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Physicochemical properties and storage stability of margarine containing *Opuntia ficus-indica* peel extract as antioxidant



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ABSTRACT

This study falls within the framework of the industrial exploitation of by-products of the prickly pear (*Opuntia ficus-indica*). The study aims to evaluate the use of hydro-ethanolic extract of prickly pear peels as a substitute of vitamin E used as antioxidant in margarine preservation. The extract was rich in total phenolics (1512.58 mg GAE/100 g DM). HPLC–DAD–ESI–MSⁿ analyses allowed the identification of sixteen compounds belonging to hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. The extract displayed a reducing power and an antiradical activity that were respectively similar to and lower than the two antioxidant standards quercetin and butylated hydroxyanisole. Tests conducted at laboratory and pilot scales showed that the margarines elaborated with peel extract were more resistant to oxidation than the margarine reference with vitamin E. In addition, neither the physicochemical nor the microbiological properties were modified. Prickly pear peels contain bioactive substances that could be used in different food sectors.

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1. Introduction

Fruits are integrated into the daily food forever. They are, with their peel, flesh and seed, an inexhaustible source of nutrients and secondary metabolites.

Opuntia ficus-indica (OFI) is native to Mexico but has been domesticated in other countries and proliferates today on dry and stony lands of the five continents. The first economic importance of this plant relies on the production of edible fruits. It is mainly consumed fresh or converted into drinks (nectars, juice), jams or marmelades. This food transformation generates a large amount of by-products (seeds and peels) (Habibi, 2004). Peels represent a large proportion of the whole fruit (from 40% to 50%) and constitute a source of bioactive compounds, notably phenolics, flavonoids and betalains (Arrizon, Calderon, & Sandoval, 2006; Kuti, 2004). In a context of resource preservation, the characterisation and isolation of bioactive compounds from by-products together with their re-valorization in the food, cosmetic or pharmaceutical industry give rise to an increasing social, economic

and scientific interest. Phenolic compounds represent very interesting molecules due to their ability to reduce or prevent oxidation that can be caused by different factors (oxygen, light, different metals) and which leads to the formation of highly reactive molecules harmful for the animal or plant cell.

Oxidation is a phenomenon that is widely spread in both food (lipid oxidation) and organism (oxidative stress). Among food products, margarine is a typical example because 82% of its content consists of fat which is the first target of oxidation (Karleskind, 1992). Indeed, lipid oxidation is a major cause of degradation of margarine during its manufacture and its conservation. The most noticeable consequence is the development of unpleasant odours. These odours often lead to the rejection of the product by the consumer (Prior, 2003). To prevent oxidation of margarine, industries add synthetic antioxidants such as Tocoblend (vitamin E) which is the most widely used. The trend today is to use natural substances that play the same role without changing the properties of the product. One of the most appropriate approaches is the exploitation of extracts of food waste. In this context, the study aimed to substitute vitamin E with a hydro-ethanolic OFI peel extract in the margarine. To reach this objective, the OFI peel extract was first characterised for its content in total phenolics, its composition in individual phenolics and its antioxidant activity.

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Then, margarines elaborated with the OFI peel extract were analysed and compared to control margarines containing vitamin E, with regard to resistance to oxidation, physicochemical and microbiological properties, at laboratory and pilot scales.

2. Materials and methods

2.1. Plant material

Sample composed of 32 healthy berries of *O. ficus-indica* (OFI) was harvested, on August 2012, in mountainous area (alt. 855 m) located at 60 km southwest of Bejaia (Algeria). The orange variety, most abundant in the region, was selected for the study.

The harvested fruits were washed with water to remove all impurities (dust, glochids) then dried at room temperature during 30 min. Their physical characteristics were then determined including dimensions (length and diameter) and fruit weight before and after peeling. Peels were crushed and stored at -20°C .

The OFI variety studied is ovoid, 4.58 ± 0.15 cm wide and 7.42 ± 0.77 cm long. Its average weight is of 85.29 ± 6.93 g, the edible part is about 47.84 ± 5.36 g, while the skin is about half of the whole fruit (37.13 ± 8.41 g).

2.2. Chemical analyses

2.2.1. Determination of moisture

The moisture content was determined by drying peels (2 g) at 105°C until a constant weight was reached. Then the moisture content was calculated from the following equation: Moisture = $1 - (\text{Dry Weight}/\text{Fresh Weight})$ (AOAC, 1980).

2.2.2. Extraction of phenolic compounds

Phenolic compounds were extracted twice by maceration of 2×50 g of OFI peels in 2×100 ml of organic solvent (70% ethanol). The mixtures, protected from light, were subjected to stirring for 2 h at room temperature and then filtered. The solvent was evaporated under vacuum by rotary evaporator type Heidolph (Germany). One of the obtained extracts was reconstituted in methanol for the determination of total phenolic content, the identification and quantification of phenolic compounds and the evaluation of the antioxidant and antiradical activities. The second extract was reconstituted in water and was followed by a lyophilization for margarine preparation. Both extracts were stored at -20°C .

2.2.3. Determination of total phenolic content

Total phenolic compound content was determined using the method of Velioglu, Mazza, Gao, and Omah (1998). Two hundred and fifty microlitres of the methanol extract were mixed with 1.5 ml of Folin–Ciocalteu (10%). After 5 min, 1.5 ml of sodium carbonate (6%) were added. The mixture was incubated in the dark for 60 min. The absorbance was read at 760 nm against a blank. Results are expressed as mg gallic acid equivalent per 100 g of dry matter (mg GAE/100 g DM).

2.2.4. Identification and quantification of phenolics by HPLC–DAD–ESI–MSⁿ analysis

The phenolic compounds, contained in the methanol extract, were separated and analysed on a HPLC–DAD–ESI–MSⁿ system consisting in a binary solvent delivery pump (Ultimate 3000, Thermo Scientific–Dionex, Germany), connected to a diode array detector (Ultimate 3000, Thermo Scientific–Dionex, Germany) and a LTQ Orbitrap spectrometer (Thermo Scientific, Germany), equipped with an atmospheric pressure ionisation interface and operated in ESI negative and positive ion modes. The LC separation was

realised on a C18 LichroCART (250×4.6 mm, $5 \mu\text{m}$) column (Merck, Germany) by using a gradient elution from 1% to 90% MeOH/ACN/Formic acid (50/50/1) in 92 min with a flow rate at $500 \mu\text{l}/\text{min}$. For the mass spectrometer, the spray voltage was 5 kV and the temperature of the heated capillary was set to 300°C . The flow rates for the sheath gas, auxiliary gas and the sweep gas were set to 40, 10 and 10 arbitrary unit min^{-1} respectively. The capillary voltage was 36 V, the split lens was -44 V and the front lens was -3.25 V. The data were processed using the XCALIBUR software program.

Compound quantification was based on the area under peak determined at 280, 320 and 350 nm for hydroxybenzoic acid derivatives, hydroxycinnamic acids and flavonoids respectively. Hydroxy-benzoic acid derivatives were expressed relative to calibration curves with gallic acid, whereas hydroxycinnamic acid derivatives and flavonoids were expressed relative to ferulic acid and rutin respectively.

2.2.5. Evaluation of the antioxidant and antiradical activities

2.2.5.1. Reducing power. The reducing power of OFI peel extract was determined by the method of Oyaizu (1986). Five hundred microlitres of the methanol extract was mixed with 1.25 ml of phosphate buffer (pH 6.6, 0.2 M) and 1.25 ml of ferricyanide potassium (1%). The mixture was incubated at 50°C for 20 min and then 1.25 ml of trichloroacetic (10%) was added. Then, 1.25 ml of this mixture was combined with the same volume of distilled water and 0.25 ml of ferric chloride (FeCl_3) (0.1%). After 30 min incubation in the dark, the absorbance was read at 700 nm against a blank. This activity was tested at different concentrations of the extract ranging from 0.14 to 14.23 mg/ml. Quercetin and butylated hydroxyanisole (BHA), from 0.1 to 0.6 mg/ml, were used as standards. The concentration that reduces by half the oxidised iron (EC_{50}) was calculated for each sample.

2.2.5.2. Scavenger effect on DPPH. The activity of DPPH radical was determined according to the method described by Brand-Williams, Cuvelier, and Berset (1995). Sixty microlitres of the methanol extract were added to 2.44 ml of DPPH solution. The mixture was placed in the dark at room temperature for 60 min. The absorbance was read at 515 nm.

To determine the concentration that reduces by half the DPPH (EC_{50}), different concentrations of the extract were tested (from 28.5 to 142.3 mg/ml). For comparison, the same standards (quercetin and BHA) were also tested at different concentrations (from 0.1 to 1 mg/ml).

2.3. Incorporation of OFI peel extract in margarine

2.3.1. At laboratory scale

To determine the concentration required to be incorporated in the margarine, three concentrations of peel extracts were tested (50, 100 and 150 ppm). Margarines were manually produced at laboratory scale. Lipid (82%) and liquid (18%) phases were prepared. The lipid phase contained palm oil, sunflower oil and equivalent hydrogenated soybean and the liquid phase: β -Carotene (12 mg/kg), aroma (diacetyl: 25 ppm), salt (0.60%), lactic acid (0.5 ml/kg) and potassium sorbate (300 mg/kg).

The lyophilized extract was incorporated in the liquid phase. After dosage of the two phases, the emulsion passed through agitation and cooling before being divided into 125 g tubs. In parallel, a margarine reference was prepared, in the same conditions, with vitamin E at 100 ppm.

2.3.2. At pilot scale

Margarine preparation at pilot scale was done with the optimal amount of extract that was defined at laboratory scale. The

Table 1
Germs sought in margarines developed and the analytical conditions.

Germs	Middle	Inoculation	Incubation	Result	Method
Aerobic germs	PCA	In mass	30 °C/72 h	White colonies	ISO 4833
Yeasts	Sabouraud agar	In mass	25 °C/5 J	Round and opaque colonies	ISO 21527-2
Coliformes	VRBL agar	Double layer	44 °C/24–48 h	Red–violet colonies	ISO 7251
<i>Staphylococcus aureus</i>	BP	In mass	37 °C/24–48 h	Colonies with a black halo	ISO 6888-1
<i>Salmonella</i>	SFB	In mass	37 °C/24–48 h	Colonies blue–green and black center	ISO 6579

ingredients that went into the composition of these margarines were identical to those used at the first scale. The weight of a tub of margarine was 500 g. The final products were stored at 4 °C.

2.3.3. Determination of the oxidative stability of margarines (Rancimat test)

The Rancimat test is an accelerated technique most commonly used for assessment of the oxidative stability of edible fats, oils and fat-containing foods. The higher the oxidative stability value, the more stable the material is (Farhoosh, 2007).

To determine the oxidative stability of the prepared margarines, 3 g of sample were put in accelerated oxidation conditions: temperature set at 98 °C and air flow to 10 l/h. As a result, volatile compounds are formed and trapped in the tube containing distilled water (60 ml) that induces the increase of its conductivity. The induction period and the oxidative stability of the samples are given in hours. It is determined from the inflection point of the curve of conductivity (ISO International Standard, 2006). The analysis was repeated 3 times for each sample.

2.3.4. Determination of physicochemical properties of the margarines developed

2.3.4.1. *pH*. Margarines pH were determined directly on the aqueous phase.

2.3.4.2. *Moisture*. The moisture content was determined on 3 g of margarine brought to 100 °C until constant weight (ISO International Standard, 1998a).

2.3.4.3. *Salt content*. Salt content was obtained following the conventional Mohr method (AOAC, 1990) by dissolving 5 g of margarine in 100 ml of boiling distilled water. The mixture was then titrated with a solution of silver nitrate AgNO₃ (0.1 N) in the presence of a few drops of potassium chromate until the colour changes to red brick. The sodium chloride (NaCl) content is given by the following equation: $RS (\%) = [(58.5 \times V \times N) / (M \times 1000)] \times 100$, where: RS is the Rate of salt (%), V_0 the volume (ml) of the AgNO₃ solution, M the sample mass (g) and N the normality of the AgNO₃ solution. The molar mass of NaCl is 58.5 g/mol.

2.3.4.4. *Determination of solid content (solid fat content)*. To determine the level of solids in the margarine, each sample was melted in an oven at 100 °C and then filtered. The filtrate obtained was then poured into three tubes up to 2 cm. The tubes were incubated separately at three different temperatures: 20 °C/20 min, 30 °C/20 min and 40 °C/20 min. Values were read using a nuclear magnetic resonance (NMR) apparatus (type minispec mq 20, Germany), processed and the final results were given in percentage of solids (ISO International Standard, 1995).

2.3.4.5. *Determination of melting point*. The first steps of determining the melting point of margarine are similar to those described above. The capillary tubes were placed in cold water then heated (0.5 °C/min) until the level of the fat matter rises in the capillary tube. The melting temperature was noted (ISO International Standard, 2002a, 2002b).

2.3.4.6. *Determination of the peroxide index*. The peroxide index determination involves mixing 5 g of melted margarine with 12 ml of chloroform, 18 ml of acetic acid and 0.5 ml of potassium iodide solution. After 1 min, 75 ml of distilled water and a few drops of starch (colour indicator) were added. The mixture was then titrated with sodium thiosulfate solution (0.01 N) until the colour changes to pale yellow. A blank was prepared in the same conditions (ISO International Standard, 1998b).

The peroxide index is expressed in meq O₂/kg calculated using the following equation:

$$PI = [V/M] \times 1000,$$

where V is the volume (ml) of sodium thiosulfate and M the sample mass (g).

2.3.5. Determination of microbiological properties of the margarines developed

Microbiological analyses were performed on final products in order to determine their hygienic quality. They include the enumeration of microorganisms and looking for some pathogens (aerobic bacteria, yeast and molds, faecal coliforms, *Staphylococcus aureus* and *Salmonella*). Table 1 summarises the germs sought and the conditions used.

2.4. Statistical analysis

All data are means \pm SD of three measurements. Differences between groups were tested by an analysis of variance (ANOVA) using the STATISTICA software (5.5). The P values of ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Humidity of the OFI peel

The humidity rate of the OFI peel was $85.76 \pm 0.10\%$. This value is close to that obtained by Ramadan and Mörser (2003) (84.7%). It also falls within the range registered by Maataoui and Hilali (2002) in Moroccan fruits varying from 83.5% to 86% and Chougui et al. (2013) in Algerian fruits (89.6%).

3.2. Antioxidant content of OFI peel

The total phenolic content was 1512.58 ± 31.5 mg GAE/100 g DM. This value was much higher than those registered for Italian (760 mg/100 g DM) and Mexican (194–362 mg/100 g DM) OFI cultivars, but similar to Tunisian cultivars (estimated from 1650 to 1850 mg/100 g DM when considering a humidity rate at 85%) (Cardador-Martínez, Jiménez-Martínez, & Sandoval, 2011; Moussa-Ayoub, El-Samahy, Rohn, & Kroh, 2011; Yeddes, Cherif, Guyot, Sotin, & Ayadi, 2011).

These differences could be attributed to the type of variety, geographical origin of fruits, maturity stage, storage conditions and also to the extraction and analytical protocols.

From the available literature it appears that, OFI peel is particularly rich in total phenolic in comparison to other fruit and

vegetable peels. Indeed, OFI peel phenolic content is 2 times higher than fruit peel of the related species *Opuntia stricta* and several times higher than commonly consumed fruits and vegetables like lemon (435.76 mg/100 g DM), pineapple (120.87 mg/100 g DM), potato (240 mg/100 g DM) and tomato (462 mg/100 g DM) (Al-Weshahy, El Nokty, Bakhete, & Rao, 2010; Kalaiselvi, Gomathi, & Uma, 2012; Li et al., 2005; Ramandeep & Geoffrey, 2004; Yeddes et al., 2011). This makes OFI peel a very interesting source for bio-active compound re-valorization.

3.3. Identification and quantification of the phenolic compounds

The OFI hydro-ethanolic extract was analysed on HPLC–DAD–ESI–MSⁿ in negative ion mode. Sixteen compounds were separated and characterised regarding their retention time, λ_{\max} and m/z for monoisotopic and fragment ions (Fig. 1 and Table 2). In addition, the structure of the main molecules identified in OFI peels is described in Fig. 2. Compounds **1** and **5** exhibited λ_{\max} at 275 nm and a $[M-H]^-$ at m/z 255.0490 and 239.0543 respectively (Fig. 1A). These data, together with their fragmentation pattern

respectively, two hydroxybenzoic acids previously reported in OFI cladodes (Ginestra et al., 2009; Nordal et al., 1966). Compounds **2**, **4**, **6**, **7** and **15** displayed λ_{\max} at 325 nm with a shoulder at 300 nm which is characteristic of hydroxycinnamic acid derivatives (Fig. 1B). Compounds **6** and **7** displayed both a $[M-H]^-$ at m/z 355.1012, with fragments at m/z 295, 193 and 175 which corresponded to two isomers of the previously identified feruloyl glucose (Sun et al., 2013). Compounds **8–14** and **16** exhibited λ_{\max} at 254 and 353 nm typical of flavonoids (Fig. 1C). Compound **16** was identified as isorhamnetin as it displayed the same retention time and $[M-H]^-$ at m/z 315.0488 as an authentic standard. Compounds **8** and **9** were defined as two isomers with regard to their identical $[M-H]^-$ at m/z 769.2140. The fragments at m/z 623 $[(M-H)-146]^-$, 477 $[(M-H)-(146+146)]^-$ and 315 $[(M-H)-(146+146+162)]^-$ suggested the presence of two rhamnosides and one hexose linked to isorhamnetin. Compounds **10** and **11** were also isomers with a similar $[M-H]^-$ at m/z 755.1982. The fragments at m/z 623 $[(M-H)-132]^-$, 477 $[(M-H)-(132+146)]^-$ and 315 $[(M-H)-(132+146+162)]^-$ indicated that isorhamnetin is linked to one hexose, one rhamnose and one pentose. The two compounds **12** and **13** were defined as isomers with a $[M-H]^-$

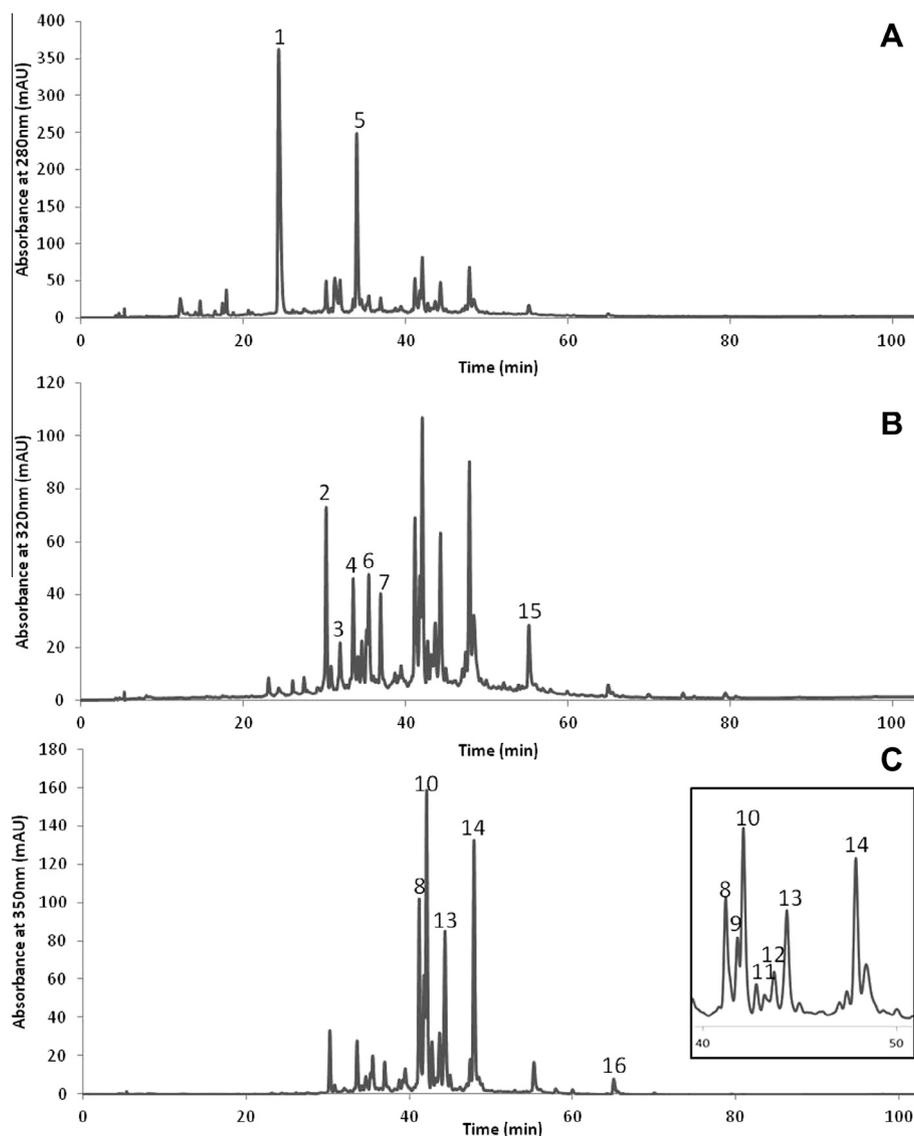


Fig. 1. HPLC chromatograms of soluble phenolics from the OFI peel extract. The profiles were recorded at 280 nm (A), 320 nm (B) and 350 nm (C). For readability, the zone between 40 and 50 min has been enhanced (Insert). Peak numbers match those of Table 2.

Table 2
Metabolites identified and quantified by HPLC-DAD-ESI-MSⁿ analysis of OFI peel extract.

Rt (min)	Peak	λ_{\max} (nm)	[M–H] [–]	MS/MS [M–H] [–]	Formula	Appm	Proposed molecule	Concentration (mg/100 g DM)
24.4	1	275	255.0490	193, 179, 165, 149	C ₁₁ H ₁₂ O ₇	5.0	Piscidic acid	271.3 ± 2.6
30.2	2	300sh, 325	959.3186					–
31.9	3	293	365.0614					–
33.6	4	300sh, 329	489.1583	235, 329, 445				–
34.0	5	275	239.0543	179, 149	C ₁₁ H ₁₂ O ₆	4.9	Eucomic acid	121.6 ± 1.0
35.5	6	300sh, 325	355.1012	295, 193, 175	C ₁₆ H ₂₀ O ₉	4.6	Feruloyl glucose	10.5 ± 0.7
36.9	7	300sh, 325	355.1012	295, 193, 175	C ₁₆ H ₂₀ O ₉	4.6	Feruloyl glucose	8.2 ± 0.6
41.2	8	254, 353	769.2140	623, 477, 315	C ₃₄ H ₄₂ O ₂₀	2.8	Isorhamnetin-Glc-Rha-Rha	40.5 ± 0.6
41.7	9	254, 353	769.2140	623, 477, 315	C ₃₄ H ₄₂ O ₂₀	2.8	Isorhamnetin-Glc-Rha-Rha	21.5 ± 0.5
42.1	10	254, 353	755.1982	623, 477, 315	C ₃₃ H ₄₀ O ₂₀	5.0	Isorhamnetin- Glc-Rha-Pen	61.7 ± 0.7
42.7	11	254, 353	755.1982	623, 477, 315	C ₃₃ H ₄₀ O ₂₀	5.0	Isorhamnetin- Glc-Rha-Pen	8.9 ± 0.6
43.7	12	254, 353	609.1428	477, 315	C ₂₇ H ₃₀ O ₁₆	4.2	Isorhamnetin- Glc-Pen	9.2 ± 0.2
44.3	13	254, 353	609.1428	477, 315	C ₂₇ H ₃₀ O ₁₆	4.2	Isorhamnetin- Glc-Pen	28.8 ± 0.3
47.8	14	254, 353	623.1570	477, 315	C ₂₈ H ₃₂ O ₁₆	5.5	Isorhamnetin- Glc-Rha	43.6 ± 2.1
55.2	15	300sh, 329	431.0953	193, 165				–
65.0	16	254, 359	315.0488		C ₁₆ H ₁₂ O ₇	5.3	Isorhamnetin	2.2 ± 0.1

Peak number is as in Fig. 1. λ_{\max} is the absorbance maxima in the UV/visible range. sh, shoulder. The reported fragments were observed in source and verified through MS/MS analysis of the parental ion in negative mode. Appm, deviation of the observed ion mass from the corresponding calculated monoisotopic mass. Glc: Glucose; Rha: Rhamnose; Pen: Pentose.

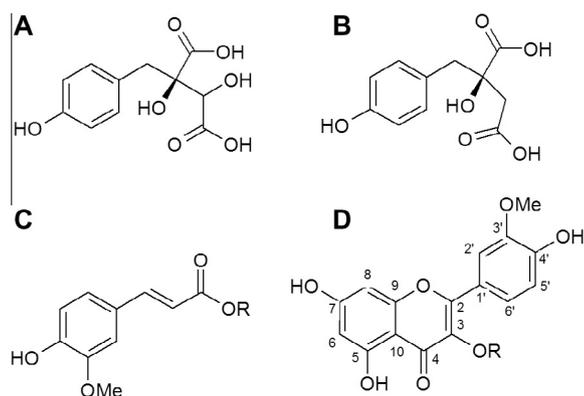


Fig. 2. Structures of the main compounds identified in OFI peel extract. (A) Piscidic acid, (B) eucomic acid, (C) feruloyl glucose, (D) glycosylated isorhamnetin. R denotes glycosyl ester. Carbon numbering has been added on the isorhamnetin structure.

m/z 609.1428. With regard to their fragmentation profiles, those compounds would correspond to isorhamnetin linked to one hexose and one pentose. Compound **14** had a [M–H][–] at *m/z* 623.1570 with fragments suggesting isorhamnetin with one hexose and one rhamnose (De Leo, Bruzual De Abreu, Pawlowska, Cioni, & Braca, 2010). Recent studies have already described the occurrence and predominance of isorhamnetin glucoside and pentoside isomers in different organs of *Opuntia* sp. including cladodes and fruits (Ginestra et al., 2009; Yeddes et al., 2011). Other flavonoids core have also been described, including quercetin and kaempferol derivatives (Ginestra et al., 2009; Yeddes et al., 2011), however none of them could have been detected in our OFI peel extract. From a quantitative viewpoint, piscidic and eucomic acids were the two most concentrated molecules in OFI peel, corresponding to 271.3 and 121.6 mg GAE/100 g DM respectively. These concentrations are 2 and 7 times higher than those found in OFI cladodes (Ginestra et al., 2009). The concentrations of the various flavonoids vary between 2.2 and 61.7 mg/100 g DM, isorhamnetin diglucoside pentoside (compound **10**) being the major compound. The total concentration of the isorhamnetin glycosides in OFI peels was 216.4 mg/100 g DM, which corresponded to half the concentration found in cladodes (Ginestra et al., 2009), but was similar and 4 times higher than in peels of Italian and Tunisian OFI cultivars,

respectively (Moussa-Ayoub et al., 2011; Yeddes et al., 2011). In addition, this concentration was 3 and 10 times higher than in peels of the related cactus species *Opuntia macrorhiza* and *O. stricta* (Moussa-Ayoub et al., 2011; Yeddes et al., 2011). Isorhamnetin derivatives and more generally flavonoids have been described in fruit pulps but at concentration several order of magnitude lower than in fruit peel (Yeddes et al., 2011). Compared to hydroxycinnamic acids and flavonoids, hydroxycinnamic acids, represented by the two feruloyl glucose isomers, were weakly concentrated in OFI peel (18.7 mg/100 g DW in total). This is in agreement with the hydroxycinnamic concentrations found in peels of Tunisian OFI cultivar (Yeddes et al., 2011). Interestingly, almost all the identified molecules have known antioxidant and/or antiradical properties (Ae, Arihara, Okada, Yoshihara, & Johansen, 1990; Takahira, Kusano, Shibano, Kusano, & Miyase, 1998).

3.4. Antioxidant and antiradical activities

The antioxidant and antiradical activities of peel extract were evaluated using two methods, respectively the reducing power and the DPPH assay, and compared to those of the two reference antioxidants quercetin and BHA. Regarding the reducing power, the OFI peel extract led to a reduction of the oxidised iron with an EC₅₀ measured a 1.03 mg/ml (Table 3). This concentration was higher but close to those of quercetin (0.40 mg/ml) and BHA (0.55 mg/ml). Regarding the antiradical assay, the concentration needed to reduce the DPPH radical by 50% (EC₅₀) was 77.81 mg/ml (Table 3). This value was about 2 orders of magnitude higher than the EC₅₀ calculated for quercetin (0.54 mg/ml) and BHA (0.78 mg/ml) indicating that the OFI extract was by far less effective than quercetin and BHA regarding radical scavenging. Taken together, it appears from these results that the OFI extract exhibits a higher antioxidant activity than antiradical potential.

Table 3
Reducing power and inhibition of the radical DPPH by the OFI peel extract and the standards (quercetin and BHA) (mg/ml).

Sample	Reducing power (EC ₅₀)	DPPH (EC ₅₀)
Extract	1.03 ± 0.23 ^a	77.81 ± 2.17 ^a
Quercetin	0.40 ± 0.02 ^b	0.54 ± 0.00 ^c
BHA	0.55 ± 0.01 ^b	0.78 ± 0.00 ^b

Values with the same letters have no significant difference. a > b > c at *P* ≤ 0.05.

3.5. Margarines developed with OFI peel extract

3.5.1. Rancimat test

The OFI peel extract concentration required to be incorporated in margarine was determined by the Rancimat test. Oxidation is a reaction which leads to the appearance of various primary and secondary degradation products, the development of rancid flavour and the alteration of the product. The purpose of the test is to predict the oxidative stability of the fat matter. The Rancimat results are represented as a curve (conductivity as a function of time). The induction period is determined from the inflection point of the conductivity curve (Hidalgo, Leon, & Zamora, 2006).

3.5.1.1. At laboratory scale. Under the same conditions, four different types of margarines were elaborated with OFI peel extract (ME1_a: 50 ppm, ME1_b: 100 ppm, ME1_c: 150 ppm) and with vitamin E (reference ME0: 100 ppm). An example of the Rancimat results is illustrated in the Fig. 3A.

The induction time obtained for the margarines, were 15.69 ± 0.43 h (ME1_a), 15.74 ± 0.11 h (ME1_b) and 15.48 ± 0.15 h (ME1_c), while for ME0, the induction time was 14.58 ± 0.24 h. It appears that the oxidation is decreased significantly ($P < 0.05$) in the margarines containing the peel extract by comparison to the one containing vitamin E. The concentration of the peel extract in the margarine did not impact significantly the oxidation. A small tendency to a higher oxidation was observed with the margarine

containing 150 ppm of peel extract. This phenomenon could be explained by the pro-oxidant properties of phenolics at high concentration, already reported by some authors (Hagerman et al., 1998; Huang, Ou, & Prior, 2005).

Compared to the reference ME0, margarines developed with peel extract showed clearly a longer induction time and therefore better resistance to oxidation even at half the concentration of vitamin E usually incorporated in margarine manufacture.

Among the 3 concentrations of OFI peel extract tested, the lowest amount (50 ppm) was chosen to prepare margarines at pilot scale in parallel with margarine reference.

3.5.1.2. At pilot scale. Margarines of 250 g were prepared and the Rancimat test performed on the two samples. The induction times were 17.32 ± 0.32 and 15.05 ± 0.04 h respectively for ME1_a and ME0, which confirms, at pilot scale, the effect of the OFI peel extract in limiting the margarine oxidation already shown at laboratory scale. An example of the Rancimat results is illustrated in the Fig. 3B.

3.5.2. Physicochemical properties of margarines developed at pilot scale

Beside the differences highlighted in the Rancimat test, the physicochemical properties of margarines ME1_a and ME0 developed at pilot scale were determined in accordance with the ISO standards (Table 4).

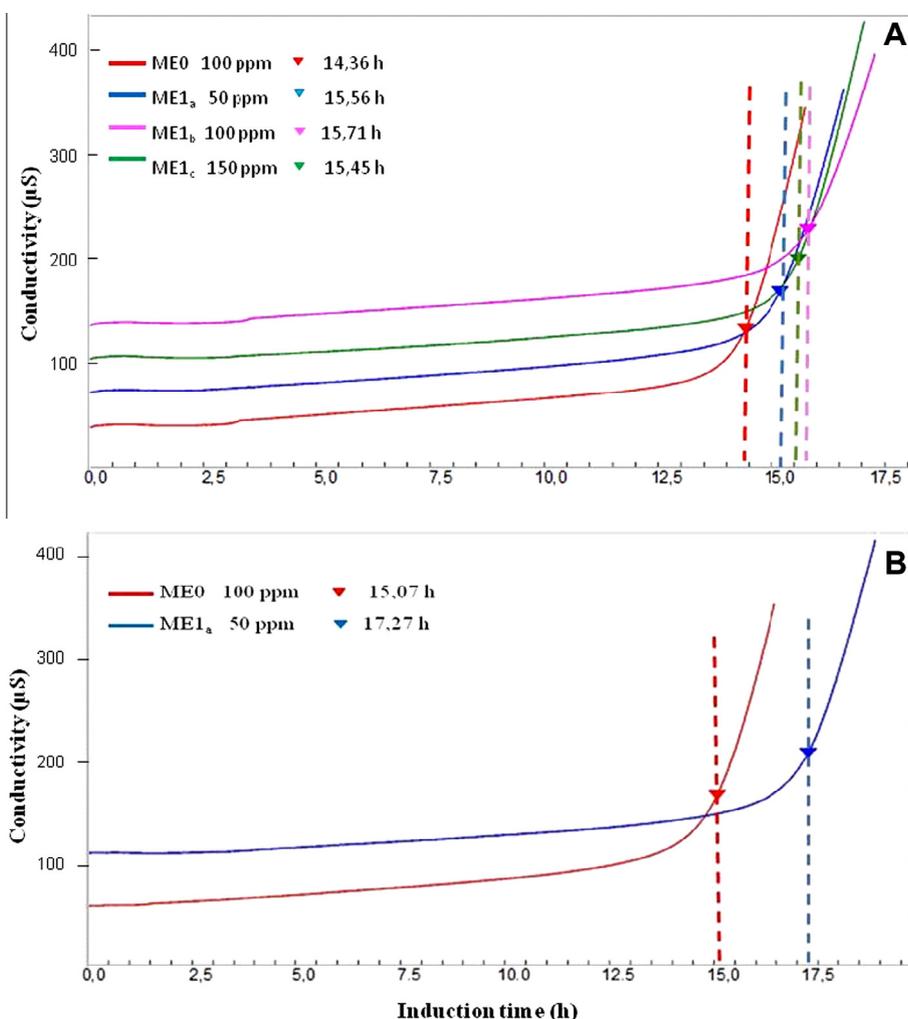


Fig. 3. Example of the induction time for margarines developed at laboratory (A) and pilot (B) scales at 98 °C. ME0: margarine reference (100 ppm of vitamin E); ME1: margarine elaborated with peel extract (a: 50 ppm, b: 100 ppm, c: 150 ppm).

Table 4
Physicochemical and microbiological properties of margarines developed at pilot scale.

Parameter	ME0	ME1 _a	Standards
pH	4.00 ± 0.00 ^b	4.50 ± 0.00 ^a	4–5.5
Humidity (%)	20.95 ± 0.49 ^a	17.11 ± 0.41 ^b	Max 16
Salt content (%)	0.22 ± 0.02 ^b	0.34 ± 0.03 ^a	0.1–0.4
Melting point (°C)	32.95 ± 1.48 ^a	33.70 ± 0.98 ^a	28–34
Peroxide index (meq/kg)	0.39 ± 0.01 ^a	0.38 ± 0.05 ^a	Max 5
Aerobic germs*	<10 ²	<10 ²	<10 ²
Faecal coliforms*	Absence	Absence	Absence
<i>Staphylococcus aureus</i> *	<10	<10	10
Yeasts*	<10	<10	10
<i>Salmonella</i> *	Absence	Absence	Absence

Values with the same letters have no significant difference. $a > b$ at $P \leq 0.05$.

ME0: 100 ppm of vitamin E; ME1_a: 50 ppm of OFI peel extract.

* CFU/g.

The pH values of the aqueous phase, the relative humidity and the salt content of the two margarines ME1_a and ME0 exhibited significant differences. With the exception of the relative humidity, all the values were however within the standard range (Table 4). Regarding the relative humidity, and according to ISO 662 (1998–90–1), the moisture content of margarine should not exceed 16%. However, companies tolerate rates up to 18%. Margarines ME1_a and ME0 had rates of $17.11 \pm 0.41\%$ and $20.95 \pm 0.49\%$ respectively, which indicates that ME0 was an outlier. This may be due to a defect in the volume of aqueous phase added during the production of margarine.

The two margarines exhibited a similar peroxide index (0.39 ± 0.01 Meq O₂/kg for ME0 and 0.38 ± 0.05 Meq O₂/kg for ME1_a) far below the maximum values allowed by the international standards (5 Meq O₂/kg). The peroxide index is one of the most widely used tests for oxidative rancidity in oils and fats. It is the amount of peroxide oxygen per 1 kg of product. It is a very useful test and a satisfactory sensitivity to appreciate the early stages of oxidative damage (Karleskind, 1992).

In addition to the physicochemical properties, two indicators of the organoleptic properties of the two margarines were measured i.e. the melting point and the Solid Fat Content (SFC).

The melting point gives an indication of the temperature at which the margarine should be smooth in the mouth. The melting point international standard range of margarine are between 28 and 34 °C which implies that margarine can melt rapidly in the mouth and be firm at room temperature to resist to mechanical work during its spreadability. The melting points of the margarines developed were not significantly different and within this the international range since ME0 and ME1_a fused at 32.95 ± 1.48 and 33.70 ± 0.98 °C respectively.

The SFC is the percentage of solidified triglycerides in oil at a given temperature. SFC is an important indicator of several characteristics of a product, including its appearance and organoleptic properties. It can be used as a measure of the degree of crystallisation of fats during treatment (Bongers & Almeida-Rivera, 2011).

The SFC rates of the two samples varied with the temperature. Indeed, it decreases with increasing temperature. The values obtained for the ME0 and ME1_a are close whatever the temperature. This proves that both margarines had the same composition and therefore their preparation was made according to the good manufacturing practices. At 20 °C, the SFC were $12.20 \pm 1.69\%$ and $12.50 \pm 0.87\%$ respectively, at 30 °C, they were $5.15 \pm 0.77\%$ and $5.40 \pm 0.52\%$. At 40 °C, the values were both very low ($1.15 \pm 0.01\%$ and $0.93 \pm 0.47\%$, respectively). These results were in agreement with those obtained by Karleskind (1992). At 37 °C SFC must be less than 6%. In the present study, margarines had already a SFC lower than 6% at 30 °C which indicates that these margarines melts easily in the mouth.

3.5.3. Microbiological properties of margarines developed at pilot scale

The risk of microbiological contamination of margarine comes mainly from the aqueous phase. This phase is more susceptible to microbial contamination than the oil phase; the milk can serve as culture medium for microorganisms accidentally introduced.

Bacteria, yeasts and molds can deteriorate the quality of margarine by releasing among, others, free fatty acids, aldehydes and ketones that can cause unpleasant odours (Karleskind, 1992). Five germs can infect the quality of margarine and are regularly sought.

According to the obtained data, faecal coliforms and *Salmonella* were absent; aerobic bacteria, *S. aureus* and yeasts were less than 10 CFU/g (Table 4). This indicates that the margarine production was completed in controlled conditions and therefore their hygienic quality was guaranteed by compliance with predetermined quality criteria.

In this study, we demonstrate that the use of the OFI peel extract successfully reduces the lipid oxidation in the elaborated margarines, and consequently extends the shelf life of these products, more effectively than vitamin E, one of the most important antioxidant known in nature.

We showed here that the OFI peel extract is rich in total phenolics which constitute well described antioxidant compounds (Cardador-Martínez et al., 2011; Moussa-Ayoub et al., 2011). Indeed, according to the literature, phenolics are referred to as multiple-function antioxidants. Firstly, they are primary antioxidants through their ability to react directly with free radicals, such as lipid, alkoxyl and peroxy radicals, and convert them to more stable, non-radical products. Secondly, they are secondary antioxidants which can inhibit lipid oxidation by several different mechanisms including chelation of transition metals, oxygen scavenging, synergism between antioxidants and singlet oxygen quenching reductants (McClements & Decker, 2000).

HPLC–ESI–MS analyses allowed identifying and quantifying at least 16 phenolics in OFI peel extract. This extract is composed by a majority of simple phenols (piscidic acid, eucomic acid, ferulic acid derivatives) which exhibits *p*-hydroxyl and a varying number of additional hydroxyls essential for mediating the antioxidant potential of a compound (Ali et al., 2013; Andjelkovic et al., 2006). Among those simple phenols, ferulic acid is known to have a high antioxidant and antiradical potential with the ability to inhibit lipid peroxidation (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999; Yashin, Yashin, Wang, & Nemzer, 2013). Piscidic acid, the major phenolic compound found in the OFI extract is also known to exhibit antioxidant properties and constitutes also a strong chelator of iron (Ae et al., 1990; Takahira et al., 1998). To our knowledge, no data is available yet on the antioxidant properties of eucomic acid, however its high structural similarity with piscidic acid suggests that it may share piscidic acid properties. In addition, OFI peel extract contained substantial concentrations of flavonoids represented by different isorhamnetin glycosides. Flavonoids are likely to intervene at different steps of the peroxidation process, through their radical scavenging properties and also their ability to chelate free iron (Leake, 1998). Isorhamnetin glycoside has previously been demonstrated to exhibit a great antioxidant potential resulting in a strong protective effect against lipid peroxidation induced by H₂O₂ (Boubaker, Sghaier, Skandrani, Ghedira, & Chekir-Ghedira, 2012). Several structural features are thought to explain the antioxidant properties of flavonoids and particularly isorhamnetin glycosides (Fig. 2). These comprise (i) the presence of the C2–C3 double bond combined to the C4 carbonyl function on the C ring (Boubaker et al., 2012; Rice-evans, Miller, & Paganga, 1996), (ii) the hydroxyl groups at position C5, C7 and C4' (Edenharder & Grunhage, 2003; Mathiesen, Malterud, & Sund, 1997) and (iii) the presence of sugar moiety since glycosylation was previously shown to enhance the antioxidant potential of the flavonoid myricetin (Hayder et al., 2008). Taken together, it

could be postulated that the high antioxidant potential of OFI peel compared to vitamin E, resulted from the addition of the antioxidant properties of each individual phenolic compound discussed above and/or could benefit from a synergistic effect due to the combination of several antioxidant phenolics as was previously observed by several authors (Borchers, Keen, & Gerstwiin, 2004; Dai & Mumper, 2010; El Darra et al., 2012; Sun & Ho, 2005).

Furthermore, the incorporation of the OFI peel extract in the margarines did not greatly affect their quality. Indeed, the physicochemical, organoleptic and microbial properties of the OFI enriched margarines were weakly or not affected when compared with the vitamin E margarines. In addition, these properties fell within the standard international range.

4. Conclusion

The present study was designed to replace the commonly used vitamin E as an antioxidant in margarine preservation, by extracts obtained from by-products represented in this case by the OFI peels.

OFI peel extract presented significant levels of phenolic compounds that play an important role against oxidation as shown by the reduction power of ferric iron which was close to those of quercetin and BHA widely used in food preservation.

The three different concentrations of OFI peel extract, incorporated in margarine, showed higher performance than vitamin E which usually used at 100 ppm. The lowest concentration of extract (50 ppm), tested at pilot scale, has extended the shelf life of margarine without any effect on its physicochemical (pH, solids content, melting point, peroxide index...) and hygienic properties. This final product was as good as the margarine reference.

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