oil filtration is desirable in order to extend the olive oil's shelf-life. However, this conclusion should be assessed considering that olive oil filtration implies a drastic removal of moisture, and the elimination of water soluble phenolics along with it. (Bakhouche *et al*., 2014). The phenolic compounds which provide the highest antioxidant activity in olive oil, such as hydroxytyrosol and tyrosol, are the most water-soluble ones (Capasso *et al*., 1992; Bakhouche *et al*., 2014). Therefore, the filtration of virgin olive oil implies a drastic loss in its antioxidant power, with negative influence both on its oxidative stability and nutritional benefits. It is advisable to consider all of the above to decide the most suitable handling of olive oil according to its destination.

The structure and dynamics of colloidal dispersions in veiled virgin olive oil (VOO) have been the subject of a study (Papadimitriou *et al.,* 2013). The authors used confocal microscopy and three different dispersive techniques for characterizing samples of turbid VOO, among which the best was the "Small Angle Light Scattering Apparatus" (SALSA). The SALSA data showed a colloid diameter range from 1.5 to 14 μm. The authors indicated that the resolution limit for the method used was 15 μm, with higher colloids not being detected. Some new details concerning colloidal globules found in olive oil have been reported recently (Cayuela and Caballero-Guerrero, 2019). These colloidal globules are formed by amphiphilic molecules, among which proteins, sugar glycosides, and glycoproteins are found, according to the contents reported for fresh olive oils (Koidis and Boskou, 2006; Lercker *et al*., 1994), and to organic fragments of diverse complexity. It is important to note that free fatty acids, which determine olive oil acidity, are also amphiphilic. The shorter their chain and the greater their unsaturation, the greater their affinity for water (Nelson and Cox, 2017). Total unsaturated fatty acids in olive oils range from 77% to 88% and monounsaturated fatty acids averaging around 75% (García-González *et al*., 2013). However, up to now, the relation of fatty acids to the colloidal globules of olive oil has not been characterized. Here is a proposal on the hypothesis to a link of free fatty acids to the pseudo-wall of the colloidal globules, suggesting that adsorption of free fatty acids in the interface of water globules may result in a decrease in their chemical reactivity.

3. EVOLUTION OF THE ACIDITY OF VEILED OLIVE OILS

The literature on the evolution of acidity in the conservation of veiled and filtered olive oils (Tsdimidou *et al*., 2005; Fregapane *et al*., 2006; Stefanoudaki *et al*., 2010) is consistent with the possible link of 'free' fatty acids to the globules as amphiphilic molecules, as explained below. Their binding to the pseudo-walls of such globules may involve some restraint of their chemical reactivity. This hypothesis is consistent with a brief report on the idea that the scattered matter of veiled oil drops its acidity level (Lercker *et al*., 1994), although these referred authors did not give reasons.

The segregation of the molecules framing the pseudo-wall of the colloidal globules may explain the subsequent decantation of water and sedimentation of solids. This is consistent with the slight increase in acidity noted during the storage of cloudy olive oils (Tsdimidou *et al*., 2005; Fregapane *et al*., 2006; Stefanoudaki *et al*., 2010). Also, according to our microscopy study, the solid particles seem to lodge inside the colloidal globules, but not spread into the triglyceride matrix. Under microscopy observation, the repulsion exerted by the triglyceride mass seems to force the droplets of water through the amphiphilic compounds of the pseudo-wall, towards the inside of the globules. Therefore, the colloidal globules collapse, due to the coalescence of microscopic droplets of water is the logical necessary outcome. Thus, the globules collapse implies the disassembly of their amphiphilic compounds. The solid particles previously suspended in a watery medium inside the colloidal globules must also be released during the proves. Consequently, the solid matter may begin the process of precipitation. From these results, a logical hypothesis is that the free fatty acids released from the pseudo-walls from the collapse of the globules, then begin exerting their characteristic chemical activities.

4. CONCLUSIONS

Colloidal globules have been reported in forming part of the sol-emulsion colloid characteristic of

unfiltered virgin olive oils, as well as in the product before a rest period in tanks. In this study it is hypothesized that the adsorption of free fatty acids on the pseudo-wall of these globules may reduce the functional olive oil acidity. Therefore, the fatty acids resulting from the triglyceride hydrolysis, are not free in the veiled olive oil but 'captive' in the pseudo-walls. It is reasonable that this fact can reduce its chemical activity, as may be the case with its prooxidant activity. Thusly, it is logical to believe that when the fatty acids adhere to the globules pseudo-walls, they may have less influence on the perception of acidity by the consumer of these cloudy virgin olive oils. The official analysis method of olive oil acidity can't detect this last effect since it is a titration test. If this is true, veiled fresh virgin olive oils can offer natural modulation of their acidity, while preserving said physical state. To the best of our knowledge, these ideas have yet to be considered in scientific literature.

ACKNOWLEDGMENTS

This work has been produced without specific funding. The author would like to thank the Spanish Council for Scientific Research for supporting their activity as staff. The author sincerely thanks Mrs. Belén Caballero-Guerrero, responsible for the Unity of Microbiology at the Instituto de la Grasa (CSIC), for the microscopy study. And finally, the author would like to thank Dr. Eddy Plasquy, producer of the virgin olive oil brand 'Del Cetino', for his commentary on the Brownian movement characteristic of emulsions.

5. REFERENCES

Amararene A, Gindre M, Le Huérou JY, Nicot C, Urbach W, Waks M. 1997. Water Confined in Reverse Micelles: Acoustic and Densimetric Studies. *J. Phys. Chem. B* **101**, 10751-10756. https://doi.org/10.1021/jp972718f

Bakhouche A, Lozano-Sánchez J, Ballus CA, Martínez-García M, González Velasco, M, Olavarría-Govantes A. 2014. Monitoring the moisture reduction and status of bioactive compounds in extra-virgin olive oil over the industrial filtration process. *Food Control* **40**, 292-299. https://doi.org/10.1016/j.foodcont.2013.12.012

Barjol JL. 2013. Introduction, in Aparicio R, Harwood J (Eds.) *Handbook of Olive Oil. Analysis and Properties, second ed.*, Springer, New York, pp. 1-17. https://doi.org/10.1007/978-1-4614-7777-8_1

Capasso R, Evidente A, Scognamiglio F. 1992. A simple thin layer chromatographic method to detect the main polyphenols occurring in olive oil vegetation waters. *Phytochem. Analysis* **3** (6), 270–275. https://doi.org/10.1002/pca.2800030607

Cayuela-Sánchez JA, Caballero-Guerrero B. 2019. Fresh extra virgin olive oil, with or without veil. *Trends Food Science Tech.* **83**, 78–85. https://doi.org/10.1016/j.tifs.2018.11.014

Ciafardini G, Zullo BA, Iride A. 2006. Lipase production by yeasts from extra virgin olive oil. *Food Microbiol.* **23** (1), 60–67. https://doi.org/10.1016/j.fm.2005.01.009Chaiyasit W, Elias RJ, McClements DJ, Decker EA. 2007. Role of physical structures in bulk oils on lipid oxidation. *Crit Rev Food Sci Nutr.* **47**, 299-317. https://doi.org/10.1080/10408390600754248

Einstein A. 1905. Über die von der molekularkinetischen Theorie der Wärme geforderte Bewegung von in ruhenden Flüssigkeiten suspendierten Teilchen. *Ann. Phys.* **17**, 549. https://doi.org/10.1002/andp.19053220806

Einstein A. 1956. Investigations of the Theory of Brownian Movement. Dover Publications, 119 pp.

European Commission, 1991. Commission Regulation (EEC) Ner 2568/91 of 11 July 1991, on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. 128 pp.

Frega N, Mozzon M, Lercker G. 1999. Effects of free fatty acids on oxidative stability of vegetable oil. *J. Am. Oil Chem. Soc.* **76**, 325. https://doi.org/10.1007/s11746-999-0239-4

Fregapane G, Lavelli V, Leon S, Kapuralin J, Salvador MD. (2006). Effect of filtration on virgin olive oil stability during storage. *Europ. J. Lipid Sci. Tech.* **108**, 134–142. https://doi.org/10.1002/ejlt.200501175

García-González DL, Infante-Domínguez C, Aparicio R. 2013. Tables of olive oil chemical data, in Aparicio R, and Harwood J (Eds.) *Handbook of olive oil: Analysis and properties*, Springer, New York, pp. 739–768.

†Gómez- Herrera C. 2007. Matter transfer during virgin olive oil elaboration. *Grasas Aceites* **58** (2), 194–205. https://doi.org/10.3989/gya.2007.v58.i2.85

Koidis A, Boskou D. 2006. The contents of proteins and phospholipids in cloudy (veiled) virgin olive oils. *Europ. J. Lipid Sci. Tech.* **108**, 323–328. https://doi.org/10.1002/ejlt.200500319

Lercker G, Frega N, Bocci F, Servidio G. 1994. "Veiled" extra-virgin olive oils: Dispersion response related to oil quality. *J. Am. Oil Chem. Soc.* **71**, 657–658. https://doi.org/10.1007/s11746-999-0239-4

Nelson DL, Cox MM. 2017. Lehninger principles of biochemistry. 7th. ed. W. H. Freeman and Co. New York. 1340 pp.

Papadimitriou V, Dulle M, Wachter W, Sotiroudis TG, Glater O, Xenakis A. 2013. Structure and dynamics of veiled virgin olive oil: Influence of production conditions and relation to its antioxidant capacity. *Food Biophys*. **8** (2), 112–121. https://doi.org/10.1007/s11483-013-9286-3

Seddig M. 1908. The measurement of temperature dependency of Brown's molecular movement. *Physik. Zeitschrift* **9**, 465-468.

Smoluchowski M. 1906. Zur kinetischen Theorie der Brownschen Molekularbewegung und der Suspensionen. *Ann. Phys.* **21** (14), 756-780. https://doi.org/10.1002/andp.19063261405

Stefanoudaki E, Williams M, Harwood J. 2010. Changes in virgin olive oil characteristics during different storage conditions. *Europ. J. Lipid Sci. Tech*. **112**, 906–914. https://doi.org/10.1002/ejlt.201000066

Tsdimidou MZ, Georgiou A, Koidis A, Boskou D. 2005. Loss of stability of ''veiled'' (cloudy) virgin olive oils in storage. *Food Chem.* **93**, 377–383. https://doi.org/10.1016/j.foodchem.2004.09.033

GRASAS Y ACEITES 72 (2)
April-June 2021, e414
ISSN-L: 0017-3495
http://grasasyaceites.revistas.csic.es

Lista de evaluadores 2020 / Reviewers List 2020

El Consejo de Redacción de GRASAS Y ACEITES agradece sinceramente la contribución de los siguientes científicos a la revisión de manuscritos durante el año 2020.
GRASAS Y ACEITES Editorial Board gratefully acknowledges the following scientists for reviewing manuscripts during 2020.

Abdul Aziz, A.R.	Chemical Engineering, University of Malaya, Malaysia.
Abreu-Naranjo, R.	Universidad Estatal Amazónica. Pastaza, Puyo, Ecuador.
Aguirrezabal, L.	Universidad Nacional de Mar del Plata. Mar de Plata, Argentina.
Albuquerque, MRJR	Ciências Exatas, Universidade Estadual Vale Do Acaraú, Brazil.
Ali, J.A.	Petroleum Engineering, Soran University, Kurdistan Region, Iraq.
Almoselhy, R.I.M.	Food Technology, Agricultural Research Center. Giza, Egypt.
Alonso Salces, R.M.	Consejo Nal. Invest. Científicas y Técnicas. Buenos Aires, Argentina.
Altemimi, A.B.	Food Science College of Agriculture, University of Basrah, Iraq.
Antoniassi, R.	Embrapa Agroindústria de Alimentos. Rio de Janeiro, Brazil.
Ariaii, P.	Ayatollah Amoli Branch, Islamic Azad University. Amol, Iran.
Arroyo-López, F.N.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Ates, M.	Department of Biotechnology, Munzur University. Tunceli, Turkey.
Aubourg Martínez, S.	Instituto de Investigaciones Marinas, CSIC. Vigo, Spain.
Avellone, G.	Mass Spectrometry, Università degli Studi di Palermo, Italy.
Badr, A.N.	Food Toxicology, National Research Centre. Dokki, Cairo, Egypt.
Baêsso, R.M.	Laboratory Ultrasound, Nal. Inst. of Metrology. Rio Janeiro, Brazil.
Baranauskiene, R.	Kaunas University of Technology. Kaunas, Lithuania.
Bassegio, D.	Universidade Estadual do Oeste do Paraná. Cascavel, Brazil.
Basyuni, M.	Forestry, Univ. Sumatera Utara. Medan, North Sumatra, Indonesia.
Ben Mansour-Gueddes, S.	Olive Tree Institute. Sousse, Tunisia.
Benítez Cabello, A.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Białek, A.	Department Biotechnology and Nutrigenomics. Magdalenka, Poland.
Bleve, G.	Institute of Science of Food Production. Lecce, Italy.
Bodoira, R.M.	Química Orgánica y Productos Naturales. Córdoba, Argentina.
Boukandoul, S.	Agroressources Food Technology, University of Bejaia, Algeria.
Brenes Balbuena, M.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Brenna Elisabetta, M.	Politecn Milan, Chim Mat & Ingn Chim Giulio Natta. Milan, Italy.
Brescia, M.A.	Frodi dei prodotti agroalimentari, Laboratorio di Perugia, Italy.
Brito, C.	Univ. de Trás-os-Montes e Alto Douro V.Q. CITAB, Portugal.
Caggia, C.	Agricultural, Food and Environment, University of Catania, Italy.
Calvo, L.	Ingeniería de Materiales, Universidad Complutense de Madrid, Spain.
Cardozo-Filho, L.	Chemical Engineering, Universidade Estadual de Maringá, Brazil.
Carrasco Pancorbo, A.	Analytical Chemistry Sciences. University of Granada, Spain.
Carrillo Terán, W.I.	Ingeniería en Alimentos, Universidad Técnica de Ambato, Ecuador.

Castejón Ferrer, D.	RMN, Universidad Complutense de Madrid, Spain.
Cavalcanti-Oliveira, E.	Dpt. Bioquímica Universidade Federal do Rio de Janeiro, Brazil.
Cevoli, C.	Agricultural and Food, University of Bologna. Cesena, Italy.
Ceylan, Z.	Seafood Processing, Fisheries, Van YüzüncüYıl University, Turkey.
Charrouf, Z.	Faculty of Sciences, Mohammed V University, Maroc.
Chen, H.	Xichang University. Liangshanzhou, Sichuan Province, China.
Çol Ayvaz, M.	Faculty of Art and Science, Ordu University. Ordu, Turkey.
Colaço, A.C.	Universidade Federal do Rio de Janeiro. Rio de Janeiro, Brazil.
Collado-Domínguez, E.A.	Universidad Nacional de Ingeniería, Managua, Nicaragua.
Conde-Innamorato, P.	Instituto Nacional Investigación Agropecuaria. Las Brujas, Uruguay.
Corazza, M.L.	Chemical Engineering, Federal University of Paraná. Curitiba, Brazil.
Correia, C.M.	Universidade de Trás-os-Montes e Alto Douro, Portugal.
Danthine, S.	University of Liège, Gembloux Agro-Bio Tech, Belgium.
Davidovich-Pinhas, M.	Lipids and Soft Matters, Technion Israel Inst. Technology, Israel.
Domaratskiy, Ye.	Kherson State Agrarian and Economic University, Ukraine.
Domínguez, H.	Ingeniería Química Universidade do Vigo. Ourense, Spain.
Dulf, F.V.	Agricultural Sciences Veterinary Medicine Cluj-Napoca, Romania.
El-Hadad, S.S.	National Research Center, Moscow State University. Giza, Egypt.
Eljasik, P.	West Pomeranian University of Technology, Poland.
Elloumi, O.	University of Sfax, Institut de l'Olivier (IO). Sfax, Tunisia.
Espert, M.	Institute of Agrochemistry and Food Technology. Paterna, Spain.
Ezquerra Brauer, J.M.	Investigación y Posgrado Alimentos, Universidad de Sonora, Mexico.
Famiani, F.	Alimentari e Ambientali, Università degli Studi di Perugia, Italy.
Fernández Bolaños, J.M.	Facultad de Química, Universidad de Sevilla, Spain.
Gaeta, S.	Università Del Piemonte Orientale. Novara, Italy.
García García, P.	Instituto de la Grasa, CSIC. Sevilla, Spain.
García González, D.L.	Instituto de la Grasa, CSIC. Sevilla, Spain.
García González, M.C.	Departamento de Ingeniería Química, Universidad de Sevilla, Spain.
García Muriana, F.J.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Garrido Fernández, A.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Gavrus, A.	Mechanical Engineering, Univ. Bretagne Loire INSA. Rennes, France.
Gecgel, U.	Tekirdağ Namik Kemal University. Süleymanpaşa, Tekirdağ, Turkey.
Gentscheva, G.	Bulgarian Institute General and Inorganic Chemistry. Sofia, Bulgaria.
Gharby, S.	Université Ibn Zohr, Faculté Polydisciplinaire de Taroudant, Maroc.
González, M.T.	Planta Piloto Ingeniería Química CONICET. Bahía Blanca, Argentina.
Gonzales-Barron, U.	Investigação de Montanha, Instituto Politécnico de Bragança, Portugal.
Górnaś, P.	Horticulture, Latvia University of Agriculture. Dobele, Latvia.
Guerberoff, G.K.	Química Orgánica, Universidad Nacional de Córdoba, Argentina.
Guillaume, D.	School of Medicine-Pharmacy, CNRS-UMR. Reims, France.
Guillén, M.D.	Univ. Basque Country UPV EHU, Fac. Pharmacy. Vitoria, Spain.
Hasan, H.T.	Pharmacognosy and Medicinal Plants, University of Baghdad, Iraq.
Hatzakis, E.	Food Science Technology, The Ohio State University, United States.
Hernández-Hernández, C.	Instituto N.I. Forestales, Agrícolas y Pecuarias. Tabasco, Mexico.
Hidalgo García, F.J.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Hong, J.	Hagerman Fish Culture Experiment Station, University Idaho, USA.
Hussain, A.I.	Applied Chemistry, Government University Faisalabad, Pakistan.
Ibrahim, I.A.A.E-H.	Pharmacology and Toxicology, Zagazig University, Egypt.
Irianto, H.E.	Marine and Fisheries Product Processing. Central Jakarta, Indonesia.
Irshad, M.	Chemistry, University of Kotli Azad Jammu & Kashmir, Pakistan.
Izquierdo, I.G.	Universidad Nacional de Mar del Plata. Balcarce, Argentina.
Jachmanián, I.	Área Grasas y Aceites, Univ. de la República. Montevideo, Uruguay.
Jafari, S.M.	Food Materials & Process Design Engineering, Univ. Gorgan, Iran.

Jaime Vernon-C, E.	Universidad Autónoma Metropolitana-Iztapalapa, México.
Jain, S.	Department of Chemistry, University of Lucknow, India.
Jalilian, J.	Plant Genetics, Faculty of Agriculture, Urmia University, Iran.
Jaroszewska, A.	West Pomeranian University of Technology in Szczecin, Poland.
Jiménez-Araujo, A.J.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Karabagias, I.K.	Laboratory of Food Chemistry, University of Ioannina, Greece.
Khademian, R.	Imam Khomeini International University. Qazvin, Iran.
Killeen, D.	New Zealand Inst. for Plant & Food Res. Ltd. Nelson, New Zealand.
Kiralan, M.	Department of Food Engineering, Balıkesir University, Turkey.
Knothe, G.	Agricultural Utilization Research Service. Peoria, United States.
Kobori, C.N.	Universidade Federal de São João del-Rei. Rodovia, Brazil.
Kowalska, D.	Institute of Food Sciences, Dept. Chemistry. Warsaw, Poland.
Kowalska, M.	Chemical Enginnering and Commodity Sciences. Radom, Poland.
Kozłowska, M.	Food Sciences, University of Life Sciences. Warsaw, Poland.
Kumral, A.	Department of Food Engineering, Uludag University, Turkey.
Lama-Muñoz, A.	Environm. and Materials Engineering, University of Jaén, Spain.
Lavi Arab, F.	Immunology BuAli, Mashhad University of Medical Sciences, Iran.
Le, V.V.M.	Food Technology, Ho Chi Minh City Univ. Technology, Viet Nam.
Lee, J.S.	Universiti Malaysia Sabah. Kota Kinabalu, Malaysia.
López López, A.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Lopusiewicz, Ł.	West Pomeranian University of Technology. Szczecin, Poland.
Makeri, M.U.	Food Sciences, Ahmadu Bello University Zaria, Nigeria.
Malacrida, C.R.	Ciências e Letras, Universidade Estadual Paulista. Assis, Brazil.
Marangoni, A.G.	Food, Health and Aging, University of Guelph. Canada.
Mariod, A.A.	Science and Arts, University of Jeddah. Alkamil, Saudi Arabia.
Maroušek, J.	Technology and Businesses in České Budějovice, Czech Republic.
Martínez-Force, E.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Martínez-Pineda, M.	Health and Sports Science, University of Zaragoza. Huesca, Spain.
Martins, A.	Food Processing and Nutrition Group (IGO). Braga, Portugal.
Mashigo, M.	Tshwane University of Technology. Arcadia, Pretoria, South Africa.
Mateos, R.	Instituto de Ciencia y Tecnología de Alimentos y Nutrición, Spain.
Mehran, Mehdi	University of Kashan. Kashan, Iran.
Membrillo del Pozo, A.	Bromatología y Tecnología Alimentos, Univ. de Córdoba, Spain.
Mendez-Encinas, M.A.	Food and Development. Hermosillo, Sonora, Mexico.
Méndez López, L.	Instituto de Investigaciones Marinas, C.S.I.C. Vigo, Spain.
Meral, R.	Faculty of Engineering, Van Yuzuncu Yıl University. Van, Turkey.
Mitchell, A.E.	Food Science, University of California. Davis, United States.
Moreno Navarrete, J.M.	Institut d'Investigació Biomèdica de Girona, Spain.
Moreira, D.K.	Instituto de Saúde Coletiva, Universidade Federal da Bahia, Brazil.
Morsy, N.F.S.	Faculty of Agriculture, Cairo University. Giza, Egypt.
Mouhamad, R.S.	Soil and Water Resources Sciences Technology. Baghdad, Iraq.
Muangrat, R.	Food Process Engineering, Chiang Mai University, Thailand.
Muñoz García, J.	Ingeniería Química, Universidad de Sevilla, Spain.
Muthukumar, S.P.	Central Food Technological Research Institute. Mysore, India.
Muzurović. E.	Clinical Medical School, University of Montenegro. Montenegro.
Nazarudin, M.F.	Universiti Putra Malaysia. Serdang, Selangor, Malaysia.
Nie, K.	Beijing University of Chemical Technology. Beijing, China.
Novo, L.P.	Centro Multidisciplinar da Barra, Univ. do Oeste da Bahia, Brazil.
Nunes, M.C.	Landscape Environment, Agriculture and Food. Lisbon, Portugal.
Ogunniyi, D.S.	Department of Chemical Engineering, University of Ilorin, Nigeria.
Okuro, P.	Food Engineering, University of Campinas. Campinas, Brazil.
Oliveira, J.V.	Food Engineering, Universidade Federal de Santa Catarina, Brazil.

Olivo, M.	Univ. Estadual Paulista Júlio de Mesquita Filho. Botucatu, Brazil.
Oriakhi, K.	Medical Biochemistry, University of Benin. Edo, Nigeria.
Ortiz-Moreno, A.	Instituto Politécnico, EscuelaNacional Ciencias Biológicas, México.
Oucif, M.	University Centre Ahmed Zabana of Relizane. Relizane, Algeria.
Özcan, M.M.	Faculty of Agriculture, University of Selcuk. Konya, Turkey.
Pachari Vera, E.	Food Industry, Univ. Nacional de San Agustín de Arequipa, Peru.
Palada, M.C.	Central Philippine University. Iloilo City, Philippines.
Panagou, E.Z.	Human Nutrition, Agricultural University of Athens. Athens, Greece.
Pang, M.	Hefei University of Technology. Hefei, Anhui, China.
Papadaki, A.	Food Science and Technology, Ionian University. Kefalonia, Greece.
Pascoal, C.V.P.	Federal Institute Science Technology of Ceará. Maracanaú, Brazil.
Patkar, G.	School of Computer Science, Vellore Institute of Technology, India.
Pejin, B.	Organic Chemistry, University of Belgrade, Republic of Serbia.
Pérez Álvarez-C, M.P.	Universidad Católica Santa Teresa de Ávila. Ávila, Spain.
Pérez Vich, B.	Instituto de Agricultura Sostenible, CSIC. Córdoba, Spain.
Pérez-Camino, M.C.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Pintea, A.M.	Agricultural Sciences and Veterinary Medicine. Timisoara. Romania.
Piravi Vanak, Z.	Food Industry and Agriculture Research Institute. Karaj, Iran.
Plastina, P.	Università della Calabria. Cosenza, Italy.
Prabhuji, S.K.	Biotechnology and Molecular Biology Centre. Gorakhpur, India.
Pujiarti, R.	Faculty of Forestry, Universitas Gadjah Mada. Yogyakarta, Indonesia.
Quiles Chuliá, M.D.	Tecnología de Alimentos, Universitat Politècnica de València, Spain.
Rachana, R.	Jaypee Inst. of Information Technology. Noida, Uttar Pradesh, India.
Ramadan, M.F.	Agricultural Biochemistry Dpt., Zagazig University. Zagazig, Egypt.
Ramos-Escudero, F.	Invest. Nutrición, Universidad San Ignacio de Loyola. Lima, Peru.
Ramos-Romero, S.	Cell Biology, Faculty of Biology, University of Barcelona, Spain.
Rodríguez Gómez, F.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Rodríguez Gutiérrez, G.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Romero, M.P.	Tecnología de Alimentos, ETSEA, Universidad de Lleida, Spain.
Romero Barranco, C.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Romero Guzmán, M.J.	Food Process Engineering, Wageningen University. Netherlands.
Rousseaux, M.C.	Transferencia Tecnológica de La Rioja. Anillaco, La Rioja, Argentina.
Rozès, N.A.L.	Biotecnologia Aliments, Universitá Rovira i Virgil. Tarragona, Spain.
Rudzińska, M.	Food Science and Nutrition, Poznan Univ. of Life Sciences, Poland.
Şahin, S.	Istanbul University-Cerrahpaşa. Avcilar, Istanbul, Turkey.
Sánchez Álvarez, A.J.	Instituto de la Grasa, CSIC. Sevilla, Spain.
Sani, B.	Agronomy Shahr-e-Qods Branch, Islamic Azad Univ. Tehran, Iran.
Santana, Á.L.	Food Science and Technology, University Nebraska-Lincoln, USA.
Santos Garcia, V.A.	Food Engineering, University of São Paulo. Pirassununga, Brazil.
Sanz, T.	Instituto de Agroquímica y Tecnología de Alimentos. Valencia, Spain.
Sanz Murías, M.L.	Instituto de Química Orgánica General, CSIC. Madrid, Spain.
Sawyer, D.B.	Maine Medical Center Research Institute. Scarborough, Maine, USA.
Schaal, G.	Laboratoire des Sciences de l'Environnement Marin. Plouzané, France.
Seiquer, I.	Estación Experimental Del Zaidín (CSIC). Armilla, Granada, Spain.
Severino, L.S.	Fisiologia da Produção Algodão, Embrapa. Campina Grande, Brazil.
Shahidi, F.	Memorial University of Newfoundland St. John's, Canada.
Shahsavari, N.	Crop Production, Islamic Azad University. Tehran, Iran.
Shinagawa F.	Food Science Laboratory Engineer Elopak. Oslo, Norwegen.
Silambarasan, R.	J.K.K. Nataraja College of Engineering. Namakkal, Tamil Nadu, India.
Šimat, V.	Department of Marine Studies, University of Split, Croatia.
Simionatto, E.	Universidade Estadual de Mato Grosso do Sul. Naviraí, Brazil.
Sinanoglou, V.J.	Design of Food Processes, University of West Attica. Egaleo, Greece.

Skaltsa, H.	Pharmacognosy, School of Pharmacy, University of Athens, Greece.
Santos, F.N. Dos	ThoMSon Mass Spectrometry Laboratory. Campinas, São Paulo, Brazil.
Stanescu, M.D.	Applied Chemistry, Politehnica University. Bucharest, Romania.
Spano, G.	Scienze degli Alimenti e dell' Ambiente, University of Foggia, Italy.
Soto, C.	Centro Regional de Estudios en Alimentos Saludables, Chile.
Téllez-Pérez, C.	Tecnológico de Monterrey, Escuela de Ingeniería. Queretaro, Mexico.
Torres, M.	Agronomy/Biochemistry INTA. E. Agropecuaria San Juan, Argentina.
Trujillo-Cayado, L.A.	Departamento de Ingeniería Química, Universidad de Sevilla, Spain.
Valšíková-Frey, M.	Slovak University of Agriculture in Nitra. Nitra, Slovakia.
Vanhanen, L.P.	Agriculture and Life Sciences, Lincoln University, New Zealand.
Vargas-Bello-Pérez, E.	Veterinary and Animal Sciences, University of Copenhagen, Denmark.
Velasco, L.	Instituto de Agricultura Sostenible, CSIC. Córdoba, Spain.
Vélez-Erazo, E.M.	School of Food Engineering, University of Campinas, Brazil.
Večeřa, R.	University Palacky Olomouc. Olomouc, Czech Republic.
Vicario, I.M.	Lab. Color y Calidad de Alimentos, Universidad de Sevilla, Spain.
Villardi, H.G.D.	Chemistry School, Federal University of Rio de Janeiro, Brazil.
Vintila, I.	University Dunarea de Jos Galati, Food Science. Galati, Romania.
Visioli, F.	Functional Foods, Estudios Avanzados Alimentación. Madrid, Spain.
Vivas-Cuellar, M.C.	Universidad Nacional de Ingeniería, Lima, Perú.
Wang, C.	Chemical Industry of Forest Products. Nanjing, Jiangsu, China.
Wang, Y.	Food Science, Jinan University. Guangzhou, Guangdong, China.
Weiland C.	Ciencias Agroforestales, Universidad de Huelva. Huelva, Spain.
Wit, M. de	University of the Free State. Bloemfontein, South Africa.
Wojciechowicz-Budzisz, A.	Wrocław University of Environmental and Life Sciences, Poland.
Wojtowicz, E.	Prof. Wacław Dąbrowski Institute of Agricultural. Poznań, Poland.
Wu, Z.	Jiangxi Univ. of Traditional Chinese Medicine. Nanchang, China.
Yu, X.	Food Science, Northwest A&F University. Shaanxi, China.
Yunus, R.	Plantation Studies Universiti Putra Malaysia. Serdang, Malaysia.
Zahran, H.A.	Fats and Oils, National Research Centre. Dokki, Cairo, Egypt.
Zakaria, R.	Food Engineering, University Putra Malaysia. Serdang, Malaysia.
Zambelli, A.	Universidad Nacional de Mar del Plata. Balcarce, Argentina.
Zekri, N.	Lab. Spectroscopy, University Med IV Ibn Battouta. Rabat, Morocco.
Zhang, H.	Wilmar Biotechnology R&D Center Co. Pudong, Shanghai, China.
Zou, Y.	Wilmar Biotechnology Research Development. Shanghai, China.

Grasas y aceites

International Journal of Fats and Oils

Volumen 72 | Nº 2 | **April-June 2021** | Sevilla (España) | ISSN-L: 0017-3495

Sumario

GOBIERNO DE ESPAÑA | MINISTERIO DE CIENCIA E INNOVACIÓN | CSIC

http://grasasyaceites.revistas.csic.es

editorial.csic.es