Journal of Chemistry

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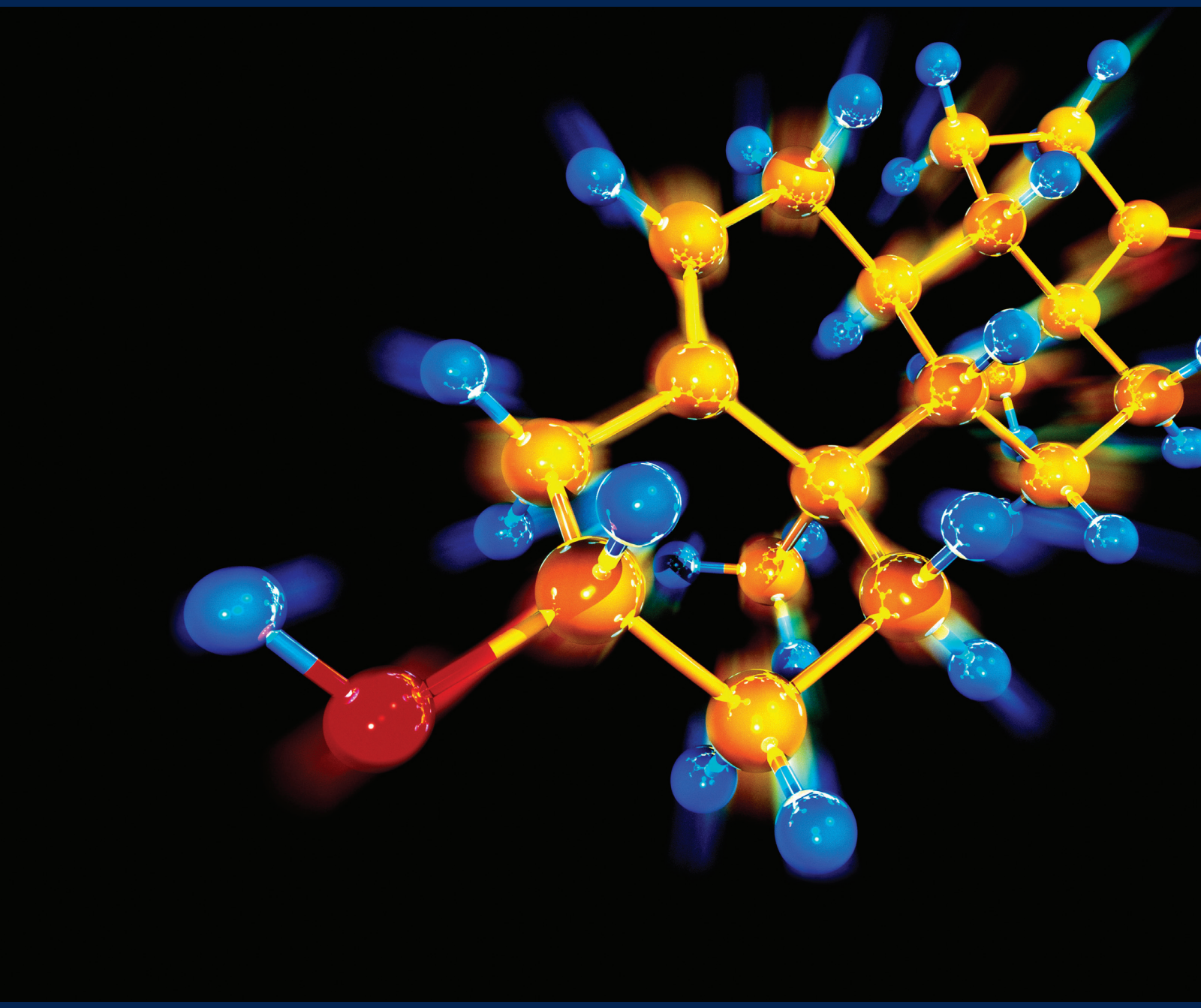
Guest Editors: Souhail Besbes, Hamadi Attia, and Christophe Blecker



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Editorial

Adding Value to Agricultural Products and Agrifood Byproducts by Highlighting Functional Ingredients

Souhail Besbes,[1,2] Hamadi Attia,[1] and Christophe Blecker[3]

[1] *University of Sfax, National School of Engineer, Laboratory of Valorization, Analysis and Food Safety, Soukra Road km 4.5, 3038 Sfax, Tunisia*
[2] *Department of Applied Biotechnology, University of Sfax, Higher Institute of Biotechnology, Soukra Road km 4.5, 3038 Sfax, Tunisia*
[3] *University of Liege, Gembloux Agro-Bio Tech. Laboratory of Food Science and Formulation, 2 Passage des Déportés, 5030 Gembloux, Belgium*

Correspondence should be addressed to Souhail Besbes; besbes.s@voila.fr

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Functional ingredients (FIs) serve to introduce and improve quality attributes of food products such as sensory appreciations, nutritional properties, health benefits, and microbiological stability. Furthermore, they serve as the basis for formulation of functional foods and technofunctionality. They encompass a wide array of phytochemicals (such as polyphenols, terpenes, and alkaloids), fibers, proteins, peptides, polysaccharides, minerals, vitamins, and so forth. They have the intrinsic ability to impact taste, flavor, appearance, color, texture, water and fat binding, counteracting fat separation, foaming and emulsifying properties, preservation, antioxidant activity, metabolite disorder, hypertension, heart disease, glycemia, and cancer. Low cost agricultural agroresources and agrifood byproducts are rich in these ingredients, and indeed they add value to the production of FIs.

FIs have been the subject of several research works and industrial developments in the last decade because of their high-tech wide applications. This special issue presents a collection of ten research papers concerning the extraction and recovery of FIs of plant and animal origin.

We sincerely hope that this collection will be of great interest to researchers and industrials and participates in the development and promotion of new ideas in the field of functional ingredients.

In this special issue, three works were focused on the use of agave as source of functional ingredients, especially soluble and insoluble fibres. A. Bouaziz et al. determined optimum conditions leading to the highest yield of soluble and insoluble fibres from Tunisian *Agave americana* L. using surface response methodology. Insoluble fibres concentrate showed a high WHC and OHC (8.66 g water/g sample and 5.6 g oil/g sample, resp.). They suggested that this material could be used as a functional ingredient in food to avoid syneresis for formulated products and to stabilize foods with high fat content. They conclude that it is promising to focus on the possibility to incorporate these fibres concentrates in food applications.

M. A. Bouaziz et al. studied chemical composition and some functional properties (water holding capacity, oil holding capacity, swelling power, and emulsifying capacity and gelling properties) of leaves powder and water extracted inulin from Tunisian *Agave americana* L. leaves. Results showed that agave leaves powder and inulin could be used as functional ingredients in food formulations. This promotes the value addition potential *of Agave americana* L. leaves. And, as an application, textural properties of *Agave* inulin-pectin mixed gels were examined using instrumental Texture Profile Analysis (TPA).

A. Chávez-Rodríguez et al. showed that the Agave *tequilana* powder may be considered as an interesting source of dietary fiber used as food ingredients in food and nutraceutical industries. And, as food application, they employed the response surface methodology to optimize the microencapsulation conditions of *Agave tequilana* Weber var. azul juice

using whey protein isolate by a spray drying technique. They optimized the process to obtain maximum powder yield but they also search the maximum solubility and bulk density and the minimum hygroscopicity and water activity to warrant functionality and preservation of the final product.

In this special issue, five work articles cover the topic of extraction and characterization of antioxidant from vegetable and animal origin. Antioxidants, which can link reactive free radicals, are supposed to play an important role in human health and prevent the rancidity and lipid oxidation in food systems. A. Sila et al. reported that the barbel muscle protein hydrolysate displayed a high angiotensin-I-converting enzyme (ACE) inhibitory activity and it exhibited an important radical scavenging effect and reducing power. Barbel muscle protein hydrolysate can be used in food systems such as meat products as a natural ingredient with high antioxidative properties. Furthermore, these bioactive substances can be used into functional foods or nutraceuticals.

F. Mraihi et al. compared phenolic contents and antioxidant activities of methanol extracts of *Crataegus azarolus* and *Crataegus monogyna* fruits cultivated in Tunisia. The richest composition in antioxidant compounds (phenolics, proanthocyanidins, and flavonoids and anthocyanins) and the higher antioxidant capacity activity of *Crataegus* can promote the use of these fruits in various fields such as functional food formulation and pharmaceutical industry.

W. Kchaou et al. analysed antioxidant activities, using several methods, of extracts from three selected Tunisian cultivars of date's by-products (*Phoenix dactylifera L.*). Results showed that the best antioxidant activity was obtained for Allig extract, followed by Bejo and Deglet Nour. Total phenolics, total flavonoids, carotenoids, and tannins were determined spectrophotometrically in these three date extracts. This study demonstrates the potential antioxidant activity with Tunisian date byproducts leading to the use these natural extracts as food additives as an alternative to synthetic compounds.

A. Romojaro et al. showed that the addition of fruits of *Rosa canina* and *Quercus ballota* and leaves of *Sanguisorba minor* to vegetable oils increased their oxidative stability. These underused edible plants could be considered as natural source of antioxidants and encourage their use by enriching vegetable oils with low content of natural antioxidant, such as sunflower oil, in order to avoid or decrease the use of synthetic antioxidant.

A review article by D. Ackar et al. discusses if we can consider cocoa and chocolate as potential functional food. They reported that these contain some components such as polyphenols and methylxanthines which could contribute to the health impact of these foods. They suggest that additional researches must be undertaken to elucidate the extent of polyphenols and methylxanthines health impact and their possible synergy in chocolate, with respect to energy contribution.

I. Felfoul et al. studied the effect of milk fat substitution by a (W1/O/W2) multiple emulsions, based on olive oil, on milk behavior during rennet coagulation. The substitution of milk fat by emulsified olive oils in milk could be considered as an option to obtain cheese with healthier saturated/unsaturated fat balance.

A. Chikhoun et al. investigated the effect of sugar cane molasses in chemical composition sensory characteristics of madeleines, minicroissants, and buns incorporated with interesterified oil. Results showed the possibility to give value addition to this sugar manufactory byproduct by its use in formulation of highly appreciated pastry products.

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Souhail Besbes
Hamadi Attia
Christophe Blecker

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Research Article

Development of Sugar Cane Molasses in Formulations of Madeleines, Mini Croissants, and Buns Incorporated with Interesterified Oil

Anis Chikhoune,[1,2] Fatiha Bedjou,[3] Sabrina Oubouzid,[2] Rosa Boukefoussa,[2] Bilal Bechri,[2] Houria Tarmoul,[2] Toufik Abdeladim,[4] Abderrahmane Tounsi,[4] Mourad Hamitri,[4] Said Chikh,[4] and Louiza Kouadri[4]

[1] *Organic Materials Laboratory, Department of Food Sciences, Abderrahmane Mira University, 06000 Bejaia, Algeria*
[2] *Nutrition and Food Technologies Laboratory, Department of Food Technologies, INATAA, University of Constantine, Ain El-Bey Road, 25000 Constantine 1, Algeria*
[3] *Ethnobotany Laboratory, Department of Biochemistry, Abderrahmane Mira University, 06000 Bejaia, Algeria*
[4] *Department of Research, Development and Quality Control, CEVITAL, 06000 Bejaia, Algeria*

Correspondence should be addressed to Anis Chikhoune; anis.chikhoune@gmail.com

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Interesterification becomes a very powerful tool in food industry. A blend of coconut oil and palm stearin is enzymatically interesterified by lipase (EC 3.1.1.3) in an aquarium reactor. The interesterified blend obtained is then incorporated in madeleines, mini croissants, and mini rolls. Physicochemical parameters' assessment for molasses used is in good agreement with the international standards. Fatty acid composition of the interesterified blend and sugar content of molasses were assessed by gas chromatography (GC) and high performance liquid chromatography (HPLC). A sensory evaluation of the madeleines, mini croissants, and buns has been carried out by untrained tasters, with a statistical analysis by a principal component analysis (PCA). Chromatographic characterization by Gas Chromatography revealed fatty acids, ranging from C6: 0 to C22: 0. Liquid sugar's content by high performance liquid chromatography revealed three main sugars: sucrose, glucose, and fructose. Results of the sensory analysis showed the good quality of the prepared products.

1. Introduction

Enzymatic interesterification has become an established technology within the oil-and-fat-processing industry and is one of the few successful applications of immobilized enzymes. Modification and optimization of structure and properties of fat products are among aims of this technique. A special interest is to obtain triglycerides with specific fatty acids to specific positions within the triglyceridic molecule. The enzymatic catalysis is carried out with lipases [1].

As ubiquitous enzymes, lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications. Lipases have surprising flexibility to catalyze the acylation and deacylation of a wide range of natural and unnatural substrates, which find a number of attractive applications in organic chemistry, pharmaceuticals, cosmetics and leather processing, and so forth, [2]. In July 2002, Archer Daniels Midland Company (ADM) built the first commercial units in USA for EIEO using Lipozyme TL IM from Novozymes [3].

Lipases are part of the family of hydrolases that act on carboxyl ester bonds. The physiologic role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. In addition to their natural function

of hydrolyzing carboxyl ester bonds, lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media [4].

These lipases are immobilized in order to separate them from the products of reaction and to allow their reuse. The catalytic reaction does not modify the oxidative stability of oils and there is no influence on the degree of triglycerides saturation. The use of specific lipases makes it possible to manufacture products that chemical methods are unable to produce, typically fats of confectioneries with specific properties, made up of symmetrical triglycerides, or nutritional fats having specific fatty acids in Sn-2 [5]. Therefore, there is a great interest in producing *trans* free products by interesterification of liquid oil with fully hydrogenated fats. The advantages of enzymatic interesterification are mild conditions and nutritional improvement or maintenance in the product [6].

Interesterified fats and oils are incorporated in margarine [7] and Frankfurters [8] and are widespread used manners nowadays.

The baking of food is a very long and established practice with some of the earliest reports coming from ancient Egypt. The introduction of oils and fats was not only found to improve ingredients and product handling but also improved texture and mouthfeel of the baked product [9]. Fats in baked systems improve tenderness, moistness, and mouthfeel. In general, higher percentages of fat produce more tender cakes. The finer the fat dispersion, the greater the crumb softness of cake [10].

Sponge cakes, like madeleines, represent a more diverse group of products than bread and other fermented products. They may be classified as intermediate-moisture foods. Croissants, buttery flaky viennoiserie pastries, are included in pastry baking products, which have a very wide range of shapes and uses, with many local and regional variations. There are similarities between biscuits, pastries, and sponge cakes in that both sheeting and blocking/forming/moulding are employed in order to achieve the desired end results. The first significant process in the manufacture of any baked product is the blending together of the ingredients used in the recipe. A number of significant changes take place during this stage, and they begin with the solubilisation, hydration, and dispersal of the various ingredients and their components [7, 11].

Large amount of waste is produced by the food industry, in addition to being a great loss of valuable materials. Many of these residues, however, have the potential to be reused into other production systems [12]. That is the case of molasses, a solution containing sugar and nonsugar components [13] and a palatable source of fermentable carbohydrates, which typically has relatively high concentrations of calcium, potassium, and sulphur but contains relatively little crude protein, therefore allowing it to be a food supplement in the diets of animals. Therefore, fermentation industries use molasses due to its low cost, wide availability, and being a rich resource as it is the main raw material in producing alcohol during fermentation process. [14–16]. It is also a suitable carbon source for some yeasts' fermentations like Kombucha fermentation on sweetened black tea [17].

Many researchers have reported that sugarcane extracts have good antioxidant activity, case of pigments from molasses alcohol wastewater [18]. To our knowledge, no other reports on the developing of molasses in human diets have appeared in the literature so far.

In this work, we are interested in incorporating an enzymatically interesterified oil (EIEO), manufactured in a local food industry (CEVITAL SPA), and other usual oils in the formulation of madeleines, mini croissants, and buns, and also in developing a local by-product (molasses) from a local sugar refinery (CEVITAL SPA).

2. Materials and Methods

2.1. Materials

2.1.1. Interesterification of the Blend. Refined, bleached, and deodorized coconut and palm stearin oils were subjected to an enzymatic interesterification. The Lipozyme TL IM, a commercial, dry, light brown, and silica-granulated lipase with a particle size in the range of 0.3–1.0 mm, derived from *Thermomyces lanuginosus* with the transferred gene for the production expressed in *Aspergillus* sp., which rearranges the fatty acids with a certain sn-1,3 specificity, was obtained from Novozymes A/S (Bagsvaerd, Denmark). It was immobilized on a fixed bed in an aquarium reactor. Continuous interesterification is used in this case. The oil is interesterified while passing down through the catalyst bed. The flow rate (1500 Kg/h) controls the residence time of the oil in the reactor, which in turn controls the level of interesterification. The interesterification is conducted in a solvent-free medium at a temperature of 70°C and an interesterification rate of 2 kg oil/kg enzyme/hour. The input pressure of the reactor was <1.5 bar.

2.1.2. Molasses By-Product Origin. Molasses, a by-product obtained from the process of sugar's refining, is provided by a local refinery (CEVITAL). It was put in a sterile and hermetically closed flask.

2.2. Manufacture Process of Madeleines, Mini Croissants and Buns. The formulation of madeleines, buns, and mini croissants was carried out in a local bakery.

The control formulation of the madeleines was carried out in a local bakery. This formulation contains a liquid sugar (inverted liquid sugar). The model formulation was incorporated with molasses (as a sugar substitute) and the blend interesterified. Table 1 shows the major ingredients used in the formulation of both control and model madeleines. Their preparation was carried out following the procedure shown in Figure 1.

For the formulation of buns and mini croissants, the flour is put in a stainless steel tank. After that, sugar, salt, and the enzymatically interesterified oil (EIEO) are added and mixed together with a stirrer. Water is then added according to the dough. Once formed, 500 g of pastry margarine is incorporated and finally cut into the desired shapes to obtain buns and mini croissants. They are allowed for a rest of few

Table 1: The composition of control and model madeleines formulated.

Formulation	Ingredients	Quantity
Control	Flour	1 kg
	Milk	500 mL
	Chemical yeast	33 g
	Oil (regular)	500 mL
	Aroma (vanilla)	15 g
	Inverted liquid sugar	715 g
	Eggs	20
Model	Flour	1 kg
	Milk	500 mL
	Chemical yeast	33 g
	EIEO	500 mL
	Aroma (vanilla)	15 g
	Molasses	700 g
	Eggs	20

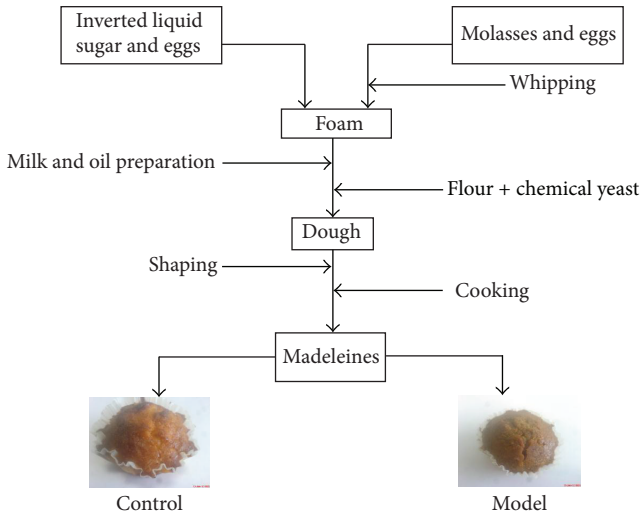


Figure 1: Manufacture process of madeleines (control and model) in a local bakery.

hours. Control formulation contains a regular oil (sunflower oil/palm oil) and model one contains the blend interesterified oil (EIEO). Table 2 shows the different ingredients used in the formulation (control and model).

2.3. Baking Conditions

2.3.1. Madeleines. Baking of madeleines was done through oven slabs made of stainless-steel containing 9 plates at 180°C during 33 min for control madeleines and 21 min for model madeleines.

2.3.2. Mini Croissants and Buns. After leaving the baking yeast act, mini croissants and buns were placed in a sole oven at 180°C for 60 min.

Table 2: The composition of formulated control and model mini croissants and buns.

Formulation	Ingredients	Quantity
Control	Flour	2 kg
	Inverted liquid sugar	300 g
	Baking yeast	20 g
	Oil (sunflower oil/palm oil)	200 cL
	Baking improver	30 g
	Salt	40 g
Model	Flour	2 kg
	Inverted liquid sugar	300 g
	Baking yeast	20 g
	EIEO	200 cL
	Baking improver	30 g
	Salt	40 g

Table 3: Principles of physicochemical parameters assessed for molasses according to [19].

Analysis	Principle
Brix	Refractometry is based on the angle of refraction (bending) of a beam (ray) of light when it strikes a different medium. the refractometer displays the results in refractometric dry substance or traditionally Brix and results are expressed as °Bx.
Polarization	The polarimeter measures the optically active substances (e.g., sucrose) in a solution sample. The results are expressed as °Z.
Purity	It is calculated by a software on the basis of Brix and polarization values. Results are expressed as %.
pH	The technique used by the pH meter is called potentiometry, which is based on the potential difference between a pair of electrodes placed into a solution from which the activity of an ion (pH) can be determined.

2.4. Physicochemical Parameters' Assessment for Molasses. The principle of the different physicochemical parameters assessed for molasses is presented in Table 3.

2.5. Determination of Molasses' Sugar Content by HPLC. Separation and identification of the three sugars glucose, fructose, and sucrose was carried out on a Bio-Rad Aminex HPX-87C (300 × 7.8 mm) column, equipped with a refractive index detector (Agilent Technologies 1200 Series, USA) according to [20]. Injection (5 μL) was performed in the split mode. The mobile phase used was water with a flow rate of 0.6 mL/min and the column was set at 80°C. The analyses of sugars were carried out in duplicate.

2.6. Determination of Fatty Acids Composition. Fatty acids composition was determined using GC (Agilent Technologies 6890 series, USA) according to [21]. Fatty acid methyl

esters (FAMEs) were with potassium methoxide and separated on a 60 m × 0.25 mm × 0.25 μm capillary column, equipped with a flame ionization detector (FID). Injection (1 μL) was performed in the split mode. Hydrogen was the carrier gas with of a flow rate of 1 mL/min. The temperature program was isothermal. The injector and detector temperature were 270°C. The analyses of FAMEs were carried out in triplicate for each sample.

2.7. Sensory Evaluation. Subjects, a number of 55 tasters for madeleines and 30 tasters for mini croissants and buns, were staff of an agribusiness food industry (CEVITAL) and University of Bejaia, Algeria.

For madeleines coded 8 from I to III (the two studied formulation processes and madeleines from a local market), were presented simultaneously to the subjects together with water. A hedonic test performed with the 55 untrained tasters was used to characterize madeleines in terms of the attributes, appearance, flavor, and crispness.

For mini croissants and buns, coded samples of 3 number; coded from A to C (the two studied formulation processes and the other samples from a local market), were presented simultaneously to the subjectstogether with slices of apples. A hedonic test performed with the 30 untrained tasters was used to characterize mini croissants and buns in terms of the attributes, aspect, mellowness and flavor.

2.8. Statistical Analysis. Statistical analysis of the data obtained from the sensory evaluation was performed by a principal component analysis (PCA) using STATISTICA Software Version 5.5 (StatSoft, France).

3. Results and Discussion

3.1. Physicochemical Parameters' Assessment for Molasses and Sugar Content by HPLC. The molasses trade commonly uses the term Brix as an indicator of specific gravity and represents an approximation of total solids content. Brix is a term originally initiated for pure sucrose solutions to indicate the percentage of sucrose in solution on a weight basis. However, in addition to sucrose, molasses contains glucose, fructose, raffinose, and numerous nonsugar organic materials [15].

The average value obtained for the Brix (83.84 ± 3.58)°Bx is in a good agreement with that reported by [22] for a sugar cane molasses but higher than that obtained by [23] for grape, mulberry, and carob molasses, in range of 73.9–75.0, 65.7–67.0, and 71.2–72.3°Bx, respectively, mainly due to their glucose, fructose and sucrose contents. This can be explained by the fact that sucrose is in a higher amount in sugar cane molasses, as displayed in Figure 2, and according to [24] molasses contains generally 80–85% solids in solution.

Polarization value (52.85 ± 3.19)°Z obtained is in a good agreement with that reported by [22]. According to [13], the degree of polarization is proportional to the concentration of the optically active substances (such as sucrose) in a sample solution.

The average purity value (65 ± 3.43)% obtained for molasses is less than the maximum established by [19]; this can be easily explained from the state of exhaustion of molasses. According to [24], it is an accepted fact that some sugar is bound to be inevitably lost in final molasses and every endeavor is necessary to bring down this sugar loss by observing certain precautions not only at the final crystallisation station but also in the operations proceeding this stage.

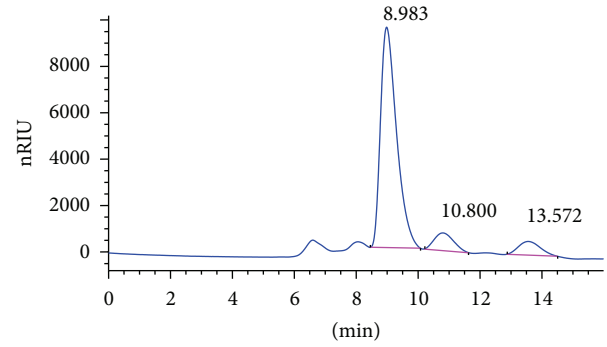


Figure 2: Chromatogram of molasses obtained by high performance liquid chromatography.

The average pH value (4.98±0.15) obtained for molasses is higher than that established by [22] but in a good agreement with that reported by [25]. According to [26, 27], there are some macromolecules and organic acids that could influence the pH of the molasses.

3.2. Molasses' Sugar Content by HPLC. Results of assessment of the sugar content are displayed in Figure 2.

The chromatogram shows three peaks corresponding to sucrose (8.98 min), glucose (10.80 min), and fructose (13.51 min) and sucrose is the main sugar present in the molasses studied. In addition to these three peaks, two peaks which are not identified in the chromatogram indicate that there are other reducing and/or nonreducing sugars not identified by the HPLC. These results are in close agreement with those reported by [25] where sucrose was the main sugar in the composition of the molasses (68.36%), followed by glucose (18.50%) and maltose (13.14%).

All types of molasses contain relatively large amounts of total sugars or carbohydrates and these compounds constitute the majority of the feeding value of molasses [15]. According to [22], sugars which account for more than half of the dissolved solids in molasses are mainly sucrose and reducing sugars, that is, the glucose and fructose. In process operations, some reducing sugars are formed due to decomposition of sucrose while some amount of the total reducing sugar gets destroyed.

3.3. Fatty Acids Composition. Fatty acids composition of the blend used is displayed in the chromatogram shown in Figure 3 and Table 4.

From Figure 3 and Table 4, it can be noticed that the predominant fatty acids in the EIEO are palmitate (C16: 0), the *cis*-oleate (C18: 1 n9), laurate (C12: 0), myristate (C14: 0), linoleate (C18: 2), and stearate (C18: 0). The percentage

Table 4: Fatty acids composition of the enzymatically interesterified oil (EIEO) studied, detected by gas chromatography.

FAC	PO (%)	EIEO (%)	SFO (%)
Caproic acid C6: 0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Caprylic acid C8: 0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Capric acid C10: 0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lauric acid C12: 0	0.00 ± 0.00	10.53 ± 0.08	0.00 ± 0.00
Myristic acid C14: 0	1.08 ± 0.18	5.00 ± 0.06	0.00 ± 0.00
Palmitic acid C16: 0	44.28 ± 0.91	44.61 ± 0.59	06.13 ± 0.28
Heptadecenoic acid C17: 1	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 0.00
Stearic acid C18: 0	4.48 ± 0.02	4.89 ± 0.04	03.83 ± 0.10
cis-Oleic acid C18: 1 n9	38.52 ± 0.70	25.26 ± 0.07	23.23 ± 0.46
cis-Oleic acid C18: 1 n7	0.77 ± 0.01	0.41 ± 0.19	00.60 ± 0.32
Linoleic acid C18: 2	10.46 ± 0.01	6.38 ± 0.22	64.96 ± 0.97
Linolenic acid C18: 3	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 0.00
Arachidic acid C20: 0	0.37 ± 0.18	0.00 ± 0.00	00.25 ± 0.15
Gadoleic acid C20: 1	0.00 ± 0.00	0.00 ± 0.00	00.00 ± 0.00
Behenic acid C22: 0	0.00 ± 0.00	0.00 ± 0.00	00.69 ± 0.00

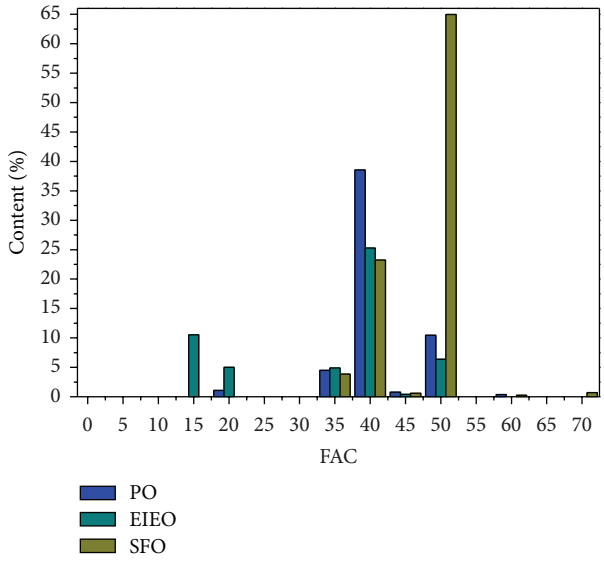


Figure 3: Fatty acids composition of the blend studied (EIEO).

of saturated and unsaturated fatty acids ranges as follows: 49% of SFA and 31% of UFA. The predominance of SFA is noticed, especially with a high content of palmitic acid. This is explained by the fact that the EIEO contains palm stearin, which is rich in palmitate.

For PO (palm oil), the major fatty acids are palmitate (C16: 0), the *cis*-oleate (C18: 1 n9), and linoleate (C18: 2). The lower levels of fatty acids are obtained for stearate (C18: 0), myristate (C14: 0), and *cis*-oleate (C18: 1 n7). The percentage of saturated and unsaturated fatty acids ranges as follows: 48% of SFA and 48% of UFA.

For SFO (sunflower oil), there is a very high content of linoleate (C18: 2) and *cis*-oleate (C18: 1 n9). Palmitate (C16: 0), stearate (C18: 0), and *cis*-oleate (C18: 1 n7) are present in small quantities. The percentage of saturated and unsaturated fatty acids ranges as follows: 10% of SFA and 88% of UFA.

According to [28], FA vary according to the oil source. This variation is due to the varietal selection and the extraction conditions. Oils of the lauric type (coconut, palm kernel) contain mainly the lauric acid (23.49–46.5)% and the myristic acid (15.1–18.4%). The lauric, palmitic, oleic, and stearic acids are the principal fatty acids commonly found in the African oilseeds. The secondary fatty acids are the myristic and linoleic acids. For sunflower oil, the Codex quotes a minimal linoleic acid content of 60%.

Given the known health benefits of unsaturates, monounsaturates and both *n*-6 and *n*-3 polyunsaturates should be substitutes for saturates in our diets, rather than consumed in addition [29].

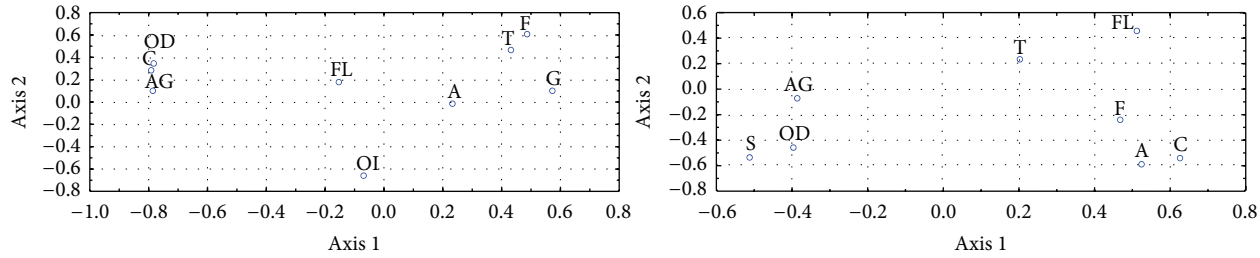
With the adoption of the Mediterranean diet, which encompasses the increased alpha-linolenic acid intake, a reduction in saturated fat and a modest increase in fiber and total carbohydrate were associated with a 72% reduction in recurrent coronary heart events in patients with prior myocardial infarction [30].

3.4. Sensory Evaluation. Axes 1 and 2 of Figure 4(a) represent the distribution of the various parameters (09) characterized by the sensory analysis of the madeleines (I, II, and III) on the unit circle.

In the unit circle, parameters (G, F, T, OD, C and AG) are well presented in the first axis than the others (A and FL). OI is not well presented in this axis. Axis 1 divided the preceding parameters very well in the following way.

(1) F and T are strongly negatively correlated with OD, C, and AG. These parameters contribute strongly to the formation of this axis.

(2) OD, C, and AG are strongly positively correlated, in particular OD and C.



(a)

Figure 4: Layout of projections for the different parameters versus axes 1 and 2 of the unit circle for madeleines (a). Layout of projections for the different parameters versus axes 1 and 2 of the unit circle for mini croissants and buns (b).

The positive correlation between T and F can be explained by the fact that the tasters use sensing as a tool to describe shape and aspect of the product.

OD, C, and AG are highly positively correlated, due to the fact that the smell and color are closely related according to the tasters, as they identify odor by color of the product. Model madeleines are compared to gingerbread from their color and odor evoking licorice.

Madeleines produced have different colors; control madeleines have a brown color, while model madeleines are darker. This color variation is due to the thermal degradation of sugars. Glucose can subsequently react under heat effect, to give products of caramelization [31] or with amino acids to give the Maillard products. These reactions are function of several parameters relating to the baking process such as temperature and moisture and also are very sensitive to the other compounds of the formulation [32].

The dark color obtained for model madeleines can be explained by the strong brownish color of the raw material (molasses), which gives its color to the dough before baking. According to [22], this color is due to the complexity of the molasses' compounds' pigmentation, mainly caramel, melanoidins, and phenolic compounds associated with molasses' colloids.

According to [33], melanoidins have commercial, nutritional, and toxicological significance as these have significant effect on the quality of food, since color and flavors are important food attributes and key factor in consumer's acceptance. Food and drinks as bakery products, coffee, and beer having brown colored melanoidins exhibited antioxidant, antiallergenic, antimicrobial, and cytotoxic properties as in vitro studies have revealed that Maillard reaction products may offer substantial health promoting effects as they can act as reducing agents, metal chelators, and radical scavengers.

The analyses of molasses' sugar content displayed the presence of mainly mono, and disaccharides (glucose, fructose, and sucrose).

According to [34] the shorter the sugar's carbon chain is, the faster the browning is. Smallest sugars are indeed better substrates because they penetrate more easily into proteins. Moreover, [35] reported that the reaction rate of the first step of nonenzymatic browning seems higher with aldoses (glucose) than with ketoses (fructose).

On the basis of tasters' assessment, sweetness is distinguished in the two types of madeleines; intensity is greater in control madeleines than in the model ones. Basically, this flavor is due to the sugar's effect, depending on sugar's type. Control has a high sweetening power due to the presence of fructose. Sweetening power of fructose is equal to 1.3 compared to that of sucrose [36].

In the case of model madeleines, the intensity is low due to the low content of reducing sugars (glucose and fructose) and high sucrose (moderate sweetness), as shown in Figure 2 but also has a slight aftertaste, probably due to the combination of sweetness and bitter flavor. According to [37], recent theoretical models of chemoreception postulate that there is a closer relationship between sweet taste receptors and those of bitter taste. Studies (structure/function) have shown that certain carbohydrates have both bitter and sweet molecular sites; therefore, they are likely to bind to the two types of receptors.

On the second axis, there is a different tendency:

(1) OI contributes strongly to the formation of this axis;

(2) parameters T, F, OD, and C contribute also to the formation of this axis;

(3) FL, G, and AG are less represented in this axis;

(4) T, F, OD and C are strongly positively correlated.

T, F, C, and OD are positively correlated, which confirm their strong positive correlation previously observed on the first axis. Flavor (FL) appears, as in the first axis, being unappreciated by tasters.

This flavor may be also due to the presence of several substances such as gums, exercising a pronounced effect on the sweet taste [22, 37].

Axes 1 and 2 of Figure 4(b) represent the distribution of the various parameters (08) characterized by the sensory analysis of the mini croissants and buns (A, B, and C) on the unit circle.

Parameters (C, A, F, FL, OD, AG and S) contribute significantly to the formation of axis 1; T contributes less.

Strong positive correlations have been identified for variables F, A, C and AG, OD, S. These two groups are negatively correlated. This can be explained as follows.

F, A, C are strongly positively correlated with each other, because the shape, appearance, and color involve generally the same mechanism of recognition which is the optical system. However the aftertaste, the odor, and flavor (AG, OD, and S) use a totally different perception mechanism which involves the bucconasal pathway. So these are two mechanisms that allow and help the taster to differentiate between the three products.

For the variable FL, it contributes like the group F, A, and C but separated from the group. This can be explained by the fact that the flavor is a complex concept for tasters although it is a parameter of differentiation.

It can also be noted that sensing T has the less contribution to the formation of the first axis this is certainly due to the fact that this attribute does not allow the taster to compare between the samples.

Along the second axis, the tendency is different. Indeed, the contribution of the different variables is totally different. Variables that contribute heavily to the formation of this axis are A and C, OD and S, and FL.

T and F contribute moderately to the formation of this axis and AG disappears completely on this axis. This can be explained as follows.

The two groups formed OD and S and A and C express the same tendency as before on axis 1. OD and S, A, and C are strongly positively correlated because they involve completely different perception systems.

On this axis, FL and F, A, and C are negatively correlated. The perception of flavor from the tasters is completely different from that of the shape, appearance, and color.

Overall, the study (PCA) of variables, the influence of some parameters is highlighted, especially the influence on the choice of tasters; they contribute also in a significant way to the formation of the two axes; therefore can be taken as choice indicators for tasters.

4. Conclusion

The aim of this study was to test the effect of incorporation of the EIEO in the manufacture of madeleines, mini croissants, and buns on one hand and the incorporation of molasses in madeleines on the other hand. The effect on the products obtained was appreciated by a sensory approach (PCA). The choice of the molasses is justified by the development of a by-product of the sugar's refining process, with a purity of $63.06 \pm 3.43\%$ and containing simple sugars (glucose, fructose and sucrose) detected by HPLC, vitamins, and minerals.

The incorporation of the EIEO in the three products studied (madeleines, mini croissants, and mini buns) revealed appreciated products' quality in different ways by the tasters (PCA). The improvement or not of a product quality cannot be revealed only by the current instrumental methods, but the sensory evaluation remains a good approach for products' quality assessment. Indeed, the choice of the variables for this study allowed an interesting appreciation of the various quality attributes.

The instrumental approach remains; however, of a great need in order to assess the effect of the ingredients used on the final products.

Abbreviation

EIEO: Enzymatically interesterified oil
ICUMSA: International commission for unification methods for sugar analysis
PCA: Principal component analysis
SFA: Saturated fatty acids
UFA: Unsaturated fatty acids
FA: Fatty acids
PO: Palm oil
EIEO: Enzymatically interesterified oil
SFO: Sunflower oil.

Sensory Evaluation

Abbreviations for Madeleines

F: Flavor
FL: Shape
G: Taste
OD: Odor
OI: Hearing
T: Sensing
AG: Aftertaste
A: Aspect
C: Color.

Abbreviations for Mini Croissants and Buns

F: Shape
A: Aspect
C: Color
AG: Aftertaste
OD: Odor
S: Flavor
FL: Flavor
T: Sensing.

Conflict of Interests

The authors have declared no conflict of interests.

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Research Article

In Vitro Antioxidant Activities of Three Selected Dates from Tunisia (*Phoenix dactylifera* L.)

Wissal Kchaou, Fatma Abbès, Hamadi Attia, and Souhail Besbes

Université de Sfax, Unité Analyses Alimentaires, Ecole Nationale d'Ingénieurs de Sfax, Route de Soukra, 3038 Sfax, Tunisia

Correspondence should be addressed to Wissal Kchaou; kchaouwissal@yahoo.fr

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Second-grade dates (*Phoenix dactylifera* L.), with hard texture, from three selected Tunisian cultivars (Allig, Deglet Nour, and Bejo) were analysed from their antioxidant activities using DPPH radical scavenging activity, FRAP assay, H_2O_2 scavenging activity, and metal chelating activity. Date extracts showed strong and concentration-dependant activity in all tested methods. The results showed that the best antioxidant activity was obtained in Allig, followed by Bejo and Deglet Nour. Total phenolics, total flavonoids, carotenoids, and tannins were determined spectrophotometrically in three date extracts. Results indicated that date contained significantly different amounts of these compounds. In fact, Allig presented the highest antioxidant compounds, followed by Bejo and Deglet Nour. Correlation analyses indicated a positive linear relationship between antioxidant activities and total phenolic and flavonoid content of date. This study demonstrates the potential antioxidant activity with Tunisian date, where we can use these natural extracts as food additives in replacement of synthetic compounds.

1. Introduction

Reactive free radicals, such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide, have been implicated in the development of many diseases such as cancer, coronary heart disease, autoimmune disease, diabetes, sclerosis, atherosclerosis, cataracts, and chronic inflammation. The damage caused by free radicals is due to their ability to inactivate many cells like protein denaturation, lipid peroxidation, membrane destabilisation, and DNA mutation which may lead to cancer [1–5].

Antioxidants, which can link reactive free radicals, are supposed to play an important role in human health and prevent the rancidity and lipid oxidation in food systems [2, 6]. Different methods were used to evaluate the antioxidant properties such as radical scavenging activity, reducing properties, metal chelating activity, hydrogen peroxide scavenging activity, or activation of various antioxidant enzymes and inhibition of oxidases [2, 4, 7].

The consumption of fruit and vegetables is associated with many benefits like anticarcinogenic, antiinflammatory, antimicrobial, anti-mutagenic, antithrombotic, neuroprotective, and antibiotic activities as well as reduction of cardiovascular diseases and cholesterol [1, 2, 5, 8, 9].

The protection offered by fruits and vegetables has been attributed to the presence of dietary antioxidants. These beneficial compounds were represented by polyphenols, flavonoids, ascorbic acid, carotenoids, and tocopherols [1].

Therefore, in recent years, the interest in the natural antioxidants has increased considerably in the human diet and the pharmaceutical products. These natural compounds can replace synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) that may exhibit toxicity and require high manufacturing costs.

Palm date (*Phoenix dactylifera* L.) has been an important crop in arid and semiarid regions of the world. This fruit has always played an important role in the nutritional, economic and social lives of the people of these regions.

Tunisia is considered to be one of the date producing countries. The amount of dates produced annually is about 145000 tonnes [10]. From this production, 30% is lost during picking, storage, commercialization, and conditioning processes [11–13]. These important quantities of by-products are generally discarded or used in animal feeding because of too hard texture, contamination with fungus, and infestation by insect or simply due to their low quality [14].

Some studies have been carried out to use these by-products to develop new products such as metabolites or biomass production [13]. In Tunisia, date by-products are classified as second-grade dates. However, besides nutritional components (carbohydrates, aminoacid, proteins, dietary fibres, etc.), they represent a potential source of various biologically active compounds (total phenolic compounds, flavonoids, carotenoids, etc.) which have antioxidant activity [8, 13, 15].

Antioxidant activity of this fruit from different origins has been studied from Algeria [16], Kuwait [17], Oman [8, 18], Iran [1, 15], and Yemen [4]. These results showed that date palm fruit possesses antioxidant properties which can vary on their phenolic content. However, there is no study, so far, that has dealt with the antioxidant activity of dates from Tunisia.

In this paper, our interest has been to study antioxidant activity and the antioxidant compounds in Tunisian dates.

The aim of this study was to evaluate the antioxidant activity of extracts from three date varieties cultivated in Tunisia using DPPH test, FRAP method, scavengers of H_2O_2, and metal chelating activities. Total phenolic, flavonoids, carotenoids, and tannins were evaluated and the correlation between antioxidant activities and total phenolic and flavonoid compounds has been reported.

2. Materials and Methods

2.1. Origin of Date Fruit. Second-grade dates (*phoenix dactylifera* L.), with texture defect (relatively hard) of the three cultivars in Tunisia, Deglet Nour, Allig, and Bejo, were obtained from Tozeur region (Tunisia). Dates were collected at "Tamr stage" (full ripeness). Ten kilograms from each variety were directly divided into bags of 500 grams and stored at −20°C prior to analysis.

2.2. Preparation of Extract. Prior to extraction processing, the date fruits were defrosted, cleaned, and pitted and the edible part of date was dried at room temperature before grinding it with a meat grinder (Moulinex, type Ne 401, france) to produce date paste.

The extraction of antioxidant compounds from all date cultivars was carried using acetone/H_2O (70 : 30, v/v) as described by Al-Farsi et al. [8], with slight modifications.

Two grams of sample was mixed for 2 h with 20 mL of solvent extract at room temperature and with continuous agitation.

The mixture was centrifuged at 5000 g for 15 min, and the supernatant was decanted. The pellets were extracted under identical conditions. Supernatants were combined and concentrated by a rotary evaporator at 40°C. The residues were dissolved in distillated water at different concentrations and used for the following experiments.

2.3. Determination of Antioxidant Components

2.3.1. Determination of Total Phenolic Contents. The polyphenols were determined by the Folin-Ciocalteu procedure according to Al-Farsi et al. [8].

200 μL of date extract (10 mg/mL) was mixed with 1.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) for 5 min at room temperature. 1.5 mL of aqueous sodium bicarbonate (60 g/L) was added, and the mixture was vortexed and allowed to stand at room temperature. After 90 min, the absorbance was measured at 725 nm. The total phenol concentration was expressed as mg of gallic acid equivalent (mg GAE) per 100 g of extract.

2.3.2. Determination of Total Flavonoids. Total flavonoid content was determined using colorimetric method described by Zhishen et al. [19].

1 mL of date extract (10 mg/mL) was diluted with 4 mL of distilled water. Then, 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of $AlCl_3$ (10%) was added and allowed to stand for 1 min. Then, 2 mL of NaOH (4%) was added and the mixture was diluted with 2.4 mL of distilled water. The solution was mixed and the absorbance was read at 510 nm after 15 min.

The total flavonoid content was calculated on the basis of the standard curve for catechin solutions and expressed as catechin equivalents.

2.3.3. Determination of Tannin Compounds. The total tannin content was estimated according to a literature procedure [20] by the Folin-Ciocalteu method, after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone, PVPP).

In brief, 1 mL of extract was added to 100 mg of PVPP. After 15 min at 4°C, the mixture was vortexed and centrifuged for 10 min at 1500 g. Aliquots of supernatant (200 μL) were transferred into test tubes and nonabsorbed phenolics were determined by Folin-Ciocalteu procedure. Calculated values were subtracted from total polyphenols contents and total tannin contents were expressed as mg gallic acid equivalents (GAE) per 100 g extract.

2.3.4. Determination of Total Carotenoids. Total carotenoids were measured according to the method of Al-Farsi et al. [8] with slight modifications. In brief, 2 g of date was extracted in 25 mL of acetone/ethanol (1 : 1, v/v) containing 200 mg/mL butylated hydroxytoluene (BHT). After extraction, the homogenate was centrifuged at 1500 g for 15 min at 4°C. The supernatant was collected and the residue was reextracted using the same method until exhaustion of colour. Finally, the combined supernatants were made up to 100 mL with the extraction solvent. Absorbance was measured at 470 nm using a spectrophotometer (SHIMADZU, UV mini 1240, Japan). Total carotenoids were calculated

using the following equation and expressed as milligrams per 100 g of fresh weight:

$$\text{Total carotenoids} = \frac{\left(\text{Ab} \times V \times 10^6\right)}{\left(A^{1\%} \times 100\, G\right)}. \quad (1)$$

Ab is the absorbance at 470 nm, V is the total volume of extract, $A^{1\%}$ is the extinction coefficient for a 1% mixture of carotenoids at 2500, and G is the weight of sample (g).

2.4. Evaluation of Antioxidant Activities

2.4.1. DPPH Radical Scavenging Activity. The antiradical activity of date sample, based on the scavenging activity of the stable free radical, DPPH (2,2-diphenyl-2-picrylhydrazyl), was estimated according to the method of Bertoncelj et al. [9].

Date extracts were dissolved in water at concentrations from 5 to 50 mg/mL. 0.1 mL of each solution was mixed with 1.9 mL of a solution of DPPH in absolute ethanol (130 μM) and 1 mL of acetate buffer solution (100 mM, pH 5.5).

Absorbance at 517 nm was determined after 90 min at room temperature in the dark.

The percentage inhibition activity was calculated from $[(A_0 - A_1)/A_0] * 100$. A_0 is the absorbance of the control, and A_1 is the absorbance of the extract.

2.4.2. FRAP Assay. Ferric reducing antioxidant power (FRAP) was determined in the extracts according to Bertoncelj et al. [9].

The principle of this method is based on the ability of the extract to reduce a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous colored form (Fe^{2+}-TPTZ) in the presence of antioxidants.

The FRAP reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6, and was prepared freshly and warmed at 37°C. 200 μL of sample (10 mg/mL) was mixed with 1.8 mL of FRAP reagent. After incubation for 10 min at 37°C, the absorbance was measured spectrophotometrically at 593 nm against a blank solution containing distilled water. A calibration curve is prepared using an aqueous solution of $FeSO_4$ as standard.

2.4.3. Scavenging Activity of Hydrogen Peroxide. The scavenging activity of date extracts on hydrogen peroxide was determined according to the method of Kumaran and Karunakaran [21].

A solution of hydrogen peroxide (2 mmol/L) was prepared in phosphate buffer (pH 7.4).

1.2 mL of each solution at different concentrations from 2 to 50 mg/mL was added to 1.2 mL of hydrogen peroxide solution. A blank solution was prepared in the same way but without H_2O_2.

The absorbance of hydrogen peroxide was measured spectrophotometrically at 230 nm after incubation during 10 min.

The percentage inhibition activity was calculated using the following formula:

$$\%\text{scavenging activity} = \left[\frac{(A_0 - A_1)}{A_0}\right] * 100, \quad (2)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract.

2.4.4. Metal Chelating Activity. The metal chelating capacity was determined by the method of Lee et al. [22].

Briefly, 1 mL of date extracts with different concentrations (10–150 mg/mL) was mixed with 3.7 mL of methanol, and 0.1 mL of a solution of 2 mmol/L $FeCl_2$ was added. After that, the reaction was mixed with 0.2 mL of 5 mmol/L ferrozine and the mixture was shaken and left standing for 10 min at room temperature.

Then, absorbance of solution was measured spectrophotometrically by measuring the formation of ferrous ion-ferrozine complex at 562 nm.

The metal chelating activity was calculated from $[(A_0 - A_1)/A_0] * 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the extract.

2.5. Statistical Analysis. All values are the means of three independent replications. Data are presented as mean ± standard deviation ($X \pm$ SD). Ducan's test and one-way analysis of variance (ANOVA) were used to compare results with $\alpha = 5\%$ (SPSS program, Windows 11.0).

3. Results and Discussion

3.1. Antioxidant Compounds

3.1.1. Total Phenolic Compounds. The amount of total phenolic contents was determined in the different extracts of three date varieties in Tunisia using the Folin-Ciocalteu method.

As shown in Table 1, there were significant differences ($P < 0.05$) among the different varieties of date. The result, expressed as gallic acid equivalent, ranged from 240.38 mg GAE/100 g extract to 505.49 mg GAE/100 g extract (Table 1).

The highest phenolic compound was obtained in Allig variety and the lowest was found in Deglet Nour variety.

These results are comparable to those obtained by previous studies despite using different phenolic acid standards for quantification. In fact, Al-Farsi et al. [8] reported that the total phenolic content ranged from 134 to 280 mg of ferulic acid equivalents (FAE)/100 g in fresh date varieties in Oman. Besides, the total phenolic compounds of two date varieties studied by Wu et al. [23] were 576 and 661 mg GAE/100 g. These results are comparable with those found in the present study.

However, Mansouri et al. [16] studied the phenolic profiles of seven different varieties of Algerien date. They found that phenolic content varied between 2.49 and 8.36 of GAE/100 g fresh weight. These levels are much lower compared to those found in this study. On the other hand, Biglari et al. [1] reported that total phenolic compounds

Table 1: Content of total phenolic, total flavonoid, total carotenoid, and tannins in different date extracts.

Sample	Phenolic content[a]	Total flavonoid[b]	Total carotenoid[c]	Tannins[a]
Allig	505.49 ± 3.36[a]	213.76 ± 1.52[a]	8.14 ± 0.16[b]	390.20 ± 4.22[a]
Deglet Nour	240.38 ± 1.12[b]	58.92 ± 2.13[b]	4.02 ± 0.45[c]	142.67 ± 2.42[b]
Bejo	391.94 ± 5.18[c]	150.11 ± 0.66[c]	12.41 ± 0.59[a]	285.80 ± 6.31[c]

Data are expressed as means ± SD ($n = 3$). Means, in the same column, with different letters are significantly different ($P < 0.05$).
[a]Milligrams of gallic acid equivalent per 100 g extract. [b]Milligrams of catechin equivalent per 100 g extract. [c]Milligrams per 100 g fresh weight.

varied between 2.89 and 141.35 mg GAE/100 g dry weight of Iranian date.

These results strongly suggest that date fruit may contain a higher level of phenolic compounds among fresh and dried fruits. These compounds are commonly found in plants and they have been reported to have a strong antioxidant activity. So, phenolic compounds are supposed to have multiple biological effects, such as anti-inflammatory, antimicrobial, antimutagenic activities. These constituents can also protect from coronary heart disease and cancer [9, 20, 21, 24].

The total phenolic content could be regarded as an important indication of antioxidant properties. This activity is mainly due to their redox properties; they can scavenge reactive oxygen species, neutralize free radicals, and decompose peroxides [3, 21].

The discordance in total phenolic content of date between different studies is due to variety, growing condition, maturity, season, agronomical differences, fertilizer, soil type, genomics, moisture content, climate, storage conditions, methods of extraction, and standards used [1, 13, 15, 18, 25–27].

This large variability can also be explained by the methods and the choice of solvent and the differences in extractability and solubility of phenolic compounds in different solvent. However, a mixed polarity solvent could extract more phenolic compounds, such that addition of water to 50% in acetone can increase extraction of condensed tannins and total phenolic content [2, 28].

3.1.2. Total Flavonoids. Total flavonoid content was determined by aluminium chloride methods and expressed as mg catechin/100 g extract.

From the results summarized in Table 1, significant differences ($P < 0.05$) between all types of date were observed. The results obtained showed that the total flavonoid content of three varieties of date varied considerably from 58.92 mg catechin/100 g extract to 213.76 mg catechin/100 g. Allig variety contained the highest amount of flavonoids (213.76 mg catechin/100 g) followed by Bejo and Deglet Nour varieties (150.11 mg catechin/100 g and 58.92 mg catechin/100 g, resp.).

The total flavonoid content determined in this study confirms previous results reported by Al-Mamary et al. [4] on different types of date cultivated in Yemen. They showed that the total flavonoid content ranged from 170 to 290 as quercetin equivalent/100 g palm date, Tamr.

On the other hand, these results are significantly higher than the values reported for different types of Iranien dates. However, Biglari et al. [1] reported that the total flavonoid content varied from 1.62 to 81.79 mg catechin equivalents/100 g dry weight of sample.

The difference between total flavonoid content in this study and other types of dates could be due to different factors such as the solubility and the extractability of the flavonoids in solvent, use of different analytical methods, and use of different standards [28].

In general, flavonoids are one of the most important phenolics which contribute to the antioxidant activity. These compounds possess many chemical and biological activities such as radical scavenging properties [1, 21].

It is possible that other phenolic compounds could also contribute to the antioxidant properties of these types of dates.

3.1.3. Determination of Tannins. The determination of tannin contents in acetone/H_2O extracts from three varieties of dates, Allig, Bejo, and Deglet Nour, was estimated using the Folin-Ciocalteu method and the results were expressed as gallic acid equivalents.

As displayed in Table 1, a significant difference ($P < 0.05$) between all types of date extracts was observed. In fact, we can conclude that Allig, Bejo, and Deglet Nour were rich in tannins, but we found that the best compounds were obtained in Allig extract.

So, this variety contained a significantly higher amount of tannins (390.20 mg GAE/100 g extract) than Bejo and Deglet Nour (285.80 mg GAE/100 g and 142.67 mg GAE/100 g, resp.).

These results are similar to those obtained in phenolic contents and suggest that tannins play an important role in the total phenolic compounds of date fruit.

3.1.4. Total Carotenoid Content. The total carotenoid content in the different varieties of date was presented in Table 1. Of the varieties studied, significant differences ($P < 0.05$) between all types of date were observed. Bejo had the highest amount of carotenoids (12.41 mg/100 g) followed by Allig (8.14 mg/100 g) and Deglet Nour (4.02 mg/100 g).

These levels are higher compared to those found in previous studies. In fact, Al-Farsi et al. [8] mentioned that carotenoids ranged from 1.31 to 3.03 mg/100 g in three varieties of fresh date grown in Oman (Fard, Khasab, and Khalas). This variation is probably due to the existing differences between the variety, growing condition, maturity, storage, and analysis conditions.

By comparison with other fruits, dates can be considered a good source of carotenoids. Moreover, Al-Farsi et al. [8] showed that carotenoid content in eight fruits studied

Table 2: Comparison of antioxidant properties of date extracts.

Samples	DPPH IC_{50} (mg/mL)	Metal chelating activity IC_{50} (mg/mL)	H_2O_2 IC_{50} (mg/mL)	FRAP ([$FeSO_4$] mmol/100 g extract)
Allig	16.70 ± 0.07^a	85.19 ± 0.19^a	10.51 ± 0.06^a	4.98 ± 0.19^a
Deglet Nour	46.79 ± 1.48^b	91.71 ± 1.40^b	20.44 ± 0.18^b	1.96 ± 0.03^b
Bejo	25.90 ± 0.21^c	90.72 ± 0.46^b	12.50 ± 0.05^c	3.24 ± 0.02^c

Each value is expressed as mean ± SD ($n = 3$).
Means, in the same column, with different letters are significantly different ($P < 0.05$).

Table 3: Correlation between total phenolic contents (TPC) and antioxidant activities of date extracts.

Correlation	R^2
TPC versus DPPH	0.97
TPC versus FRAP	0.96
TPC versus H_2O_2	0.94
TPC versus metal chelating activity	0.74

Table 4: Correlation between total flavonoid contents (TFC) and antioxidant activities of date extracts.

Correlation	R^2
TFC versus DPPH	0.98
TFC versus FRAP	0.96
TFC versus H_2O_2	0.95
TFC versus metal chelating activity	0.72

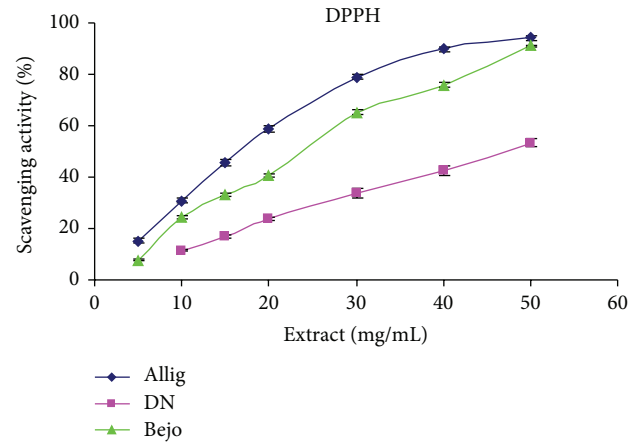

Figure 1: Effect of different concentrations on DPPH scavenging activity in different date extracts: Allig, Bejo and Deglet Nour (DN). Values are means of three replications ±SD.

ranged from 0.02 mg/100 g of fresh weight in strawberries to 2.26 mg/100 g in mandarins.

3.2. Antioxidant Activity. Antioxidant activity of three date varieties, based on DPPH, ferric reducing antioxidant power (FRAP), hydrogen peroxide scavenging activity, and metal chelating activity, was evaluated in this study.

3.2.1. DPPH Radical Scavenging Activity. DPPH is a stable organic free radical with a maximum absorption at 517 nm. The free radical scavenging activity is a commonly used method to evaluate the antioxidant activity in vitro. This method is based on the ability of antioxidant to scavenge the DPPH radical that can donate an electron or hydrogen atom.

As shown in Figure 1, it is noticed that the different extracts of date exhibited a potential free radical scavenging activity. In fact, high DPPH radical scavenging activity was observed in the following order: Allig > Bejo > Deglet Nour. These results suggest that scavenging abilities of different extracts of date against DPPH radical were concentration-dependant. So, by increasing the concentration of different extracts of date, their DPPH radical scavenging ability increases.

At the dose of 20 mg/mL, the DPPH radical scavenging activity is in the following order: Allig (58.77%), Bejo (40.78%), and Deglet Nour (23.98%).

DPPH is usually expressed as IC_{50}, the amount of antioxidant necessary for decreasing the initial concentration of DPPH by 50%. When the IC_{50} value of the sample was lower, the antioxidant activity was higher [9].

Table 2 presents the IC_{50} values of different date extracts. Significant differences ($P < 0.05$) were observed between their DPPH IC_{50} values. The highest antiradical activity was observed in the Allig extract and the lowest activity in Deglet Nour. The IC_{50} values of Allig, Bejo, and Deglet Nour were 16.70, 25.90, and 46.79 mg/mL, respectively.

The correlation between the free radical scavenging activity and total phenolic contents has been studied, and a positive correlation between them was observed ($R^2 = 0.97$) (Table 3). The high correlation coefficient reported that free radical scavenging activity may be attributed to their phenolic compounds.

Other studies showed good correlation between total phenolic contents and free radical scavenging activity [9].

The free radical scavenging activity of phenolic compounds is generally due to their redox properties, their ability to give a hydrogen using DPPH, and a single oxygen quencher. On the other hand, this activity is dependent not only on the concentration of phenolic compounds but also on the degree of hydroxylation and polymerisation [4, 7, 29–31].

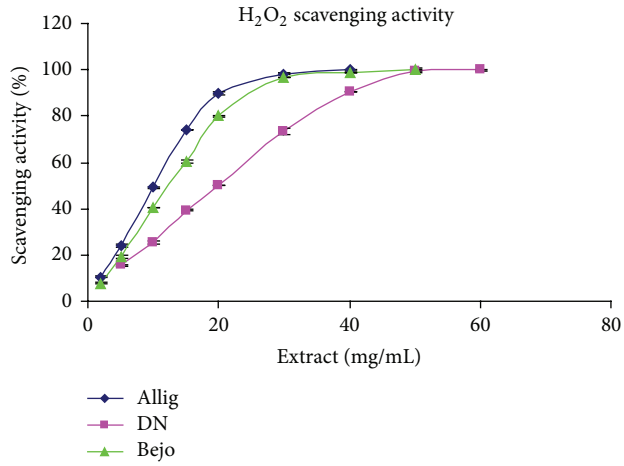

Figure 2: Effect of different concentrations on H_2O_2 scavenging activity in different date extracts: Allig, Bejo, and Deglet Nour (DN). Values are means of three replications ±SD.

Correlation between flavonoids for each variety and free radical scavenging activity was calculated. A highly significant influence was also exhibited with $R^2 = 0.98$ (Table 4). It is apparent that flavonoids were important phenolic compounds of the date contributing to the antioxidant activity. This is in agreement with the results reported by Mansouri et al. [16] and Biglari et al. [1, 15]. However, Harris and Brannan [28] mentioned that there is no positive correlation between flavonoid levels and radical scavenging activity.

In fact, the free radical scavenging activity of flavonoids is generally due to the number and arrangement of the hydroxyl groups of flavonoids [4].

3.2.2. FRAP Assay. For determination of antioxidant activity, we used the FRAP assay (ferric reducing antioxidant power). This test has been widely used to determine the antioxidant capacity of different extracts. The method is based on the ability of the sample to reduce the ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants [1, 9, 20, 27].

The averages of antioxidant activity based on FRAP assay are given in Table 2. There was a significant difference ($P < 0.05$) between all types of dates.

The FRAP activity of different extracts is in the following order: Allig (4.98 mmol [$FeSO_4$]/100 g extract), Bejo (3.24 mmol [$FeSO_4$]/100 g extract), and Deglet Nour (1.96 mmol [$FeSO_4$]/100 g extract). This large variability can be explained by the influence of different varieties.

These results are in agreement with the results determined by DPPH scavenging activities which showed that the highest antioxidant activity was obtained in Allig extract and the lowest activity in Deglet Nour extract.

A positive linear correlation between the total antioxidant activity, determined by the FRAP method, phenolic contents, and flavonoids was observed ($R^2 = 0.96$ (Table 3) and $R^2 = 0.96$ (Table 4), resp.).

The strong positive relationship indicates that phenolic and flavonoid contents were important compounds contributing to the antioxidant activities of date extract, but it is also possible that other phenolic compounds could contribute to the antioxidant capacities of date. These results are in agreement with other previous studies which presented a strong correlation between reducing power determined by FRAP assay and phenolic and flavonoid contents [1, 9, 20].

The reducing capacities are generally due to the ability of natural extract to reduce the cations by breaking the free radical chain by donating a hydrogen atom.

3.2.3. H_2O_2 Scavenging Activity. The scavenging ability of different date extracts with hydrogen peroxide is shown in Figure 2. It is noticed that all the date extracts exhibited a potential hydroxyl radical scavenging activity and this activity increases with an increasing concentration. These results revealed that the highest hydroxyl radical scavenging activity was in the Allig date extract. In fact, at the dose of 20 mg/mL, the scavenging effect of date extract with the H_2O_2 radical is as follows: Allig (89.99%); Bejo (80.14%), and Deglet Nour (50.32%).

On the other hand, Table 2 summarized the IC_{50} values from all date extracts and showed that significant differences ($P < 0.05$) were observed between them. So, the IC_{50} values from Allig, Bejo, and Deglet Nour were 10.51, 12.50, and 20.44 mg/mL, resp.).

Concerning H_2O_2 scavenging activity, a strong correlation was established with the phenolic contents of different extracts ($R^2 = 0.94$; Table 3) and the flavonoid contents ($R^2 = 0.95$; Table 4). These results suggest that phenol and flavonoid contents could be the probable contributors to their antioxidant activities and may probably be involved in removing the H_2O_2.

The present study is in agreement with many authors who have observed a direct correlation between hydroxyl radical activity and total phenolic and flavonoid contents [2, 6].

The quenching hydroxyl radical activity of different date extracts can be explained by the prevention of the propagation of lipid peroxidation process and the reduction of chain reaction due to the presence of phenolic compounds. However, hydrogen peroxide is dangerous because it can form the hydroxyl radical. This reactive free radical may be the origin of the toxic effects, the cytotoxicity of the mammalian and bacterial cells [2, 4, 6, 21]. Therefore, it is important to remove the H_2O_2 for antioxidant activity in cells or food systems.

3.2.4. Metal Chelating Activity. The metal chelating capacity is based on chelating of Fe^{2+} ions by the ferrozine reagent.

The presence of Fe^{2+}-ferrozine complex was measured by the reduction of formation of red-coloured complex [21].

In the present study, the chelating power from the three date extracts is shown in Figure 3. The results obtained indicated a concentration-dependant antioxidant capacity. The percentage of metal scavenging capacity of 50 mg/mL of Allig, Bejo and Deglet Nour was found to be 35.84%, 34.68%, and 30.81%, respectively.

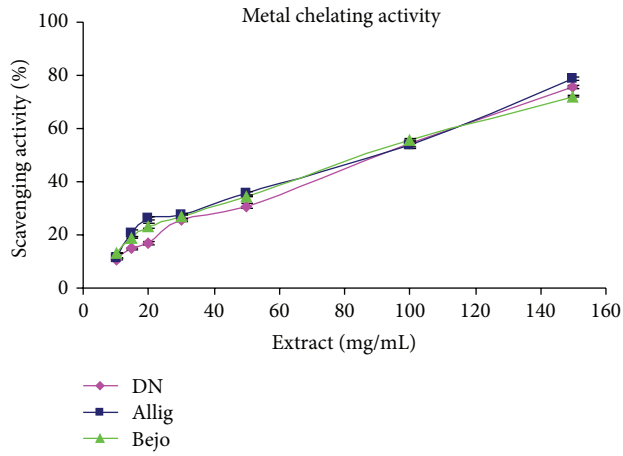

Figure 3: Effect of different concentrations on metal chelating activity in different date extracts: Allig, Bejo, and Deglet Nour (DN). Values are means of three replications ±SD.

These results were lower than that obtained by the previous activities. This is in agreement with the results obtained by Al-Mamary et al. [4] who showed that the palm date syrups have a low to intermediate iron binding capacity.

Results expressed as IC_{50} values are summarized in Table 2. Allig possessed the lowest IC_{50} value (85.19 mg/mL), but there is no significant difference between IC_{50} values from Bejo and Deglet Nour ($P < 0.05$) (90.72 mg/mL, 91.71 mg/mL, resp.).

In addition, the correlation of total phenolic contents and total flavonoids with metal chelating activity of various date extracts has been studied. Positive correlation factors were observed between chelating power and phenolic compounds ($R^2 = 0.74$; Table 3) and flavonoids ($R^2 = 0.72$; Table 4). These results suggest that metal chelating activity may be related to the presence of phenolic compounds. In fact, the metal chelating agents are effective as secondary antioxidants; they can inactivate metal ions (Fe^{2+}) and reduce the redox potential.

However, iron is essential for many activities like respiration, but it is capable of generating free radicals contributing to lipid peroxidation, protein modification, and DNA damage [4, 21].

4. Conclusions

The present study reported that Tunisian date varieties can be a good source of natural antioxidant. The antioxidant activities were evaluated using different methods, such as free radical scavenging activity, FRAP assay, H_2O_2 scavenging activity, and metal chelating activity. However, on the bases of the used methods, the antioxidant efficiency of date extracts can be arranged as follows: Allig > Bejo > Deglet Nour. This arrangement could be due to the difference in the phenolic, flavonoid, carotenoid, and tannin contents. A strong correlation was observed between antioxidant activities and phenolic and flavonoid contents.

These results signify that dates fruits can be a good source of natural antioxidant, which can play an important role in reducing oxidative stress and protecting of the human health from dangerous diseases, including cancer and liver and cardiovascular diseases.

Due to the importance of these scientific results, this work can be extended in vitro by evaluating the stabilization of edible oil with natural antioxidant and in vivo to evaluate the oxidative properties.

Conflict of Interests

According to this manuscript titled "In vitro antioxidant activities of three selected dates from Tunisia (*Phoenix dactylifera* L.)", there is no conflit of interests regarding the publication of this paper.

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Research Article

Optimization of *Agave tequilana* Weber var. *Azul* Juice Spray Drying Process

Alejandra Chávez-Rodríguez,[1] Irma G. López-Muraira,[1] Juan F. Goméz-Leyva,[1] Guadalupe Luna-Solano,[2] Rosa I. Ortíz-Basurto,[3] and Isaac Andrade-González[1]

[1] *Departamento de Estudios de Postgrado e Investigación (DEPI), Instituto Tecnológico de Tlajomulco, Km 10 Carr, San Miguel Cuyutlan, CP 45640, Tlajomulco de Zúñiga, JAL, Mexico*
[2] *DEPI, Instituto Tecnológico de Orizaba, Avenida Oriente 9 No. 852, Colonia Emiliano Zapata, CP 94320, Orizaba, VER, Mexico*
[3] *DEPI, Instituto Tecnológico de Tepic, Avenida Tecnológico No. 2595, Colonia Lagos del Country, CP 63175, Tepic, NAY, Mexico*

Correspondence should be addressed to Isaac Andrade-González; isaacag2001@yahoo.com.mx

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In this work, the response surface methodology was employed to optimize the microencapsulation of *Agave tequilana* Weber var. *azul* juice with whey protein isolated using a spray drying technique. A Box-Behnken design was used to establish optimum spray drying conditions for *Agave tequilana* juice. The process was optimized to obtain maximum powder yield with the best solubility time, hygroscopicity, bulk density, water activity, and reducing sugars. The independent parameters for the spray drying process were outlet temperature of 70–80°C, atomizer speed of 20000–30000 rpm, and airflow of 0.20–0.23 $m^3\ s^{-1}$. The best spray drying condition was at outlet temperature of 80°C, atomizer speed of 20000 rpm, and air flow rate of 0.23 $m^3\ s^{-1}$ to obtain maximum powder yield (14.65%$_{bm}$), minimum solubility time (352.8 s), maximum bulk density (560 kg m^{-3}), minimum hygroscopicity (1.9×10^{-7} $kg_{water}\ s^{-1}$), and minimum a_w (0.39). The *Agave tequilana* powder may be considered as an interesting source of dietary fiber used as food additive in food and nutraceutical industries.

1. Introduction

Agave tequilana Weber var. *azul* is an important crop in the state of Jalisco, México, for tequila production. Today, *Agave* juice is also used to make syrup and fructooligosaccharides (FOS) powder as another alternative industrial due to its high content of fructans. These *Agave* fructans consist of a complex mixture of FOS containing principally β(2-1) and β(2-6) linkages [1, 2], which can stimulate the growth of bifidobacteria as prebiotic, increase Ca^{++} absorption, and decrease blood triglyceride levels [3–6]. The prebiotic effect opens the new alternatives for *Agave* fructans as food ingredients: sweeteners, texture modifiers, and fat-replacer in food products [7]. Also, FOS are officially recognized as natural food ingredients and are classified as dietary fibers. However, the FOS are carbohydrates that undergo many changes, like the hydrolysis, Maillard-reaction, and caramelization [3].

Spray drying is one of the most important methods for obtaining powders. Although the spray drying is a fast process, changes on the spray drying conditions can affect the physicochemical and functional proprieties. For example, the spray drying of chicory inulin at a temperature range of 135–195°C resulted in significant FOS degradation (20 to 100%) and loss of its functional properties because the heat induced degradation [8, 9]. Microencapsulation of FOS in a carrier is an alternative technique that can be used to minimize degradation and loss of functional properties during spray drying [10, 11]. Spray drying is the most commonly used encapsulation method in the food industry [11] and exist carriers for several spray-dried products [12, 13]. The influence

of the main process variables, such as temperature, atomizer speed, air flow, feed flow, nature of food and its geometry, carriers types, and solution to sample ratio on the mass transfer mechanism, has been studied extensively [14–18].

Response Surface Methodology (RSM) will be a useful tool to obtain successful spray drying operating parameter, because RSM describes the effect of the test variables on the responses, determines interrelationships among test variables, and represents the combined effect of all test variables on the response [19, 20]. Moreover we studied out the effects of feed properties and drying conditions on the physical properties of the powder, like moisture content, bulk density, hygroscopicity, solubility time, and flow behavior. We found that inlet air temperature and feed flow rate are the significant parameters in case of most of the responses analyzed by [19]. It was found that the air temperature and airflow rate are the important parameters in case of most of the responses on ginger extract spray drying [21]. We found that the best drying conditions for the inulin were: 210°C and 5% and powder particles presented spherical and smooth surfaces [22].

Although the best drying conditions for inulin or FOS were reported by [22], additional work is needed to determine the optimal processing parameters to spray dry *Agave* juice. The purpose of this work was to optimize the spray drying process on physicochemical properties of *Agave tequilana* Weber var. *azul* fructooligosaccharides.

2. Materials and Methods

Seven-year-old *Agave tequilana* Weber var. *azul* "pine" and "head" were harvested and processed (washed, squeezed, and filtrated by Agaveros Industriales of Jalisco in Jocotepec, Jalisco, Mexico) to obtain the juice, which was kept at −20°C.

2.1. Spray Drying. A pilot scale spray dryer (GEA NIRO model A/S Production minor, Columbia, Washington, D.C., USA) with a cylindrical section of the drying chamber that is 1.2 m in diameter and 1 m high and the conical section that is 0.7 m high with a bottom outlet that is 0.3 m in diameter. The rotating disc atomizer has twenty-four annular 4 × 3 mm orifices on an 18 mm thick disc with a diameter of 0.10 m. The atomizer has the capacity to evaporate 40 kg of water per hour and was used for the drying process in all experimental treatment. The outlet temperature, atomizer speed, and airflow were set to 70 and 80°C, 20000 and 30000 rpm, and 0.20 and 0.23 $m^3\ s^{-1}$.

In each treatment, the *Agave* juice was defreezed at 4°C, concentrated at 20° Brix, and mixed with 0.1% commercial whey protein isolated WPC-80 (donated by America Alimentos Company). The juice mixture was homogenized using a mixer (Glas-Col Mod. Precision Stirrer, IN, USA).

The juice-whey protein isolated mixture was placed into a stainless steel container. A plastic flexible hose was placed inside the container and connected to the inlet of a variable flow peristaltic pump (Watson Marlon, Model 504U, Falmouth, Cornwall, UK). The pump outlet was then connected to the feed hose of the atomizer. The drying time in all treatments was 45 min.

The spray-dried powders were collected, weighed, and packed in 4 L glass flasks.

2.2. Bulk Density and Solubility Time. The total of 20 ± 0.1 g of powder was transferred into a 100 mL graduated cylinder and gently dropped onto a rubber mat from a height of 0.15 m for 40 times. The bulk density was calculated by dividing the powder weight by the volume it occupied in the cylinder; samples were run in duplicate [23–26].

The spray-dried powder solubility time was determined as described by [26]. Briefly, 10±0.1 g of powder was added to 250 mL distilled water at 25°C. The mixture was then agitated on a stirring hot plate (Cimarec, Model SP131015, Thermo Scientific, San Jose, CA, United States) set at position 350 rpm and the time required for the material to completely dissolve was recorded. All samples were run in duplicate.

2.3. Hygroscopicity. The total of 1 ± 0.01 g of powder was evenly spread on a glass dish (0.09 m diameter) with a high humid air-to-powder surface area ratio. Samples were then placed in desiccators set at 25°C and 85% relative humidity using an HNO_3 solution. A 90 min sampling interval was selected to obtain the moisture sorption kinetics. The weight gain of the samples was considerably lower after 90 min [23]. Thus, the weight increase per gram of powder solids after 90 min was determined [23, 26]. All samples were run in duplicate.

2.4. Water Activity. The water activity of *Agave* juice powders was measured at 25°C using an Aqualab 3TE (Decagon, Pullman, WA, USA) calibrated with a LiCl solution with known water activity. All samples were run in duplicate.

2.5. Yield. The *Agave* juice yield of all experiments was determined. The mass of product was divided by the product of the total mass of solution dried during each experiment (8.224 kg) and the total solids concentration. The average and range of yields for a particular set of operating conditions were then calculated from the three repeat experiments. To estimate the yield and for further spray-drying analysis, only the powder collected from the sample pot was considered.

2.6. Total Reducing Sugars. Direct reducing sugars were measured by the Fehling method modified [27], without hydrolysis. All samples were run in duplicate.

2.7. Experimental Design and Optimization. The effects of the three independent processing parameters, outlet temperature (X1, °C), atomizer speed (X2, rpm), and airflow (X3, $m^3\ s^{-1}$) on the dependent variables were investigated; the response surface methodology is presented in Table 1. Box-Behnken designs are response surface designs requiring only three levels, which are coded as −1, 0, and +1. A total of 17 experiments in this study were based on three levels and the three-factor experimental design, with three replicates at the centre of

Table 1: Box-Behnken experimental design.

Std. order	Run order	Independent variables		
		Temperature (°C)	Atomizer speed (rpm)	Air flow (ms^{-1})
2	1	80 (+1)	20,000 (−1)	23 (0)
14	2	75 (0)	25,000 (0)	23 (0)
8	3	80 (+1)	25,000 (0)	24 (+1)
1	4	70 (−1)	20,000 (−1)	23 (0)
15	5	75 (0)	25,000 (0)	23 (0)
9	6	75 (0)	20,000 (−1)	22 (−1)
4	7	80 (+1)	30,000 (+1)	23 (0)
7	8	70 (−1)	25,000 (0)	24 (+1)
13	9	75 (0)	25,000 (0)	23 (0)
10	10	75 (0)	30,000 (+1)	22 (−1)
11	11	75 (0)	20,000 (−1)	24 (+1)
3	12	70 (−1)	30,000 (+1)	23 (0)
6	13	80 (+1)	25,000 (0)	22 (−1)
12	14	75 (0)	30,000 (+1)	24 (+1)
5	15	70 (−1)	25,000 (0)	22 (−1)

Values in parentheses () indicate coded levels.

the design to estimate the pure error sum of squares. The statistical software MINITAB (Release 14.1) was used for the experimental design, data analysis, and regression modeling. The independent variables were X1 (70–80°C), X2 (20,000–30,000 rpm), and X3 (0.20–0.23 $m^3\ s^{-1}$) with point center at 75°C, 25000 rpm, and 0.215 $m^3\ s^{-1}$, respectively. The experimental order was randomized. Experimental data from the Box-Behnken design was analyzed and fitted to a second-order polynomial model. Consider

$$Y_k = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i} \sum_{j=i+1} \beta_{ij} X_i X_j, \quad (1)$$

where Y_k is the predicted response, β_0 is the constant (intercept), β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the cross-product coefficient. X_i and X_j are independent variables.

3. Results and Discussion

3.1. Fitted Models and Response Surfaces. Results of the experimental design with standard deviation to quality parameters of *Agave* juice subjected to different spray drying conditions are shown in Table 2. The regression coefficients (β) of the quadratic polynomial equation for the coded independent variables, interactions upon response variables, determination coefficient, and lack-of-fit test and probability are shown in Table 3 for all the responses. The significant terms ($P \leq 0.05$) were used as a tool to check the significance of each of the coefficients of the proposed models for each response.

Analysis of variance showed that the quadratic polynomial models were highly significant ($P \leq 0.001$) for solubility (Y5) and yield (Y6) and less significant for bulk density (Y2), activity water (Y1), hygroscopicity (Y3), and reduced sugar (Y4). The coefficients of determination ($R2$) values for the response variables Y6 and Y5 were greater at 0.99 and 0.98, respectively; for variable Y2, the coefficient was 0.82; and for variables Y4, Y3, and Y1, the coefficients ranged from 0.66 to 0.59. The coefficients of variation (CV) should not be greater than 10%, but in this work, they were found in the range of 0.026–4.72% for all the responses, which indicates better precision and reliability of the experiments carried out. The lack-of-fit, which measures the fitness of the models, resulted in a significant F-value only for solubility and yield, indicating that these models were sufficiently accurate for predicting those responses. The P values of the models were 0.0001 for yield, 0.001 for solubility, 0.16 for bulk density, 0.558 for hygroscopicity, 0.484 for reduced sugar, and 0.638 for water activity, which further indicates the goodness of fit.

3.2. Response Surface Analysis of Powder Yield. Using multiple regression techniques, a response surface model was developed for powder yield as a function of the spray drying process variables. A complete-second order model (1) was tested for its ability to describe the response surface. The analysis of variance (Table 3) shows that the model is highly significant ($P < 0.0001$). Values of P less than 0.05 indicate that the model terms are significant. In this case, β_2, β_3, β_1^2, β_2^2, β_3^2, β_{12}, β_{13}, and β_{23} are significant model terms. Values greater than 0.10 indicate that the model terms are not significant. The lack-of-fit P value of 0.029 implies that the lack-of-fit is highly significant. Thus, all of the quadratic terms are significant.

The powder yield varied in the range from 24.85% to 76.70%, in our experiment runs. The quadratic polynomial model was used to fit the quadratic model for this response with $R2 = 0.990$. The ANOVA analysis for the response "powder yield" showed that atomizer speed and airflow are more significant than outlet temperature. However, in two representative plots are shown the effects of outlet

Table 2: Responses to the quality parameters investigated.

Std. order	Run order	Dependent variables											
		Activity water		Bulk density		Hygroscopicity $\times 10^{-7}$		Reduced sugars		Solubility		Yield	
		a_w	σ	(kg m^{-3})	σ	(kg$_{water}$ s^{-1})	σ	(kg L^{-1})	σ	(s)	σ	(%)	σ
2	1	0.352	0.013	0.556	0.000	2.011	0.038	0.005	0.004	73	0.000	49.70	0.043
14	2	0.418	0.047	0.556	0.095	2.139	0.011	0.008	0.003	41	0.001	24.85	0.028
8	3	0.484	0.067	0.556	0.029	2.000	0.017	0.004	0.011	36	0.001	51.70	0.011
1	4	0.353	0.019	0.588	0.000	2.006	0.010	0.004	0.007	58	0.001	34.70	0.044
15	5	0.415	0.010	0.500	0.051	2.153	0.015	0.009	0.003	46	0.001	26.35	0.020
9	6	0.415	0.023	0.588	0.023	1.967	0.053	0.009	0.003	53	0.001	36.80	0.014
4	7	0.343	0.006	0.556	0.009	1.944	0.015	0.017	0.006	66	0.001	70.45	0.005
7	8	0.323	0.022	0.667	0.067	2.047	0.021	0.016	0.007	55	0.001	70.15	0.008
13	9	0.358	0.010	0.521	0.007	2.014	0.053	0.005	0.010	50	0.000	26.25	0.004
10	10	0.339	0.009	0.541	0.000	1.928	0.040	0.015	0.004	65	0.001	76.70	0.007
11	11	0.418	0.054	0.625	0.000	2.050	0.016	0.016	0.004	53	0.001	70.80	0.004
3	12	0.361	0.016	0.541	0.000	1.967	0.044	0.013	0.009	69	0.000	76.00	0.005
6	13	0.344	0.008	0.541	0.000	2.025	0.041	0.005	0.010	76	0.002	50.60	0.008
12	14	0.323	0.099	0.556	0.023	2.158	0.020	0.008	0.003	38	0.002	73.30	0.008
5	15	0.342	0.005	0.541	0.007	2.008	0.023	0.003	0.004	44	0.001	34.45	0.028

σ : standard deviation.

Table 3: Analysis of variance of the regression coefficients of the fitted quadratic equation for the quality parameters studied.

Coefficients	Response					
	Aw (Y1)	Bulk density (Y2) $Kg\,L^{-1}$	Hygroscopicity (Y3) $Kg_{water}\,s^{-1}$	Reduced sugar (Y4) $Kg\,L^{-1}$	Solubility (Y5) s	Yield (Y6) %
β_0 (intercept)	0.397^{***}	0.5257^{***}	0.7567^{***}	7.4^{*}	7.59^{***}	5.1633^{***}
Linear						
β_1	0.018 ns	−0.016 ns	−0.0021 ns	−0.8 ns	0.5113^{*}	0.1788 ns
β_2	−0.0215 ns	−0.0204 ns	−0.0016 ns	2.4625 ns	0.0325 ns	2.6112^{***}
β_3	0.0135 ns	0.0241 ns	0.0148 ns	1.3875 ns	-1.1638^{**}	1.685^{**}
Cross-product						
β_{12}	−0.0043 ns	0.0117 ns	−0.0025 ns	0.7 ns	-0.755^{*}	-1.0275^{**}
β_{13}	0.0398 ns	−0.0278 ns	−0.0058 ns	−3.2 ns	-2.1275^{***}	-1.73^{***}
β_{23}	−0.0047 ns	−0.0055 ns	0.0133 ns	−3.375 ns	-1.13^{**}	-1.87^{***}
Quadratic						
β_{11}	−0.0226 ns	0.0167 ns	−0.0226 ns	−1.3875 ns	1.8113^{**}	1.9221^{*}
β_{22}	−0.0221 ns	0.0179 ns	−0.0206 ns	3.7375 ns	1.7037^{**}	4.4571^{**}
β_{33}	−0.0011 ns	0.0339 ns	−0.0068 ns	0.8375 ns	−0.6087 ns	3.2596^{**}
R^2	0.59	0.82	0.63	0.66	0.98	0.99
CV	0.049	0.029	0.026	4.727	0.51	0.76
Lack-of-fit	0.266	0.504	0.577	0.118	0.951	0.029
P	<0.638	<0.160	<0.558	<0.484	<0.001	<0.0001

*Significant at $P \leq 0.05$, **significant at $P \leq 0.01$, and ***significant at $P \leq 0.001$.
ns: not significant.

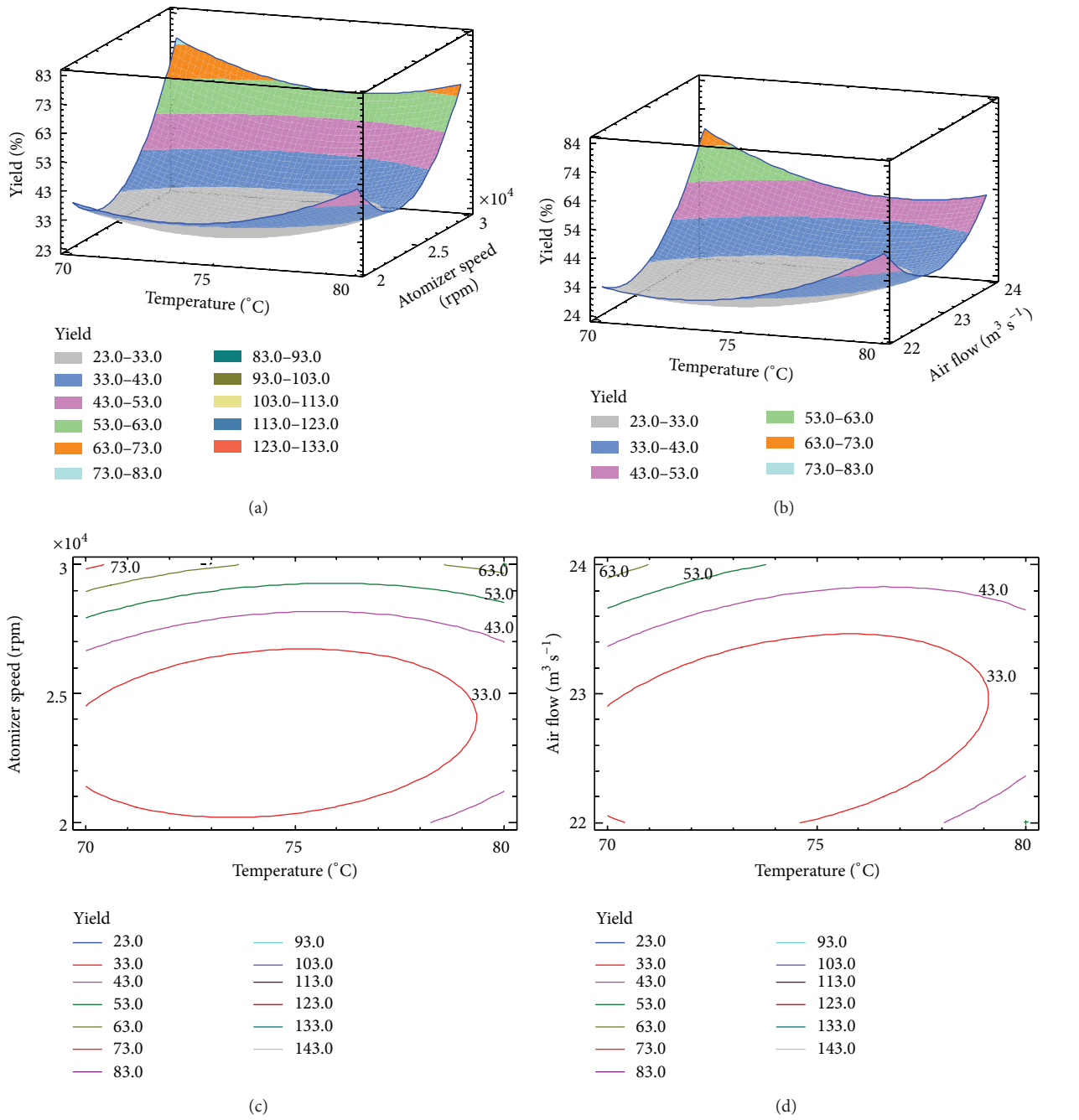

FIGURE 1: Surface plot showing (a) the effect of temperature and atomizer speed at air flow 0.215 $m^3 s^{-1}$ and (b) the effect of temperature and air flow at an atomizer speed of 25000 rpm on the powder yield.

temperature with atomizer speed and airflow (Figures 1(a) and 1(b)). Figure 1(a) shows the effect of temperature and atomizer speed on powder yield at the airflow center point (0) and Figure 1(b) shows the effect of outlet temperature and airflow. In Figure 1(a) it is evident that at lower outlet temperatures and higher atomizer speeds the powder yield presents the higher value. Evenly in Figure 1(b) we can also observe that at lower outlet temperature and higher airflow the powder yield presents the higher value. Even if the inlet temperature was constant in all experiments (180°C), the increase of feed flow due to control of the outlet temperature and the nature amorphous of spray-dried material causes significant problems with the deposition of powder on the wall of spray dryer, which reduces the powder yield [19, 22].

3.3. Response Surface Analysis of Solubility Time. Using multiple regression techniques, a response surface model for the powder solubility time, as a function of spray drying process variables, was developed. A complete second-order model

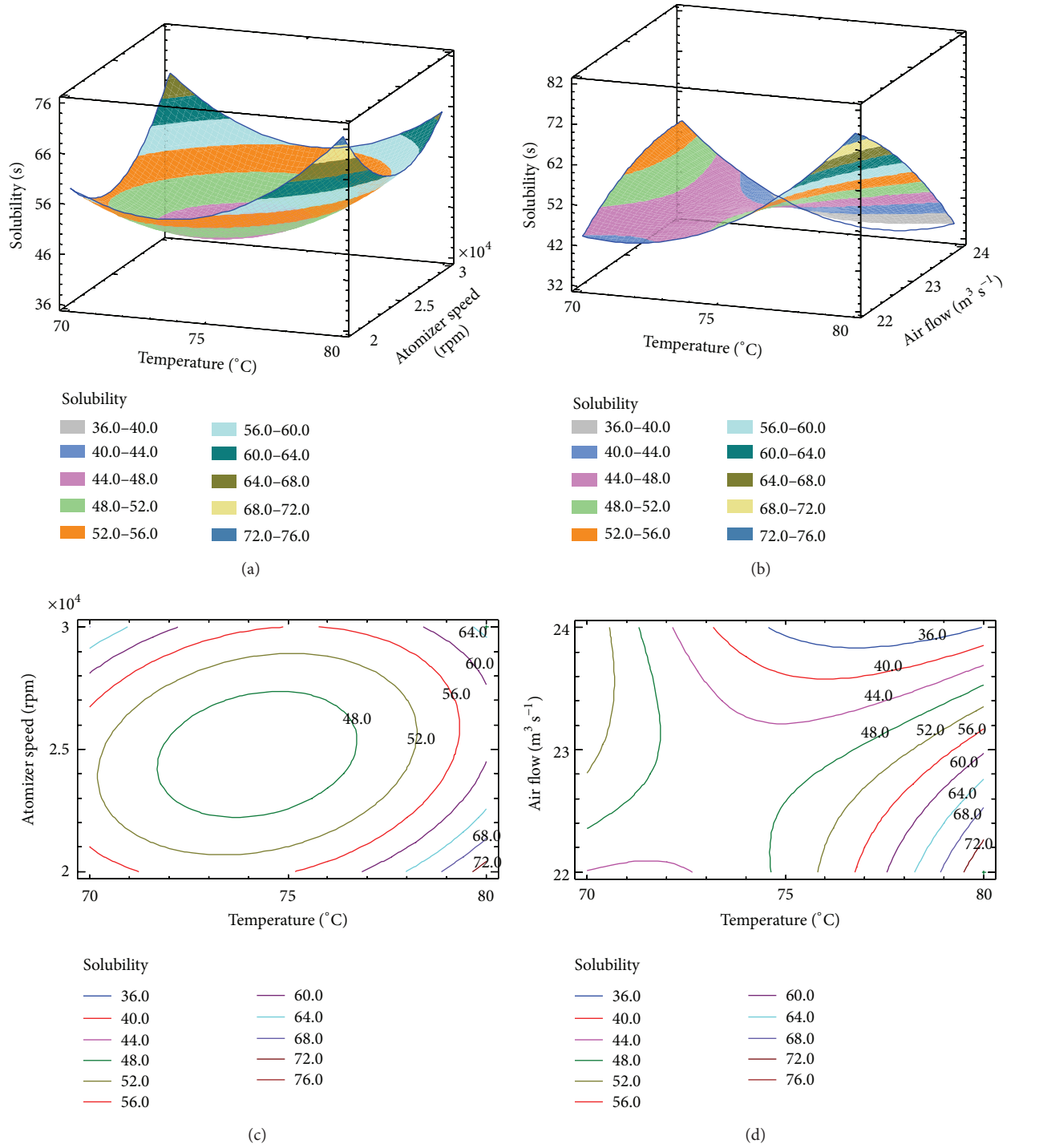

FIGURE 2: Surface plot showing (a) the effect of temperature and atomizer speed at an air flow of 0.215 $m^3\ s^{-1}$ and (b) the effect of temperature and air flow at an atomizer speed of 25000 rpm on the powder solubility time.

(1) was tested for its ability to describe the response surface. Analysis of variance (Table 2) shows that the model is highly significant ($P < 0.001$). Values of P less than 0.0500 indicate that the model terms are significant. In this case, β_0, β_1, β_3, β_1^2, β_2^2, β_{12}, β_{13}, and β_{23} are significant model terms. Values greater than 0.1000 indicate that the model terms are not significant. The lack-of-fit P value of 0.951 implies that the lack-of-fit is slightly significant. However, the determination coefficient was at 0.98.

Figures 2(a) and 2(b) present the surface and contour plots for solubility. Figure 2(a) shows the effect of temperature and atomizer speed at the airflow centre point (0.200 $m^3\ s^{-1}$) on powder solubility. It is evident from Figure 2(a) that the solubility showed an increase with an increase in outlet

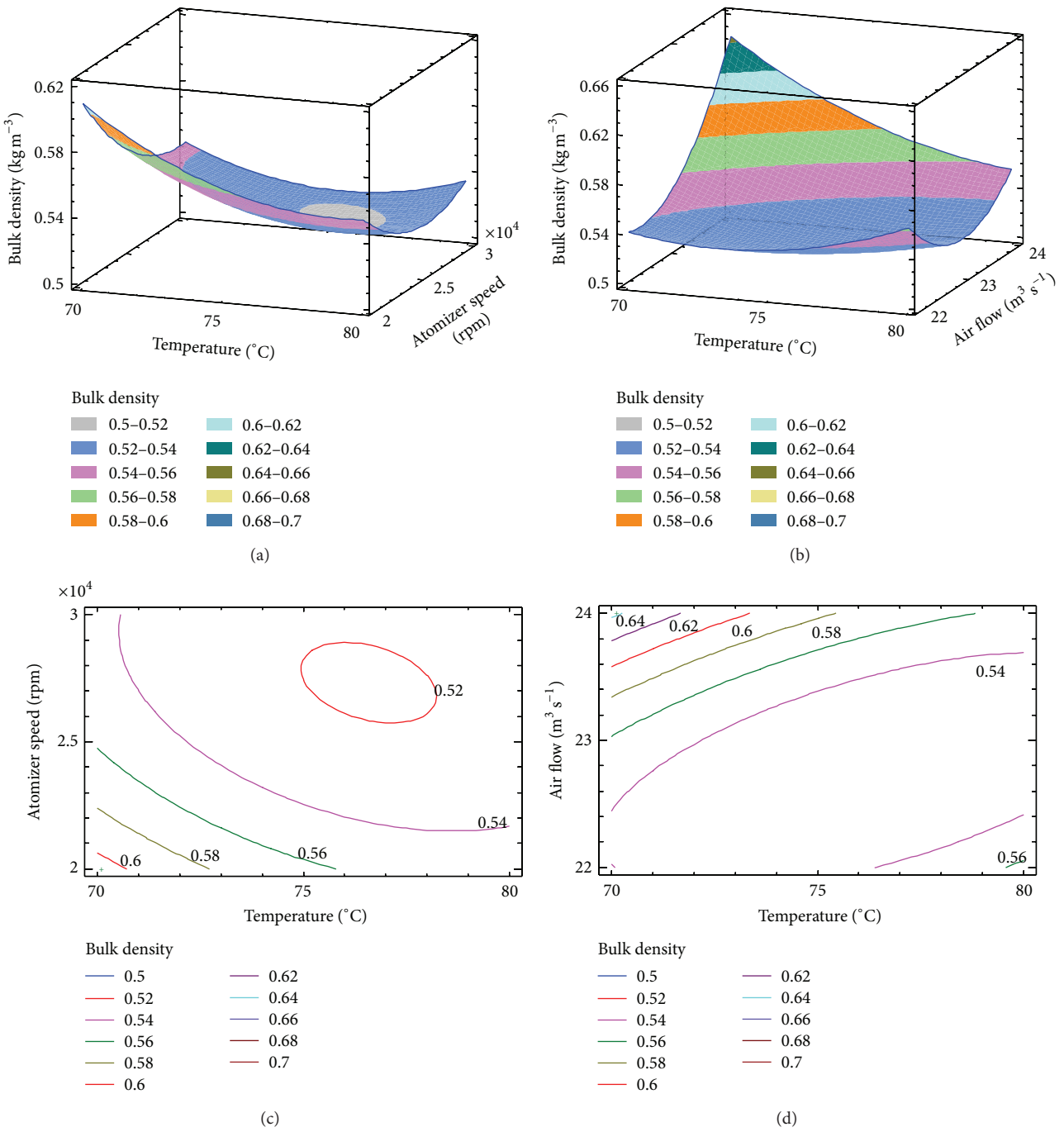

FIGURE 3: Surface plot showing (a) the effect of temperature and atomizer speed at an air flow of 0.215 m^3 s^{-1} and (b) the effect of temperature and air flow at an atomizer speed of 25000 rpm on the bulk density.

temperature and atomizer speed. However, at the centre point, the solubility decreases to a lower value at 38 s. Figure 2(b) shows that with an increase in outlet temperatures the solubility is also increased, but an increase in airflows represents a decrease in the solubility. This trend is similar to that reported by other works [24, 26].

3.4. Response Surface Analysis of Bulk Density. A surface response model was also developed for bulk density as a function of the spray drying process variables. A complete second-order model (1) was tested for its ability to describe the response surface. Analysis of variance (Table 2) showed that the model was not significant ($P < 0.05$). In this case only β_0 was significant. Values greater than 0.1000 indicate that the model terms are not significant. The lack-of-fit P value of 0.504 implies that it is not significant. Thus, it means that none of the quadratic terms were significant. However, the determination coefficient was at 0.82.

Figures 3(a) and 3(b) show the surface and contour plots for bulk density. Figure 3(a) shows the effect of temperature and atomizer speed at the airflow centre point ($0.200\ m^3\ s^{-1}$) on bulk density. It is evident from Figure 3(a) that, at lower level of outlet temperatures and atomizer speed, the bulk density reaches the highest value, as well as higher level of airflow and low level of outlet temperature (Figure 3(b)). In Figures 3(a) and 3(b), the bulk density has deceased with the increase of the outlet temperature due to an increase in the feed flow caused by outlet temperature control in each experiment [21, 26]. This same behavior was observed for hygroscopicity, water activity, and reducing sugars.

3.5. Optimization of Spray Drying Conditions. The spray drying process was optimized to the responses using a numerical optimization technique in which an equal importance of "one" was given to all three process parameters (temperature, atomizer speed, and airflow). The process was optimized to maximize powder yield, solubility, and bulk density and to minimize hygroscopicity and water activity. As a result of, the optimum operating conditions for temperature, atomizer speed, and airflow were 80°C, 20,000 rpm, and $0.230\ m^3\ s^{-1}$, respectively. The solution for the optimum spray drying conditions was found to satisfy the goal with a powder Yield same at 73.3% w/w, solubility at 35.28 s, hygroscopicity at $1.9 \times 10^{-7}\ kg_{water}\ s^{-1}$, bulk density at $560\ kg\ m^{-3}$, and water activity (aw) at 0.39.

4. Conclusions

The responses were correlated with independent variables using proper transformations of the responses, and the data points were fitted in a quadratic model with significant values of *R*2. Spray drying of *Agave tequilana* juice regardless of outlet temperature, atomizer speed, and air flow did not cause a significant difference in hygroscopicity, bulk density, water activity, and reduced sugar. However, significant effects were observed for powder yield and solubility at low or high temperatures, atomizer speeds, or airflows. Spray drying of *Agave* juice at low outlet temperatures (70°C) enhanced the cloud value with a maximum powder yield. The optimization of the drying process showed that the best conditions for pilot spray drying are inlet drying temperature of 180°C and outlet drying temperature of 80°C associated with an atomizer speed of 20,000 rpm and an airflow of $0.23\ m^3\ s^{-1}$, maintaining the material at room temperature (25°C) during the feeding of the dryer. Optimization of responses was based on minimum values of water activity and hygroscopicity and maximum values of yield, solubility, and bulk density.

Nomenclature

Y: Predicted response
X: Independent variables.

Greek Letters

β_0: Constant (intercept)
β_i: Linear coefficient
β_{ii}: Quadratic coefficient
β_{ij}: Cross-product coefficient.

Subscripts

i: Number of independent variables.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Chemical Composition, Functional Properties, and Effect of Inulin from Tunisian *Agave americana* L. Leaves on Textural Qualities of Pectin Gel

Mohamed Ali Bouaziz, Rabaa Rassaoui, and Souhail Besbes

Ecole Nationale d'Ingénieurs de Sfax, Laboratoire Analyses Alimentaires, Route de Soukra, 3038 Sfax, Tunisia

Correspondence should be addressed to Mohamed Ali Bouaziz; medalibouaziz@yahoo.fr and Souhail Besbes; besbes.s@voila.fr

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In this study, the chemical composition and functional properties of *Agave americana* L. (AA) leaves were determined. The *Agave* leaves powder had a high amount of total dietary fiber (38.40%), total sugars (45.83%), and protein (35.33%), with a relatively low content in ash (5.94%) and lipid (2.03%). The *Agave* leaves were exhibited with potential food application. The *Agave* inulin showed a significant difference compared with the commercial inulin as for aw (0.275 against 0.282), pH (5.53 against 5.98), ash (2.89% against 1.19%), protein (3.46% against 1.58%), water holding capacity (2.42% against 1.59%), solubility (73 g/L against 113 g/L), and emulsion capacity (14.48% against 21.42%), respectively. The textural properties of *Agave* inulin-pectin mixed gels were examined using instrumental Texture Profile Analysis (TPA). Firmness of the prepared *Agave* inulin-pectin mixed gels was lower than the pectin gel (0.3554 N against 5.7238 N, resp.). This reduction of firmness showed a synergetic effect between pectin and inulin. These results suggested a positive interaction between *Agave* inulin and pectin to decrease the firmness of mixed gels and open a good alternative to obtain value added products from this resource.

1. Introduction

Agave is usually thrived in semiarid regions such as Mexico, Australia, and Africa. Commonly grown species include *Agave americana* L., *Agave attenuata*, and *Agave tequilana*. Different from other *Agave* species, AA L. has a large asparagus-like flower stalk, but no piñas. Because of no piñas (a reservoir of fructans), the AA is commercially less valuable for the production of alcoholic beverages, compared to other *Agave* species such as *Agave tequilana* and *Agave attenuate*, although its leaves can be used for pulque (a beer-like drink) production. *Agave* is the biggest genus that identifies a group of desert plants belonging to the monocotyledonous family called Agaveceae [1]. This genus is characterized by spiny leaves yielding various types of fibers and composed of wild plants that do not need tender care and are traditionally used as source of fibers. The North American AA plant is a species belonging to such a genus, which is also flourishing in South of Africa as well as the Mediterranean area [2]. Various species of *Agave* are used in the traditional medicine either as medicinal plants or as good anti-inflammatory agents [3, 4]. Uribe and Saldivar [5] confirmed the anticancerogenic and antioxidant properties of the *Agave* syrup. This plant has been shown to have both antibacterial and antifungal properties [6]. Moreover, the leaf of AA base contains up to 16% of fructans. Pina and leaf base can be used for the commercial production of fructans and long-chain inulin, which can be used as vaccine adjuvant in the pharmaceutical industry [7]. This *Agave* plant is native to Mexico and other parts of the Caribbean area [8, 9]. Plants were taken from there to Europe, Africa, and the Far-East by the Spanish and Portuguese, where they naturalized rapidly, especially in the high arid regions around the shores of the Mediterranean [10].

In Tunisia, the AA is the most abundant variety of *Agave* [11]. This variety is characterized by the fact that it is a much voluminous plant with long, fleshy, rigid, hard-surfigured, and lanceolate leaves growing directly out from the central stalk to form a dense rosette. Its floral stalk, sometimes

termed the trunk, can reach 10 to 20 m of length. Evaluation of AA as a source of fiber was launched recently in Tunisia, where fibers are extracted traditionally and used for making twines and ropes [12]. The AA was much used by Tunisians for its fibers when fibers extracted by simple immersion in seawater were used to make ropes and twines for agricultural, marine purposes and known for its wealth of structural insoluble polysaccharides [13] and soluble polysaccharides [14]. Thus, it would be wise to valorise any noble fractions of AA.

On the other hand, inulin is the second polysaccharide reserve most abundant after starch. It is the main reserve carbohydrate [15–17]. It can be found, for instance, in onions (1–5% on a fresh weight basis), garlic (4–12%), banana (0.2%), and chicory roots (15–20%). Indeed, by its chemical structure, inulin is not hydrolysed or absorbed in the small intestine, and then it is considered a soluble fiber that can be incorporated into various food products. Its low sweetness and its properties similar to sucrose allow it to replace sugar in some formulations. inulin stimulates the growth of bifidobacteria, which is believed to have health-promoting functions. Many other health enhancing aspects of inulin concern diabetes, lipid metabolism, cancer prevention, and antiulcer activity [18, 19].

The technological use of inulin is based on its properties as a sugar replacer (especially in combination with high intensity sweeteners), as a fat replacer and texture modifier. For fat replacement in low-fat dairy products inulin seems particularly suitable as it may contribute to an improved mouthfeel. Also, inulin was used to improve rheological characteristics and nutritional properties of food and to be classified among functional foods [20].

Inulin gel formation is different from that obtained with hydrocolloids. inulin forms particle gels, whereas the increase of viscosity through most hydrocolloids is obtained by bonds between chains [21]. Rheological properties of inulin are quite well documented in the literature [22–24]. Interactions of inulin with some carbohydrates such as maize starch, maltodextrins, or pectin have also been analysed [24–26].

Gelling properties of pectin may be affected by many factors. Increased degree of methoxylation (DM) resulted in higher setting temperature and so more rapid gel formation for high methoxyl pectin (PHM) [27]. C. Rolin and J. de vries [28] reported that calcium addition also influences gel formation behaviour of PHM [28]. Moreover, gelling temperature increases in the presence of Ca^{2+}. Calcium content influences also the rheological behaviour of low methoxyl (LM) pectin gels by increasing G' (elastic modulus), but at Ca^{2+} levels that are too high, syneresis may occur. Contrarily to PHM, the gel temperature increases with decreasing DM. In addition, LM pectin with a blockwise distribution of free carboxyl groups is very sensitive to calcium [29].

Interactions between mixed biopolymer systems of which pectin is one component have been largely studied, such as pectin/alginate [30], pectin/starch [31], and pectin/gelatine [32] mixtures. However, few studies exist on the behaviour of mixed inulin-pectin gels. Pectin mixtures are widely used in food applications to obtain products with better properties.

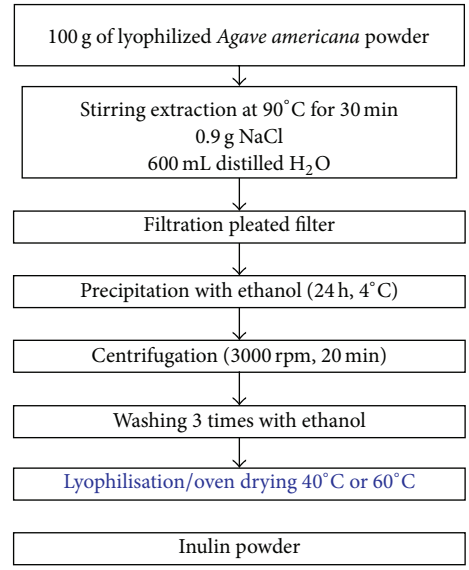

Figure 1: Extraction diagram of inulin from *Agave americana* L.

The aim of the present work is to characterize leaves powder and inulin from the AA and next to study the synergistic effect of inulin on pectin gel for food preparations.

2. Materials and Methods

2.1. Origin of Materials. AA plants cultivated organically were collected at the same time from the same cultivation zone (M'saken, Sousse, Tunisia). Leaves were obtained from plants at the same stage of maturation. In this work, the basal leaves of AA were used. 5 kg of leaves is cut into large pieces and stored at −20°C until use for the different analyses.

The pectin (high methyl pectin (PHM), medium rapid set) was supplied by Zina company, Sfax, Tunisia.

2.2. Preparation of the Sampling. At the first step, AA leaves were washed with water and the chlorophyll cuticle is removed. Then, the leaves are cut into small pieces and milled using a laboratory mixer. After that, the resulting biomass was lyophilized and stored at 4°C until the analysis.

2.3. Extraction Process. The inulin from AA leaves was extracted by mixing 600 mL of distilled water per 100 g of sample and the mixture was blended in a mechanical device made of stainless steel with 0.9 g of salt/L and then stirred at 90°C for 30 min (Figure 1). The suspension was filtered on canvas and then the supernatant was filtered under vacuum with Whatman paper. The filtrate was precipitated with ethanol (90%) overnight at 4°C and centrifuged at 3000 rpm for 20 minutes. The obtained pellet was subjected to three washes with ethanol, lyophilized or oven dryed at 40°C/60°C, and stored in desiccators until they were analysed [33, 34].

2.4. Chemical Composition. All analytical determinations were performed at least in triplicate. Values of different

parameters were expressed as the mean ± standard deviation ($X \pm SD$).

Dry matter was determined according to the Association of Official Analytical Chemists [35].

Nitrogen content of samples was determined by Kjeldahl method, following the method of the AOAC (1995) [35]. Protein content of each sample was calculated by multiplying the total nitrogen content by a factor of 6.25 [36].

Ash content was determined after incineration at 550°C, during 8 hours, using a muffle furnace (NABER, Germany). It was expressed as percent of dry weight [35].

Fat content was determined by continuous extraction with a Soxhlet on samples previously dried and ground, according to the method of the AOAC. The solvent used for this analysis is hexane [35].

Fiber was determined by the adopted method described by Prosky et al. (1988) [37]. This is an enzyme-gravimetric method officially classified by AOAC (1995) [35]. The *Agave* leaves were crushed by an electric grinder for fine particles. Then, the sampling is gelatinized with a thermostable α-amylase (A-3306) and next treated with a protease (P-3910) and amyloglucosidase (A-3042) (11 500 units/mL) to hydrolyze proteins and starch.

After enzymatic hydrolysis, the residues were recovered by centrifugation and washed with distilled water (twice), alcohol 95% (twice), and acetone (once). Finally, residues are dried and weighed. Corrections are made during the determination of protein and ash. Insoluble fiber (IF) content is calculated using the following formula:

$$\%IF = (\text{Residue} - (\text{Protein} + \text{Ash})) \times 100. \quad (1)$$

After enzymatic attack, 4 volumes of 95% ethanol were added to the supernatant to precipitate inulin. The precipitate, collected by centrifugation, was washed successively with 75% ethanol, 95% ethanol, and acetone. The dried residue was weighed. Corrections are made during the determination of protein and ash. Soluble fiber (SF) content is determined from the following formula:

$$\%SF = ((\text{Residue}) - (\text{Protein} + \text{Ashes})) \times 100. \quad (2)$$

The total dietary fiber (TF) is determined as the sum of insoluble and soluble fiber:

$$\%TF = \%IF + \%SF. \quad (3)$$

Soluble sugars are firstly extracted with 15 mL of a solution of 96% ethanol with stirring at room temperature and then centrifuged at 9418 g, 4°C for 20 min. Secondly, the resulting residue was washed with 5 mL of a solution of 80% ethanol. Then, the supernatants were collected and evaporated to obtain a volume of 1 mL. Finally, it was adjusted to obtain 10 mL with distilled water [38]. The obtained solution was analyzed by the phenol-sulfuric method [39].

Polysaccharides were determined as follows: the residue obtained from soluble sugars extraction was stored for 24 hours at room temperature to evaporate the ethanol traces. Then, 10 mL of HCl (30%) was added and the mixture was incubated in a water bath at 60°C for 2 hours and then centrifuged at 9418 g, 4°C for 30 min. The supernatant was filtered through a filter paper and then adjusted to 10 mL with distilled water. The obtained solution was analyzed by the phenol-sulfuric method [39]. The assay is performed with a mixture (v/v) of 1 mL of the solutions obtained with a solution of 5% phenol. 5 mL of concentrated sulfuric acid is then added and the mixture was placed in water bath at 25–30°C for 20 min. The optical density was measured at a wavelength λ = 490 nm with a spectrophotometer (SHIMADZU mini 1240). The concentration of soluble sugars and polysaccharides is determined against a standard curve made with glucose. Total sugars were the sum of soluble sugars and polysaccharides.

The mineral constituents (Ca, Mg, Na, K) were analyzed separately, using an atomic absorption spectrophotometer (Hitachi Z6100, Japan).

The pH was measured using a pH-meter (METTLER TOLEDO MP220) at 20°C.

The levels of soluble solids of raw material, expressed as °Brix, were measured using a refractometer (Mod. DR-101, Coseta S.A., Barcelona, Spain). Both measurements were taken at 20°C.

Water activity was measured by a NOVASINA aw Sprint TH-500 Apparatus. The measurement was performed at 25°C.

2.5. Determination of Technofunctional Properties

2.5.1. Particle Size. The measurement of particle size distribution tells us about the size of *Agave* leaves powder. This particle size was measured using a sieve with a mesh size of 200 μm (Model VE 100, Retch, Germany). The fine fraction (particle size < 200 μm) was used for analysis.

2.5.2. Water Holding Capacity and Oil Holding Capacity (WHC and OHC). The method of Moure et al. (2001) was used with a slight modification. 1 g of samples was stirred in 10 mL of distilled water or corn oil and then centrifuged at 7125 g for 20 min (JOUAN CR4 22, USA). The volume of the supernatant was measured. The water-holding capacity was expressed as the number of gram of water held by 1 g of sample. The oil-holding capacity was expressed as the number of gram of oil held by 1.0 g of sample [40].

2.5.3. Emulsion Capacity (EC). The emulsion capacity was determined by a model system described by Blecker et al. (1997). Then, sunflower oil was added to 50 mL of solutions (7% w/v) and emulsified using an Ultraturax T25 (IKa, Staufen, Germany) at 15000 rpm for 10 min. During emulsification, temperature was maintained at 0°C by immersing the reaction vessel in ice bath. The sudden increase in electrical resistance showed the phase inversion point; the oil phase becomes continuous, which can be determined by electrical conductivity measurements. Emulsion capacity is expressed in g oil g^{-1} of sample [41].

2.5.4. Swelling Power. A dispersion of 200 mg of dietary soluble fiber in 10 mL of distilled water was introduced into a graduated cylinder. After 18 hours of standing at room

temperature, the amount of water retained by the fibers was determined. The swelling is the ratio between the volume of water and the test [42].

2.5.5. Solubility. The solubility of inulin extracted from the *Agave* leaves and commercial inulin was determined as follows: at 25°C. inulin was added slowly in 10 mL of water under stirring until complete dissolution and saturation. The solubility is expressed as the mass of inulin dissolved in one liter of distilled water [43].

2.5.6. Pectin-Inulin Mixed Gel Preparation. High methoxyl pectin (PHM), inulin, and mixed gels were prepared to study the effect of *Agave* inulin on gelling properties. 15% to 30% of inulin extracted from AA was used and dissolved in 50 mL of distilled water and added with sucrose until a 55°Brix of soluble solid levels. Subsequently, the PHM (4%) was added and dissolved by stirring. The pH was adjusted to 3 using a citric acid solution (10%). The obtained solution was heated to boiling with stirring until reaching a 65°Brix of soluble solids extract. Finally, the preparation was setting into cylindrical containers (3.5 cm diameter × 3 cm height). The solutions were cooled to room temperature overnight (Figure 3). Similarly, standard solutions at 4% of pectin and 20% of commercial inulin concentrations were prepared with distilled water and compared to mixed gels (the ratio PHM/inulin mixture was 4 : 20).

2.5.7. Texture Analysis. Penetration test was performed with a Texture Analyzer (Analysis LLOYD instruments, Fareham, UK) interfaced to a personal computer (Windows-based Software NEXYGEN PLOT). Constant speed penetration tests were performed directly on cylindrical containers (3 cm diameter × 3.5 cm height). All instrumental texture analyses were conducted on chilled (25°C) samples. A cylindrical probe (25 mm of diameter) was introduced for 30 mm into the samples (the speed = 40 mm/min). The prepared gels were subjected to a test initiation of chewing (Texture Profile Analysis). From the force-versus-time curves, values for the maximum force (N) were calculated as force at a distance of 15 mm (F_{max}) and a detection limit of 0.005 kg force into two times. Triplicate measures for each gel were performed. Textural parameters considered in the present study were firmness, elasticity, cohesiveness, adhesiveness, and chewiness.

2.6. Statistical Analysis. One-way analysis of variance (ANOVA) was used to determine significant differences ($P < 0.05$) between inulin-PHM gels and PHM or inulin gels. Duncan's test was used to access the differences between gels. Statistical analyses were performed on statistical analysis package STATISTICA (Release 5.0 Stat Soft Inc. Talsa, OK).

3. Results and Discussion

3.1. Physicochemical Properties of Powder and Inulin from Agave americana L. Leaves. The extracted powder and inulin from AA leaves were illustrated in Figure 2. The proximate

(1) (2)

Figure 2: *Agave americana* leaves powder (1) and inulin (2).

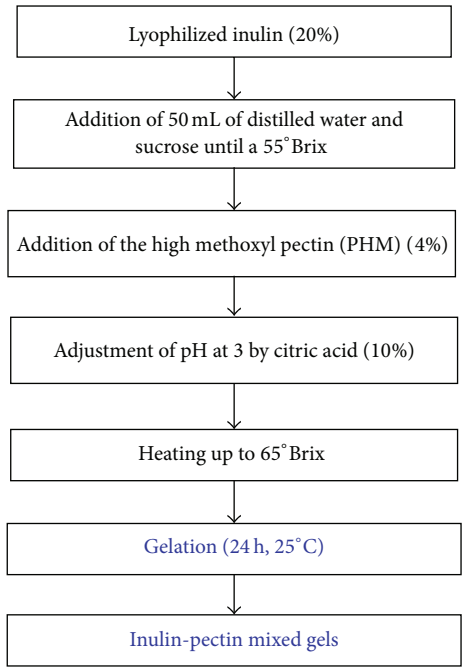

Figure 3: Diagram of inulin-pectin gels preparation.

composition of leaves powder from AA plant was presented in Table 1. Results showed a low content of the water (5.86%) which facilitates their conservation. But *Agave* is a succulent plant, and this recalls the rich succulence racket prickly when water content was approximately 92% [44].

Moreover, the total fiber content was the highest (38.40%) followed by protein content (35.33%) with a relatively low lipid (2.03%) and Ash (5.94%) levels.

The sugar fractions of *Agave* leaves were essentially formed by insoluble and soluble sugars (3.16% and 42.67% of total sugars, resp.). *Agave* leaves contained a high insoluble fiber level which confirms the appearance of the flesh filamentous leaves [45]. However, the soluble fiber fraction was lower compared to insoluble fiber fraction (9.03% against 29.37%). The soluble fraction was represented mainly by fructans [14]. The presence of this fraction confirms the choice of using leaves part of the plant for inulin extraction.

Table 1 shows the mineral composition of AA leaves powder. A predominance of potassium (1.096 mg/100 g of

Table 1: Chemical composition of *Agave americana* L. leaves powder.

Parameters	Dry matter (DM) (%)	Ash[a]	Protein[a]	Lipid[a]	Soluble sugar[a]	Insoluble sugar[a]	Soluble fibre[a]	Insoluble fibre[a]	Total fibre[a]	K	Na	Ca	Mg	pH
Lyophilised *Agave* leaves	94.14 ± 0.58	5.94 ± 0.12	35.33 ± 0.73	2.03 ± 0.06	42.67 ± 0.74	3.16 ± 0.63	9.03 ± 0.64	29.37 ± 0.59	38.40 ± 1.48	1.096 ± 0.11	0.045 ± 0.01	0.762 ± 0.13	0.092 ± 0.02	5.06 ± 0.07

[a] (g/100 g DM).
[b] (mg/100 g DM).

Table 2: Physicochemical properties of inulin obtained from *Agave americana* L. and commercial inulin (% DM).

Parameters	Yield	Aw	pH	Dry matter (%)	Ash (%)	Protein (%)
Agave americana Inulin	79.12 ± 0.50	0.275 ± 0.013[a]	5.53 ± 0.55[a]	92.19 ± 0.28[a]	2.89 ± 0.31[a]	3.46 ± 0.13[a]
Commercial inulin	* * *	0.282 ± 0.011[a]	5.98 ± 0.34[a]	91.67 ± 0.76[a]	1.19 ± 0.18[b]	1.58 ± 0.11[b]

Means in the same column with different letters are significantly different ($P < 0.05$).

Table 3: Functional properties of *Agave americana* L. leaves powder, inulin extracted from *Agaves americana*, and commercial inulin obtained by lyophilisation.

Parameters	Solubility at 25°C (g/L)	WHC (g of water/g of sample)	OHC (g of oil/g of sample)	SP (mL of water/g of sample)	Emulsion capacity (%)
Agave powder	* * *	14.60 ± 0.66[c]	9.87 ± 0.29[b]	15.20 ± 0.30[b]	17.17 ± 1.04[c]
Agave inulin	73.47 ± 0.14[a]	2.42 ± 0.18[b]	3.26 ± 0.59[a]	1.99 ± 0.13[a]	14.48 ± 0.23[a]
Commercial Inulin	113.68 ± 4.14[b]	1.59 ± 0.02[a]	3.47 ± 0.03[a]	1.08 ± 0.01[a]	21.42 ± 0.70[b]

Means in the same column with different letters are significantly different ($P < 0.05$).
WHC: water holding capacity, OHC: oil holding capacity, SP: swelling power.

AA) and calcium (0.762 mg/100 g of AA) was observed and low levels of sodium (0.092 mg/100 g of AA) and magnesium (0.045 mg/100 g of AA) similarly net *Aloe vera* [46].

The pH of AA powder was 5.06 presented in Table 1. This value was higher than other fibre products such as pomegranate bagasses powder coproduct 4.4 [47] or orange dietary fibre 4.06 or lemon albedo 3.96 [48, 49].

Furthermore, Table 2 presents the physicochemical properties of inulin obtained from AA and inulin extracted from commercial chicory. Both inulins had a very high dry matter (91-92%). Significant difference was observed between *Agave* inulin and commercial inulin pH (5.53 against 5.98, resp.) ($P < 0.05$). This result can be due to the differences between the two plant initial compositions.

The water activity of *Agave* inulin and commercial inulin ($P < 0.05$) was 0.275 and 0.282, respectively. The water activity and pH of *Agave* inulin and commercial inulin, both parameters highly related to product deterioration, indicate that the risk of deterioration (by microorganism, enzymes, or no enzymatic reactions) is minimal.

Inulin from AA was characterized by a higher protein and ash contents than the commercial chicory inulin (3.46% against 1.58% and 2.89% against 1.19%, resp.). This significant difference can probably be due to the difference between the laboratory and the industrial purification process and the botanical differences between the two studied plants AA and chicory.

3.2. Functional Properties. Table 3 showed the functional properties of AA powder, *Agave* inulin, and commercial inulin. The WHC of *Agave* leaves powder had the highest level compared with *Agave* inulin and commercial inulin (14.60 g of water/g of sample against 1.59–2.42 g of water/g of sample). This result can be explained by the high *Agave* fibre content (38.40%) and protein content (35.33%) [50–53]. The obtained WHC of Agave leaves powder was higher than these of the fibroprotein extracts from date seeds (4-5 g of water/g of sample) [52], the citrus fiber (10.66 g of water/g of fiber) [42], grapefruit fiber (9.77 g of water/g of fiber) [50] and orange fiber (11 g of water/g of fiber) [54].

OHC of *Agave* leaves powder was 9.87 g of oil/g of the sample. Considering this value of oil retention, the *Agave* leaves powder could be employed as like ingredient to stabilize the products rich in oil. These WHC and OHC were a function of size, shape, hydrophilic, and hydrophobic interactions and were affected by the presence of carbohydrates, lipids, and amino acid residues on the surface, since most nonpolar amino acid residues and polar groups are not hydrated in the interior [40, 52]. The particle size of *Agave* leaves powder and *Agave* inulin (particle size < 250 μm) affected technofunctional properties. Indeed, the very fine particles explained the importance of WHC and OHC increases. The high WHC and OHC of these *Agave* leaves powder and inulin suggest that it can be used as a functional ingredient to improve the sensory properties of the formulated product, to reduce syneresis, modify texture, viscosity, and reduce calories of foods.

The higher swelling property of *Agave* leaves powder might be attributed to its lower density and lager surface area among the fiber samples. *Agave* and commercial inulin have a lower swelling power than the *Agave* leaves powder (1-2 mL/g against 15.20 mL/g, resp.). It was suggested that the differences in hydration properties were a function of the physical structure of the fiber, which could be manipulated by processing history. Experimental procedures, including how sample was prepared, alter the physical structure of the fiber, which could affect the hydration properties [55]. This could explain the differences in hydration properties observed between *Agave* leaves powder, *Agave* inulin, and commercial inulin. Hydration properties determine the role of dietary fiber in regulating colonic function and also their physiological effects [56, 57].

Table 4: Effect of drying process on the technofunctional properties of inulin extracted from *Agave americana* leaves.

Parameters	WHC (g of water/g of sample)	OHC (g of oil/g of sample)	SP (mL of water/g of sample)	Emulsion capacity (%)
Lyophilisation	2.42 ± 0.18^{a}	3.26 ± 0.59^{a}	1.99 ± 0.13^{a}	14.48 ± 0.23^{a}
Drying oven ($T = 40^{\circ}C$)	1.62 ± 0.07^{b}	2.21 ± 0.12^{b}	1.5 ± 0.52^{a}	11.3 ± 0.03^{b}
Drying oven ($T = 60^{\circ}C$)	1.36 ± 0.01^{c}	1.90 ± 0.04^{c}	1.15 ± 0.68^{a}	10.49 ± 0.66^{c}

Means in the same column with different letters are significantly different ($P < 0.05$).
WHC: water holding capacity, OHC: oil holding capacity, SP: swelling power.

The solubility of *Agave* inulin was significantly lower than those of commercial inulin (73.47 ± 0.14 g/L against 113.68 ± 4.14 g/L) ($P < 0.05$). However, the solubility remained high for both. This high solubility in water probably affects the hydration properties of inulin.

The emulsion capacity (EC) is a molecule's ability to act as an agent that facilitates solubilization or the dispersion of two immiscible liquids. Emulsions are formed due to the presence of hydrophobic and hydrophilic groups of carbohydrate. The EC of the agave leaves powder was 17.17% and for *Agave* inulin was 14.48%, while the EC of the commercial inulin was 21.42%. Probably, a relationship was existed between emulsion properties and solubility of the studied fiber. This result suggests that the improvement of emulsification capacity could be due to the presence of soluble protein and fiber. M. Viuda-Martos et al. [47] reported similar result for pomegranate juice arils bagasse and pomegranate juice whole fruit bagasse.

3.3. Effect of Drying Process on the Technofunctional Properties of Inulin Extracted from AA Leaves. Table 4 presented the effect of varying the drying temperature on the technofunctional properties of the *Agave* inulin. If drying temperature increased, the various technofunctional properties decreased. For example, the WHC of lyophilized *Agave* inulin was higher than these obtained by drying oven *Agave* inulin. Therefore, it can be concluded that temperature of drying had an influence on the structure and hydrophobic characteristics of *Agave* inulin.

Significant difference was observed between the different drying processes (lyophilization, oven drying at 40°C and 60°C) concerning the functional properties except swelling power. For example, the OHC decreased with the increase of drying temperature. The lyophilized inulin OHC was 3.26 g of oil/g of sample against 2.21 g of oil/g of sample for the oven dried inulin at 40°C and 1.90 g of oil/g of sample for the oven dried inulin at 60°C. Freeze-drying has provided the most appreciated technofunctional inulin. Certainly, this process preserved the inulin structure.

3.4. Synergetic Effect of Agave Inulin on Textural Qualities of Prepared Mixed Gels. The synergetic effect of prepared *Agave* leaves inulin-PHM mixed gel on texture parameters was studied and compared to PHM gel, commercial inulin gel, and the commercial inulin-pectin mixed gel. Figure 4 and Table 5 exhibited the results of the textural analysis. The inulin showed a significant contribution to firmness, chewiness,

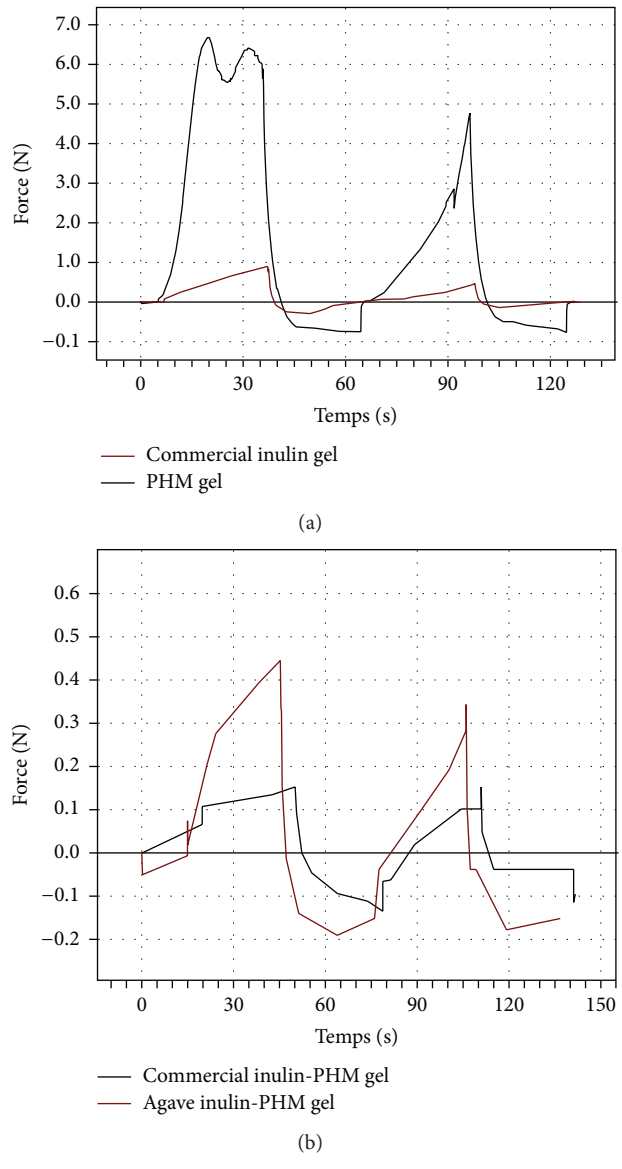

Figure 4: Texture profile of commercial inulin and PHM gels, commercial inulin-PHM, and *Agave* inulin-PHM gels.

Table 5: Texture parameters of different prepared gels with inulin and commercial high-methoxy pectin (PHM).

Parameters	Firmness (N)	Cohesiveness	Elasticity (mm)	Chewiness (N·mm)	Adhesiveness (N/mm)
Commercial Inulin	0.6836 ± 0.3068^{a}	0.3294 ± 0.0236^{a}	14.7903 ± 0.1655^{a}	4.1992 ± 0.0013^{a}	1.2318 ± 0.0583^{a}
Commercial PHM	5.7238 ± 1.3484^{b}	0.2762 ± 0.0123^{a}	14.2419 ± 0.1125^{a}	26.8461 ± 0.1425^{b}	7.4136 ± 0.0263^{b}
Commercial Inulin + Commercial PHM	0.1838 ± 0.1440^{a}	0.4138 ± 0.3784^{a}	9.2336 ± 0.3594^{b}	1.0684 ± 1.3346^{a}	0.9902 ± 0.1792^{a}
Commercial PHM + Agave Inulin	0.3554 ± 0.0550^{a}	0.3149 ± 0.0906^{a}	10.1741 ± 1.0038^{b}	1.2663 ± 0.3407^{a}	1.3051 ± 0.1636^{a}

Means in the same column with different letters are significantly different ($P < 0.05$).

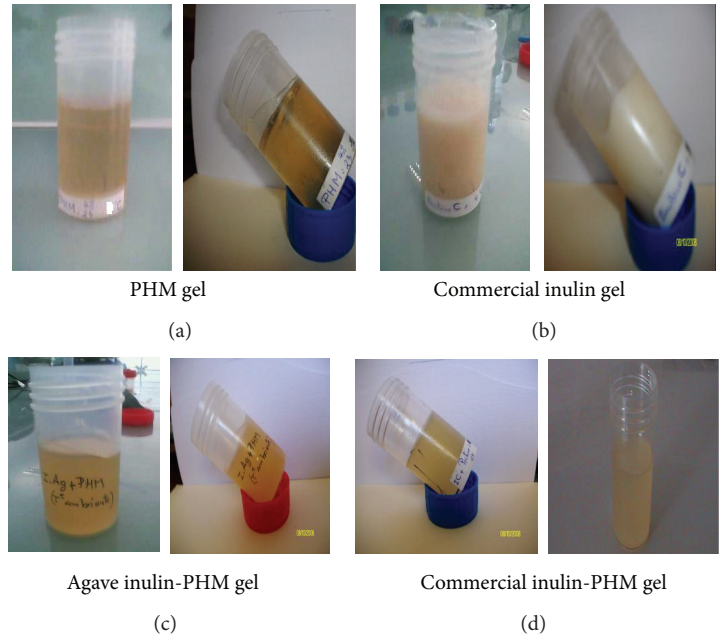

Figure 5: Different prepared gels in laboratory.

and adhesiveness of prepared inulin gels compared with commercial PHM gel.

The firmness is the force required to achieve a given deformation. No significant difference was observed between the firmness of the commercial inulin gel, the Agave inulin-PHM and the commercial inulin-PHM mixed gels. Those prepared gels were very fragile and presented a significant different firmness compared to the commercial PHM gel ($P < 0.05$). These low levels of firmness of the commercial inulin gel, the commercial inulin-PHM, and *Agave* inulin-PHM mixed gels could be explained by the presence of synergetic effect between inulin and PHM. For example, firmness of PHM-*Agave* inulin mixed gel is 0.3554 N against 5.7238 N for the PHM gel. However, firmness of PHM-*Agave* inulin mixed gel was slightly lower (0.3554 N) than these of commercial inulin gel (0.6836 N) and slightly higher than the commercial inulin-PHM mixed gel (0.1838 N). Probably, pectin reacts synergistically with *Agave* inulin which enhances the tenderness of mixed gels. This result can be explained by the presence of impurities from *Agave* inulin due to the absence of a purification step. Furthermore, firmness of the prepared gels decreased with the presence of inulin which confirms the synergy between these two hydrocolloids especially the inulin in improving the textural parameters of gels. These prepared gels were presented in Figure 5.

Adhesion was the maximum force required to remove the probe from the sample after applying a compressive force. According to the obtained results, no significant difference was shown between the adhesiveness of different prepared gels except those of PHM gel ($P < 0.05$). For example, adhesion of PHM-*Agave* inulin mixed gel was significantly lower than those of PHM gel (1.3051 N/mm against 7.4136 N/mm) ($P < 0.05$). These results confirmed the existence of synergy between principally inulin and PHM.

Cohesiveness was the ratio of the area under the curve of the second compression to the area under the curve of the first compression [58]. Table 5 indicates that the cohesion was very low in different gels. The cohesiveness levels, ranging between 0.2762 and 0.4138, were not changing significantly for the mixed gels.

Elasticity was the height at which the sample returns to its original size after compression [59]. Significant difference was shown between elasticity of commercial inulin and PHM gels and the mixed gels (*Agave* inulin-PHM and commercial inulin-PHM gels). However, the *Agave* inulin-PHM or commercial inulin-PHM mixed gels were slightly lower compared with PHM and commercial inulin gels (9-10 mm against 14 mm, resp.). These results can be explained by the synergetic effect between pectin and inulin gels.

Furthermore, these results could be explained in the fact that the *Agave* inulin contains proteins, sugars, and fibers other than inulin in low proportions. For example, the protein fraction present in the *Agave* inulin was about 3.46%; thus more residues have probably a role in gelation such as the S-S bridge. They are involved in establishing a gel network. The *Agave* inulin-PHM gel had an appreciated texture more than the commercial inulin-PHM gel and gives importance to the *Agave* inulin to play the role of a texturing in various food formulations. Yet the saturation of synergy between inulin, protein, and pectin affected the general appearance of the mixed gels and revealed the higher affinity of compounds for the pectin matrix. Similar phenomena were reported between k-carrageenan and hydrocolloid from leaves of *Corchorus olitorius* [60].

Moreover, the presence of inulin can probably cause local disruptions of the pectin gel structure and at the same time reduces the freedom of polymeric chains of pectin for searching for an ordered binding. The *Agave* inulin changed the properties of the matrix resulting in a more nonpolar matrix. This is indicated by a larger retention of the more hydrophobic compounds than the less hydrophobic compounds in the more rigid gels [61].

When solutions of two biopolymers were mixed, interactions between their chains depend on the balance between the enthalpy and the entropy changes on mixing, being, therefore, either favorable (association) or unfavorable (segregation) [62]. Almost all biopolymer mixtures exhibit segregate interactions, unless there is an electrostatic drive to association. These usually result in phase separated networks where the components tend to exclude each other from their domains [63].

4. Conclusion

The present paper reported the basic chemical and physicochemical properties of inulin from leaves of AA obtained by water extraction. Results indicated the potentiality to valorize *Agave americana* L. leaves of Tunisia, especially inulin fraction. For gelling properties, it has revealed that PHM-*Agave* inulin gel exhibited lower firmness due to the synergy between *Agave* inulin and pectin in relation to gel strength. This synergy implies that inulin could not only be an alternative to pectin in many applications but may introduce new functions to inulin. Thus AA is an interesting source of inulin though further investigation should be done in order to fully explore the potential of this studied hydrocolloid.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Optimization of Insoluble and Soluble Fibres Extraction from *Agave americana* L. Using Response Surface Methodology

Amine Bouaziz,[1] Manel Masmoudi,[1] Amel Kamoun,[2] and Souhail Besbes[1]

[1] *Université de Sfax, Laboratoire Analyses, Valorisation et Sécurité des Aliments, Ecole Nationale d'Ingénieurs de Sfax, Route de Soukra, 3038 Sfax, Tunisia*
[2] *Université de Sfax, Laboratoire de Chimie Industrielle, Ecole Nationale d'Ingénieurs de Sfax, Route de Soukra, 3038 Sfax, Tunisia*

Correspondence should be addressed to Manel Masmoudi; manel_masmoudi@yahoo.fr

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Experimental design methodology was used to determine significant factors affecting the extraction yield of soluble and insoluble fibres from *Agave americana* L. and in second time to find optimum conditions leading to the highest yield. Results clearly indicated that the temperature, the powder to water (P/W) ratio, and the agitation speed were the most important factors influencing fibres extraction yield which increased with temperature, P/W ratio, and agitation speed. Ionic strength affected significantly soluble fibre extraction yield and was the most important factor among nonsignificant ones influencing insoluble fibres extraction yield. Then, a Box-Behnken design was carried out to maximise fibres extraction. Selected optimal conditions were temperature: 90°C; P/W ratio: 0.1625; agitation speed: 400 rpm; and ionic strength: 1.5 g/L. These conditions yielded 93.02% and 80.46% of insoluble and soluble fibres, respectively. Concentrates showed high fibres purity and good functional properties.

1. Introduction

The Agavaceae is a plant family with nine genera and about 293 species. *Agave*, a monocotyledonous and monocarpic plant, is the most important genus with about 166 species [1, 2]. It is a voluminous, herbaceous, and perennial plant with long, succulent spiny leaves growing directly out from the central stalk to form a dense rosette. Its floral stalk, sometimes termed the trunk, can reach 10 to 20 m of length [3, 4].

The most important diversity center is the Mexican territory, with species spread from southwestern United States through Central America, the Caribbean and into northern South America [1]. Plants were taken from there to Europe, Africa, and the Far-East by the Spanish and Portuguese, where they naturalized rapidly, especially in the high arid regions around the shores of the Mediterranean [5]. They can prosper there due to their shallow rooting system and succulent morphology, while traditional annual crops cannot [6].

In Tunisia, the *Agave americana* L. is the most abundant variety of *Agave* but it has never been exploited, while it is worldwide used for commercial (rope, paper, fibres, pectin, mezcal, aguamiel, pulque, and tequila), ornamental (yucca, century plant and mother-in-law's tongue) and medicinal applications (steroid extraction and antibacterial salves) [2–7].

Plant's leaves are characterized by the abundance of agrate bundles of short fibres that are good holders of water, which gives the *Agave*'s leaves their rigidity and succulence [4]. Like most natural fibres, *Agave* fibrous bundles are composed mainly of α-cellulose (64.8%), lignin (15.9%), and hemicelluloses (5.1%) and also can contain low content of waxes and ash [4].

In *Agave* species (CAM plants), fructans are reserve carbohydrates and they are synthesized and stored in the stems. The main function of these fructose polymers is storage before flowering and acts as osmoprotectants during drought [8].

Nowadays, fibres have a capital interest due to their physiological and nutritional roles. Besides, high-fibres diets are associated with the prevention and treatment of some

diseases such as constipation, colon cancer, coronary heart disease, diverticulosis and diabetes [9, 10]. Dietary fibres may be divided into two parts when they are dispersed in water: soluble and insoluble fractions. The latter one is related to both water absorption and intestinal regulation, whereas, soluble fibres are associated with the reduction of cholesterol in blood and the diminution in the intestinal absorption of glucose [9].

Incorporation of dietary fibres into a wide range of products will contribute to the development of value-added foods or functional foods that currently are in high demand [11]. In addition to the physiological benefits provided by high fibre foods, studies have shown that fibres components can give textural, gelling, thickening, emulsifying, and stabilizing properties to certain foods. By understanding functional properties of dietary fibres, one can increase their use in food applications and aid in developing food products with high consumer acceptance [10].

The aim of the present work is to look for the experimental conditions leading to the maximum extraction yield of soluble and insoluble fibres fractions from basal part of leaves of *Agave americana* L. using experimental design methodology. As many factors can influence the extraction yield, a step of screening was firstly applied in order to retain statistically significant factors. Then, a response surface methodology (RSM) was undertaken to fit and exploit a mathematical model representing relationship between the response (extraction yield) and variables retained in the first step of optimization.

2. Material and Methods

2.1. Samples. *Agave americana*'s leaves were procured from Essghar region (Sfax, Tunisia). After elimination of chlorophyllous cuticle, leaves were cut into regular pieces, rinsed with water, dried for 72 h at 50°C, milled twice to obtain a very fine powder and preserved at −20°C prior to analysis and extraction.

2.2. Dietary Fibre Extraction. Figure 1 shows the extraction process from *Agave*'s leaves powder to produce DF concentrates. Hot water was used to extract DF from milled leaves in a jar, homogenised using a mechanical stirrer 2021 (Heidolph rzr, Metrohm, USA) and maintained in a thermostatic bath (Raypa, Spain); operating conditions were fixed by the experimentation's matrix. After solubilisation of free sugars and fructans, insoluble DFs were recuperated by centrifugation (6500 g, 10 min) using a 4K15 centrifuge (Sigma, Osterode, Germany) [12]. Its concentration was carried out by a succession of five rinsings (water at 40°C) and of five centrifugations until the residue was free of sugars. In the other hand, soluble DFs (fructans) were settled overnight in ethanol 95% and then recuperated by centrifugation (6500 g, 10 min). Its concentration was carried out by rinsing three times with 50%, 70% and 100% ethanol [13]. The residues obtained were freeze-dried in a Benchtop 3 L freeze dryer (Virtis, Gardiner, NY) to give the DF concentrates and stored at 3°C for subsequent analyses.

2.3. Physicochemical Analyses of Raw Material and DF Concentrates. All values given were the mean of three replications and were expressed as the mean ± standard deviation ($\overline{x}$ ± SD).

2.3.1. Dry Matter. Dry matter was determined by oven-drying at 105°C to constant weight [14].

2.3.2. pH. The pH of 10% aquatic suspension of *Agave* powder and all other solutions was determined potentiometrically with a pH meter MP 220 (Mettler toledo, Barcelona, Spain) according to NF 05-108 [15].

2.3.3. Water Activity (aw). The water activity was determined at 25°C by a Novasina AW SPRINT TH-500 apparatus.

2.3.4. Ash and Mineral Content. Ash content was determined by incinerating sample at 550°C for 8 h. The total ash was expressed as the percent of dry weight [16]. The residue was digested with a nitric/perchloric acid (2 : 1, v/v) mixture and then adjusted to 50 mL with ultrapure water [12–17]. Mineral constituents (K, Ca, Na, Mg, Zn, and Cu) were analyzed separately using an atomic absorption spectrophotometer (analytik-jena ZEEnit 700, Germany) [14].

2.3.5. Protein. Total nitrogen was determined by the Kjeldahl method [15]. Protein was calculated using the general factor (6.25).

2.3.6. Total Fat. Crude fat was estimated by Soxhlet extraction with hexane over a period of 8 h [18].

2.3.7. Reducing Sugars. Reducing sugars were determined according to Bertrand and Thomas method. This latter is well described in detail by Blanchard [19].

2.3.8. Total Sugars. Soluble sugars concentration was determined by the phenol-sulphuric acid method [20] after ethanol extraction. Insoluble sugars fraction was submitted to a hydrochloric acid digestion for 2 h at 60°C. Released sugars were also determined by the phenol-sulphuric acid method.

2.3.9. Dietary Fibres. Insoluble and soluble dietary fibres (DF) were determined according to the AOAC enzymatic-gravimetric method of Prosky et al. [21]. Briefly, samples were gelatinized in phosphate buffer with heat-stable alpha amylase (A-3306, Sigma-Aldrich Chemical Co., St. Louis, USA) (100°C, pH 6, 15 min) and then enzymatically digested with a protease (P-3910, Sigma-Aldrich Chemical Co., St. Louis, USA) (60°C, pH 7.5, 30 min), followed by incubation with amyloglucosidase (A-3042, Sigma-Aldrich Chemical Co., St. Louis, USA) (60°C, pH 4.5, 30 min) to remove protein and starch. Then, samples were filtered, washed (with water, 95% ethanol, and acetone), dried, and weighed to determine insoluble fibres content. Four volumes of 95% ethanol (preheated to 60°C) were added to the filtrate and to the water washings. Then, precipitates were filtered and

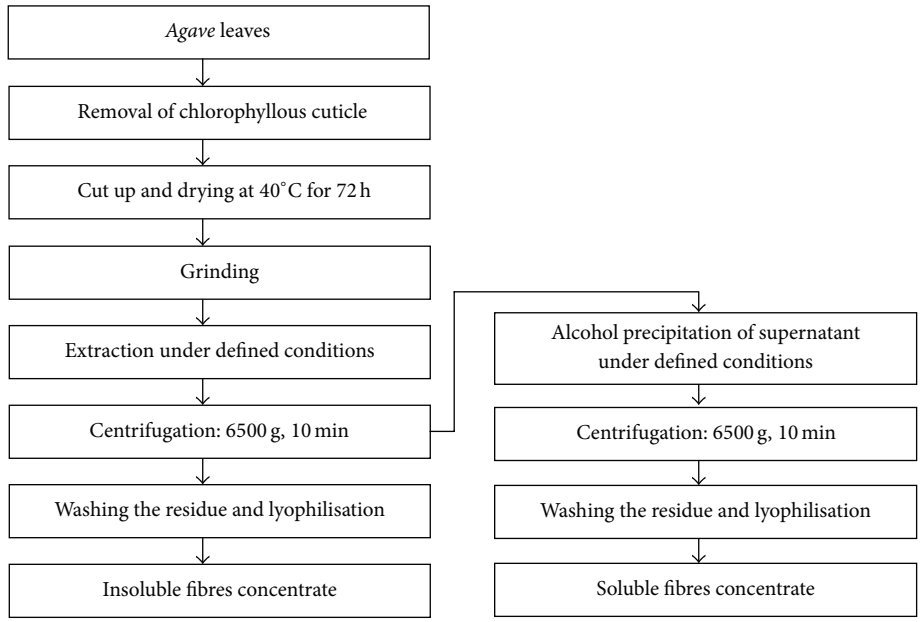

Figure 1: Elaboration process of insoluble and soluble fibres concentrates from *Agave americana* L.

washed with 78% ethanol, 95% ethanol, and acetone. After that, the residues (soluble DF) were dried and weighed. The obtained values were corrected for ash and protein. Total DF was determined by summing insoluble and soluble DF.

2.3.10. Microstructure Visualization. DF shape and the surface morphology at the micrometer scale were investigated with a scanning electron microscope (SEM), XL30 type ESEM-FEG (Philips/FEI) at 20 kV, using a working distance of 10.0 mm. DF concentrates were metalized with a gold/palladium coating of thickness $l = 4$ nm using a BALTEC MED 020 device prior to observation [7]. The detection system used was a back-scattered electron detector (BSE) [22, 23].

2.3.11. Water Holding Capacity. Water holding capacity (WHC) was determined using the method described by Macconnell et al. [24]. Hundred milligrams of DF concentrates was added to 10 mL of distilled water in a 50 mL centrifuge tube and stirred overnight at 4°C. Then the mixture was centrifuged at 14000 g for 20 min. The free water was decanted and absorbed water was then determined.

2.3.12. Oil Holding Capacity. Oil holding capacity (OHC) was measured using the method described by Caprez et al. [25]. Hundred milligrams of DF concentrates was added to 10 mL of corn oil in a 50 mL centrifuge tube. The content was stirred, and then the tubes were centrifuged at 1500 g for 30 min. The free oil was decanted and the absorbed oil was determined.

2.4. Experimental Methodology. When many factors affect a desired response, it can be an exhausting task to optimize a process [13]. Therefore, screening step seems to be indispensable to retain only significant factors influencing fibre extraction yield, while response surface methodology (RSM) can be an effective tool for optimizing the response. Screening allows the investigation of up to $N - 1$ variables with N experiments using a fractional factorial design such as Plackett-Burman design [26]. Response surface methodology is defined as a statistical method that uses quantitative data from appropriate experimental design to determine optimal conditions and predict the optimum response. Box-Behnken design (BBD), one of RSM, is more efficient and easier for arranging and interpreting experiments in comparison with others [27].

In the present study, experimental design software NEMROD-W [28] was used in order to select significant factors and look for the best experimental conditions leading to the highest fibres extraction yield.

2.4.1. Screening Step. Two Plackett-Burman designs were used to select significant factors, starting from six factors (U_1 to U_6) for the first design and eight factors (U_1 to U_8) for the second one, chosen to evaluate their effect on insoluble and soluble fibres extraction yield, respectively. Table 1 shows different factors and the two levels (+1) and (−1) of each variable used for screening.

2.4.2. Response Surface Methodology. The fibres extraction procedure was optimized using the RSM. This latter has been extensively utilized to optimize culture conditions and medium composition of fermentation process, conditions of enzyme reaction, and processing parameters in the production of food and drug [13–29]. A Box-Behnken design was chosen to look for the best experimental conditions.

Table 1: Experimental domain for screening step.

	Factor	Unit	Low level	High level
U_1	P/W ratio	g powder/mL water	0.0333	0.1
U_2	Temperature	°C	60	80
U_3	pH	—	4	9
U_4	Time	hour	0.5	2
U_5	Agitation speed	rpm	200	400
U_6	Ionic strength	g NaCl/l medium	1	2
U_7	M/E ratio	l/l	0.25	0.5
U_8	Precipitation temperature	°C	5	35

Table 2: Chemical composition of *Agave* leaves (g/100 g dry matter).

Components	Values
Dry matter (%)	16.42 ± 0.71
pH	5.03 ± 0.08
Protein	28.90 ± 1.46
Fat	5.46 ± 0.02
Ash	3.53 ± 0.33
Total carbohydrates	62.74 ± 1.38
Reducing sugars	20.06 ± 0.10
Soluble dietary fibres	7.72 ± 0.21
Insoluble dietary fibres	37.88 ± 0.49
Total dietary fibres	45.05 ± 0.96

Table 3: Mineral composition of *Agave* leaves.

Mineral	Values
K[a]	1.103 ± 0.017
Ca[a]	0.571 ± 0.016
Na[a]	0.037 ± 0.002
Mg[a]	0.086 ± 0.006
Zn[b]	1.299 ± 0.151
Cu[b]	0.307 ± 0.015

[a]g/100 g dry matter; [b]mg/100 g dry matter.

3. Results and Discussion

3.1. Chemical Composition. The main characteristics of the *Agave*'s leaves are given in Table 2. High moisture and protein and total carbohydrate contents were observed. However, low fat and ash contents were found. This plant exhibited a low pH value. This low pH could be explained by the presence of many organic acids such as malic, citric, and oxalic acids [30]. *Agave*'s leaves had high contents of soluble and insoluble fibres (7.72% and 37.88%, resp.). These values are comparable with those reported for *Opuntia ficus indica* f. *inermis* cladodes (8.78% and 30.36% of soluble and insoluble fibres, resp.) [17].

Table 3 summarized the mineral composition of the *Agave*'s leaves. Potassium is the most abundant mineral (1.103 g/100 g of dry weight). It was as twice as calcium content. Magnesium and sodium contents were, respectively, 86 and 37 mg/100 g of dry weight. However copper and zinc were found at very low level. These values were lower than those observed by Ayadi et al. [17] for *Opuntia ficus* cladodes and those found by Femenia et al. [31] in Aloe Vera.

The chemical composition of the *Agave* leaves (high amount of fibres and proteins), as well as the low cost of exploitation, can justify the search of valorization of this product. Indeed, *Agave* leaves could be considered as a potential source of dietary fibres.

3.2. Experimental Approach

3.2.1. Screening Step. Twelve experiments were carried out to select significant factors from those cited in Table 1 according to the conditions indicated in Tables 4 and 5 for insoluble and soluble fibres extraction, respectively. Response values (fibres yields) are reported in the last column of each table. These observed responses were used to compute coefficients of each model. This allowed us to write the following models:

$$\begin{aligned}\hat{y}_1 &= 63.009 + 5.193X_1 + 8.171X_2 + 0.136X_3 \\ &\quad + 0.516X_4 + 4.898X_5 + 3.336X_6, \\ \hat{y}_2 &= 51.076 + 5.623X_1 + 8.193X_2 \\ &\quad + 0.583X_3 + 0.547X_4 + 3.576X_5 \\ &\quad + 3.576X_6 + 1.099X_7 - 1.433X_8,\end{aligned} \tag{1}$$

where $\hat{y}_1$ is insoluble fibres extraction yield, $\hat{y}_2$ is soluble fibres extraction yield, X_1 is powder to water ratio (P/W), X_2 is extraction temperature, X_3 is pH, X_4 is time, X_5 is agitation speed, X_6 is ionic strength, X_7 is medium to ethanol ratio (M/E), and X_8 is ethanol precipitation temperature.

The analysis of variance showed that the regression sum of squares was statistically significant at the level of 95% for both insoluble and soluble fibres extractions. Results of this analysis for each design are summarized in Table 6. Coefficients of determination, R^2, were 0.886 and 0.977 for insoluble and soluble fibres extraction yield respectively. Thus, the predicted models well represented the observed values.

The analysis of the contrast coefficient b_j as it can be seen in Figure 2(a) showed that three factors: temperature, *Agave* powder to water ratio, and agitation speed had pronounced influence on the insoluble fibres extraction yield. Temperature and powder to water ratio improve the hydrodynamic process by increasing the diffusion of hydrophilic biomolecules to liquid extraction which allows the best fibres

Table 4: Experimental conditions of Plackett-Burman design for insoluble fibres extraction and the corresponding experimental responses.

N° exp	P/W ratio	Temperature	pH	Time	Agitation speed	Ionic strength	IF extraction yield (%)
1	0.1	80	4	2	400	2	89.87
2	0.0333	80	9	0.5	400	2	74.10
3	0.1	60	9	2	200	2	63.03
4	0.0333	80	4	2	400	1	65.67
5	0.0333	60	9	0.5	400	2	57.24
6	0.0333	60	4	2	200	2	46.57
7	0.1	60	4	0.5	400	1	66.24
8	0.1	80	4	0.5	200	2	67.26
9	0.1	80	9	0.5	200	1	68.49
10	0.0333	80	9	2	200	1	61.69
11	0.1	60	9	2	400	1	54.32
12	0.0333	60	4	0.5	200	1	41.63

Table 5: Experimental conditions of Plackett-Burman design for soluble fibres extraction and the corresponding experimental responses.

N° exp	P/W ratio	Temperature	pH	Time	Agitation speed	Ionic strength	M/E ratio	Precipitation temperature	SF extraction yield (%)
1	0.1	80	4	2	400	2	0.25	5	73.03
2	0.0333	80	9	0.5	400	2	0.5	5	65.61
3	0.1	60	9	2	200	2	0.5	35	51.85
4	0.0333	80	4	2	400	1	0.5	35	52.89
5	0.0333	60	9	0.5	400	2	0.25	35	39.67
6	0.0333	60	4	2	200	2	0.5	5	37.21
7	0.1	60	4	0.5	400	1	0.5	35	47.43
8	0.1	80	4	0.5	200	2	0.25	35	60.54
9	0.1	80	9	0.5	200	1	0.5	5	58.06
10	0.0333	80	9	2	200	1	0.25	35	45.48
11	0.1	60	9	2	400	1	0.25	5	49.28
12	0.0333	60	4	0.5	200	1	0.25	5	31.86

extraction efficiency and the highest purity [32, 33]. As for agitation speed, it accelerates the diffusion rate of molecules and homogenizes extraction medium. The other factors were statistically not significant. However, ionic strength was the most important factor from nonsignificant ones ($b_6 = 3.34$). This fact can be explained by the precipitation of protein present abundantly in *Agave* leaves leading to obtain a more pure DF concentrate. On the other hand, Figure 2(b) showed that four factors: temperature, *Agave* powder to water ratio (P/W), agitation speed, and ionic strength had pronounced effect on the soluble fibres extraction yields. Thus, it seems evident to retain these factors in order to optimize soluble and insoluble fibres extraction using the RSM.

3.2.2. Optimization by RSM. A Box-Behnken design was chosen to look for the best experimental conditions of four independent factors kept after the screening study which are X_1: powder to water ratio; X_2: extraction temperature (°C); X_3: agitation speed (rpm); and X_4: ionic strength (g of NaCl/L). For each factor, the experimental range was chosen on the basis of results of screening experiments. On the one hand, powder to water ratio and temperature domains were extended due to their high significance detected in the screening step. On the other hand, extraction time was fixed at 30 minutes and pH at its natural value to reduce the cost of this process. In addition, the precipitation of soluble fibres was carried out in ambient temperature using a 0.5 ethanol to medium ratio for economic reasons. The relationship between the extraction yield and the four variables for each response was approximated by the following second order polynomial function:

$$\begin{aligned}\hat{y} = {} & b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 \\ & + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 \\ & + b_{23}X_2X_3 + b_{14}X_1X_4 + b_{24}X_2X_4 + b_{34}X_3X_4.\end{aligned} \quad (2)$$

Twenty-five experiments were carried out to estimate the 15 model coefficients for each response according to the conditions indicated in Table 7. In order to estimate the pure

Table 6: Analysis of variance for Plackett-Burman designs.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
IF					
Regression	1549.47	6	258.245	6.5046	2.89*
Residuals	198.51	5	39.702		
Total	**1747.98**	**11**			
SF					
Regression	1538.42	8	192.303	15.8162	2.22*
Residuals	36.48	3	12.159		
Total	**1574.9**	**11**			

*Significant at the level of 95%.

Table 7: Experimental conditions of Box-Behnken design and the corresponding experimental responses.

N° Exp	P/W ratio	Temperature	Agitation velocity	Ionic strength	IF extraction yield (%)	SF extraction yield (%)
1	0.050	60	300	1.5	64.34	56.67
2	0.200	60	300	1.5	79.06	69.04
3	0.050	90	300	1.5	78.45	68.39
4	0.200	90	300	1.5	88.23	77.24
5	0.050	75	200	1.5	69.87	59.26
6	0.200	75	200	1.5	77.06	68.04
7	0.050	75	400	1.5	74.87	62.34
8	0.200	75	400	1.5	85.45	74.53
9	0.050	75	300	1.0	72.11	60.49
10	0.200	75	300	1.0	80.85	67.05
11	0.050	75	300	2.0	72.54	63.44
12	0.200	75	300	2.0	81.05	71.35
13	0.125	60	200	1.5	70.86	59.79
14	0.125	90	200	1.5	78.65	69.45
15	0.125	60	400	1.5	71.54	63.73
16	0.125	90	400	1.5	91.05	79.57
17	0.125	60	300	1.0	72.20	66.43
18	0.125	90	300	1.0	79.85	70.46
19	0.125	60	300	2.0	72.67	62.41
20	0.125	90	300	2.0	81.56	75.16
21	0.125	75	200	1.0	73.32	61.48
22	0.125	75	400	1.0	79.47	69.66
23	0.125	75	200	2.0	72.33	64.86
24	0.125	75	400	2.0	80.14	73.23
25	0.125	75	300	1.5	70.38	62.34
26	0.125	75	300	1.5	71.92	61.05
27	0.125	75	300	1.5	69.83	59.97
28	0.125	75	300	1.5	71.47	60.05
29	0.125	75	300	1.5	70.18	61.28
30	0.125	75	300	1.5	72.25	60.63

error variance, five replications were performed at the central point [13]. Response values (fibres yields) are reported in the two last columns of Table 7; these values were used to estimate the model coefficients b_j by the least square method which allowed us to write the following estimated models:

insoluble DF:

$$\begin{aligned}\widehat{y}_1 &= 71.005 + 4.960X_1 + 5.593X_2 \\ &\quad + 3.369X_3 + 3.003X_1^2 + 3.575X_2^2 \\ &\quad + 3.094X_3^2 + 2.279X_4^2 + 2.930X_2X_3,\end{aligned} \tag{3}$$

soluble DF:

$$\begin{aligned}\widehat{y}_2 &= 60.409 + 4.724X_1 + 5.144X_2 + 3.371X_3 \\ &\quad + 1.173X_4 + 2.282X_1^2 + 4.806X_2^2 + 3.298X_3^2 \\ &\quad + 3.222X_4^2 + 1.572X_2X_3 + 2.201X_2X_4.\end{aligned}$$

It can be seen from the regression equation of insoluble fibres extraction that only the linear coefficient corresponding to ionic strength was not significant. This result is in agreement with screening results. However, all quadric terms were highly significant; X_4^2 include all other quadric terms aliased with linear ones. Finally, a positive correlation between temperature and agitation velocity occurred. This result can be explained by the contribution of this interaction to break connections of fibres to other macromolecules which allows the increase of the purity of the fibre extract.

For the soluble fibres extraction, all linear and quadric terms were highly significant and had a positive effect on the response. Moreover, positive correlations occurred between temperature and agitation velocity on the one hand and temperature and ionic strength on the other hand. This latter interaction could be explained by the denaturation of protein leading to a higher purity.

The analysis of variance for fitted models showed that the regression sum of squares was statistically significant at the level of 99.99% for two responses and the lack of fit was not significant. Results of this analysis are summarized in Table 8. The coefficients of determination, R^2, for insoluble and soluble fibres extraction yields were 0.971 and 0.964, respectively. Thus, the predicted model well represented the observed values.

In order to validate the adequacy of model equations using point test method, five verification experiments were accomplished under various extracting conditions (within the experimental range). Table 9 presents design matrix along with the experimental results and theoretical values predicted by regression equations. Correlation coefficients (R^2) between experimental and predicted values for two responses were 0.967 and 0.964. There was no statistically significant difference at level of 95% between experimental and predicted values. Results clearly indicate that experimental values are in good agreement with predicted ones and also suggest that regression models are accurate and adequate for the extraction of both soluble and insoluble fibres which consolidate ANOVA results.

The relationship between responses and experimental variables can be illustrated graphically by contours plots (Figures 3 and 4). The topography of these responses is illustrated by isoresponse contours representing lines of constant response in a two-variable plane. Such plots are helpful in studying the effects of the variation of the factors in the studied domain and, consequently, in determining the optimal experimental conditions [34].

In Figure 3(a) the examination of the isoresponse contours plot showed that the yield increased when increasing temperature and/or increasing the *Agave* powder to water ratio. These effects were markedly shown for temperatures over 75°C and for P/W ratio higher than 0.145. This can be explained by the high solubility of fructans and simple sugars which increases with increasing temperature, giving a higher purity of extract. Similar results were obtained by Masmoudi et al. [13] for extracting pectin from lemon with acidified date juice. Qiao et al. [29] showed that the best yield of polysaccharide extracted from *Hyriopsis cumingii* occurs at a low ratio of water to raw material and a temperature of 80°C which is close to our results.

The positive effect of temperature above 75°C was also demonstrated in Figure 3(b). Thus, the increase of temperature and agitation speed improved the fibre extraction yield. This positive correlation can be explained by the thermodynamic effect on breaking connections of fibres to other biomolecules. In Figures 3(c) and 3(d), isoresponse curves are arranged in linear trajectory parallel to the vertical axis corresponding to the ionic strength. This fact appears as an evident, logical, and expected consequence which is in perfect harmony with the screening study.

Similar conclusions could be obtained from analyzing contours plots dressed in Figure 4. As previous discussion showed, all factors involved in the optimization study affect significantly fructans extraction. This fact is probably due to the thermodynamic and hydrodynamic phenomena. Ionic strength was significant as it intervenes by increasing purity while precipitating soluble protein.

Optimal extraction conditions: powder to water ratio of 0.1625, extracting temperature of 90°C, agitation speed of 400 rpm, and NaCl concentration of 1.5 g/L were determined using Nemrod W software. The suitability of these optimal extracting variables was also tested by executing the experiment under these conditions. Measured values belonged to the predicted intervals of both optimum insoluble and soluble fibres extraction yield (Table 10).

3.3. Characteristics of Insoluble Fibres Concentrate

3.3.1. Physical and Chemical Analysis of DF Concentrates. Analyses of lyophilised insoluble and soluble fibres are given in Table 11. Analysis showed high dry matter content (94.51% and 91.67%) and low water activity (0.225 and 0.337) for insoluble and soluble fibres, respectively, which allows a good microbiological stability and long preservation. The amount of fibres was of about 86 g/100 g and 90 g/100 g for insoluble and soluble fibres, respectively. These values are close to levels measured for DF preparations from date (ranging between

Table 8: Analysis of variance for Box-Behnken design.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
IF					
Regression	1030.01	14	73.572	36.072	<0.01***
Residuals	30.594	15	2.039		
Validity	25.539	10	2.554	2.526	15.9
Error	5.055	5	1.011		
Total	**1060.6**	**29**			
SF					
Regression	999.31	14	71.379	29.567	<0.01***
Residuals	36.213	15	2.414		
Validity	32.313	10	3.231	4.143	6.5
Error	3.899	5	0.779		
Total	**1035.52**	**29**			

***Significant at the level of 99.99%.

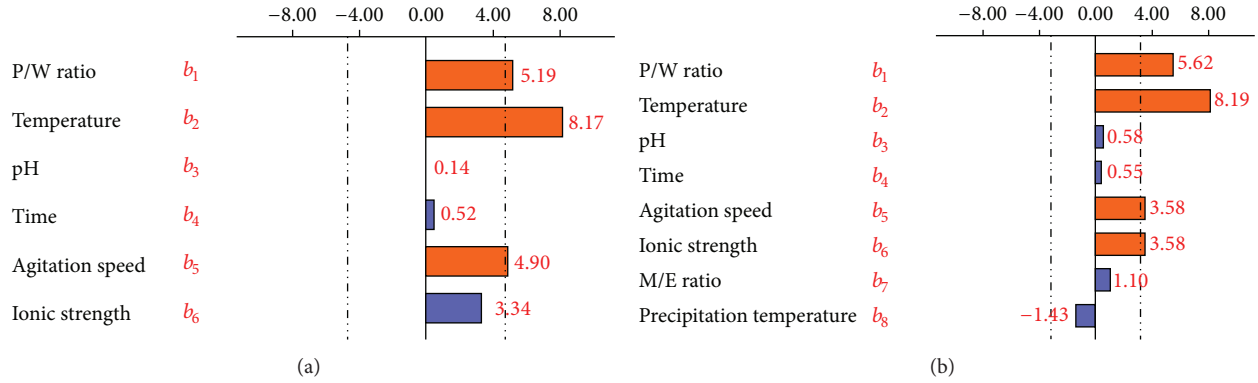

Figure 2: Graphical study of factor effects on soluble and insoluble fibres extraction (screening step): (a) insoluble fibres screening design and (b) soluble fibres screening design.

Table 9: Models validation experiments.

Exp.	IF		SF	
	yi	$\hat{y}$i	yi	$\hat{y}$i
31	67.180	67.295	56.430	57.183
32	73.350	73.081	61.790	62.495
33	72.230	75.082	63.430	64.944
34	74.370	74.747	63.910	64.478
35	71.450	72.291	61.170	63.292

yi: experimental value
$\hat{y}$i: predicted value.

Table 10: Suitability of selected optimal conditions to the measured values.

IF	Measured values: yi (%)	93.02
	Predicted value: $\hat{y}$i (%)	92.56 ± 2.43
SF	Measured values: yi (%)	80.46
	Predicted value: $\hat{y}$i (%)	81.53 ± 2.55

88 and 92% depending on the variety used for the process) and apple (89.8%), but notably higher than those of other

Table 11: Chemical composition of insoluble and soluble fibres concentrates (g/100 g dry matter).

	IF	SF
Dry matter (%)	94.51 ± 0.14	91.67 ± 0.27
a_w	0.225 ± 0.002	0.337 ± 0.002
pH	6.35 ± 0.04	5.59 ± 0.04
Total fibres	86.23 ± 1.32	89.84 ± 0.88
Ash	8.01 ± 0.05	3.25 ± 0.17
proteins	2.32 ± 0.09	1.44 ± 0.11
Fat	1.24 ± 0.04	0.097 ± 0.003
Soluble sugars	1.58 ± 0.21	4.42 ± 0.54

fruits DF concentrates reported for grapefruit, lemon, orange and mango (28–78.2%), citrus peel (57%), and mango peel (~71%) [12]. Table 10 reveals the presence of ash as a major contaminant of insoluble DF concentrate (~8%) followed by protein, soluble sugars, and fat; this may be due to the use of sodium chloride in the extraction process. For fructans, soluble sugars and ash are the main contaminant of the extract. The low level of protein obtained could be due to the repeating washings after extraction leading to the

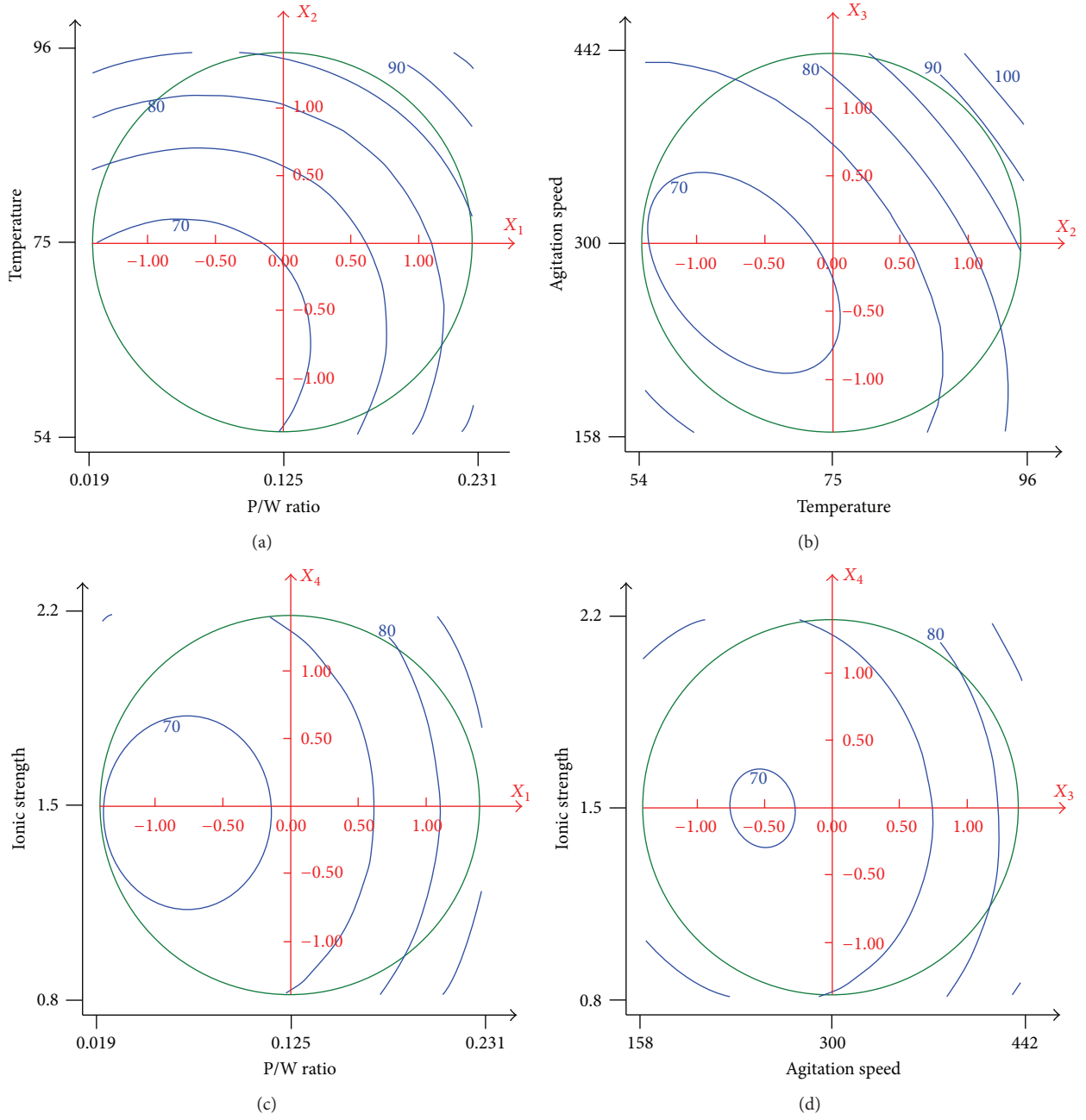

Figure 3: Contour plots illustrating the effect of (a) temperature and P/W ratio at constant agitation speed (300 rpm) and ionic strength (1.5 g/L), (b) temperature and agitation speed at constant P/W ratio (0.125) and ionic strength (1.5 g/L), (c) ionic strength and P/W ratio at constant agitation speed (300 rpm) and temperature (75°C), and (d) ionic strength and agitation speed at constant P/W ratio (0.125) and temperature (75°C) on insoluble fibres yield extracted from dried *Agave* leaves' powder.

elimination of amino acids issued from protein hydrolysis at high temperatures.

3.3.2. Water and Oil Holding Capacities (WHC and OHC). Insoluble fibres concentrate showed a high WHC (8.66 g water/g sample) (Table 12). This value is higher than those reported for other fruit fibre concentrates, such as citrus, apple, oat bran, and pear DF (3.6 to 6.8 g water/g sample) [9]. The high WHC of *Agave* insoluble fibres concentrate suggested that this material could be used as a functional ingredient in food to avoid syneresis of formulated products

Table 12: Water and oil holding capacities of *Agave* fibres concentrate (g/g of fibres concentrate).

	IF	SF
Water holding capacity	8.66 ± 0.02	2.28 ± 0.03
Oil holding capacity	5.62 ± 0.03	3.37 ± 0.01

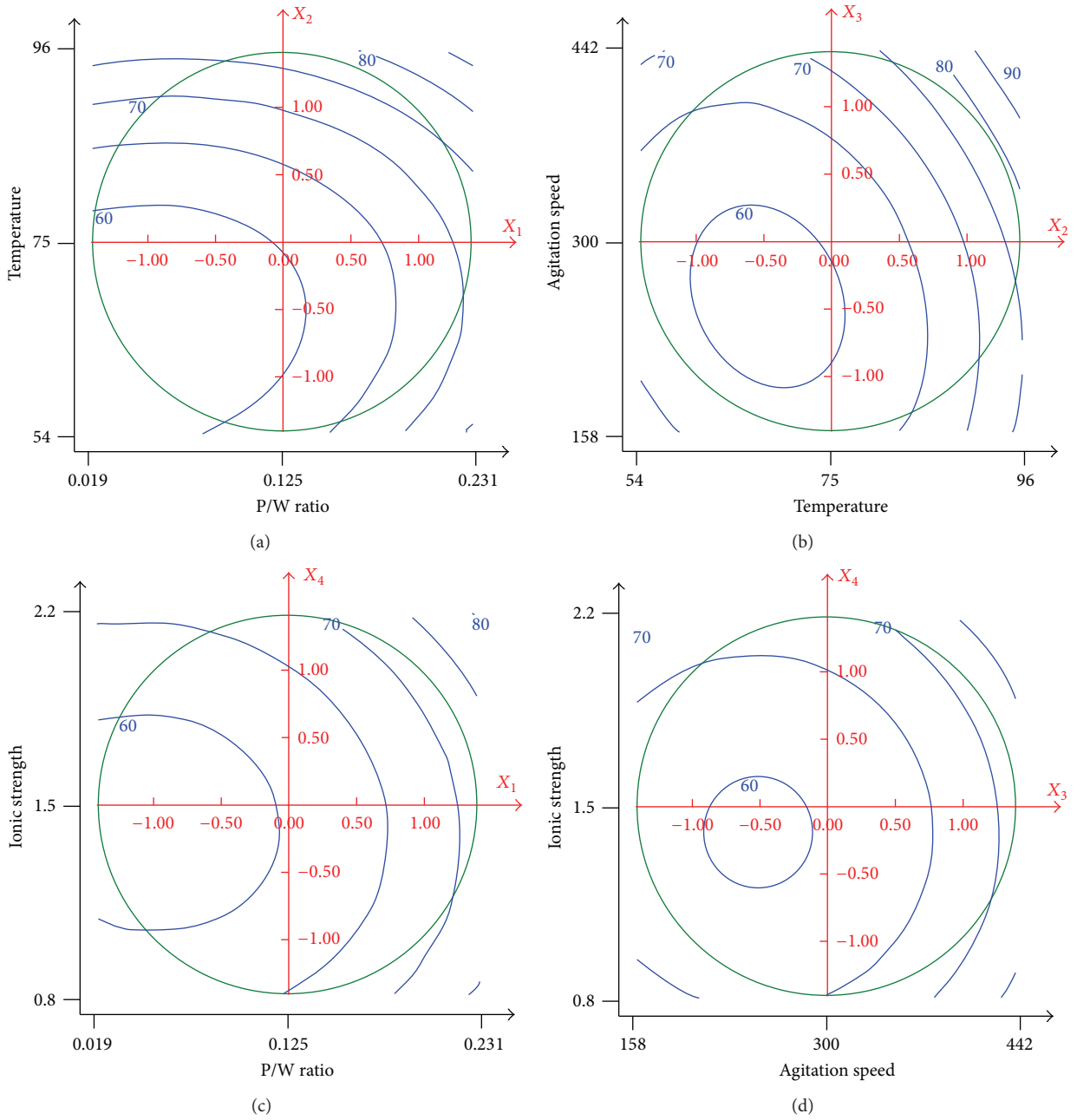

Figure 4: Contour plots illustrating the effect of (a) temperature and P/W ratio at constant agitation speed (300 rpm) and ionic strength (1.5 g/L), (b) temperature and agitation speed at constant P/W ratio (0.125) and ionic strength (1.5 g/L), (c) ionic strength and P/W ratio at constant agitation speed (300 rpm) and temperature (75°C), and (d) ionic strength and agitation speed at constant P/W ratio (0.125) and temperature (75°C) on soluble fibres yield extracted from dried *Agave* leaves' powder.

[12]. This concentrate is also characterised by a high OHC (5.6 g oil/g sample) (Table 12), superior to some agricultural byproducts and DF concentrates cited by Abdul-Hamid et al. [9, 10], for peach DF concentrate and rice bran (1.11 and 4.54 g oil/g sample). The high OHC of *Agave* insoluble DF concentrate suggested that this material could be used as an ingredient to stabilize foods with a high percentage of fat and emulsion [12]. Fructans concentrate has not good water and oil holding capacities; however, it showed a high solubility at 25°C of 86.52 ± 3.47 g/L which is comparable with commercial chicory inulin (113.68 ± 4.14 g/L).

3.3.3. Microstructure Visualization. As it can be well seen in Figure 5, *Agave* insoluble fibres have two distinct morphologies. In micrograph of Figure 5(e), hemicelluloses filaments overlap to form an amorphous nodular network and play the role of gathering and supporting elements of principal structure [7]. This latter, of 150 to 200 μm of diameter (micrographs of Figures 5(a) and 5(b)), has a cylindrical shape associating helicoidal microfibrils called tracheids (micrographs of Figures 5(c) and 5(d)). These voluminous structures have a low density and show a lot of vacuum which illustrate and justify the high water and oil holding capacities.

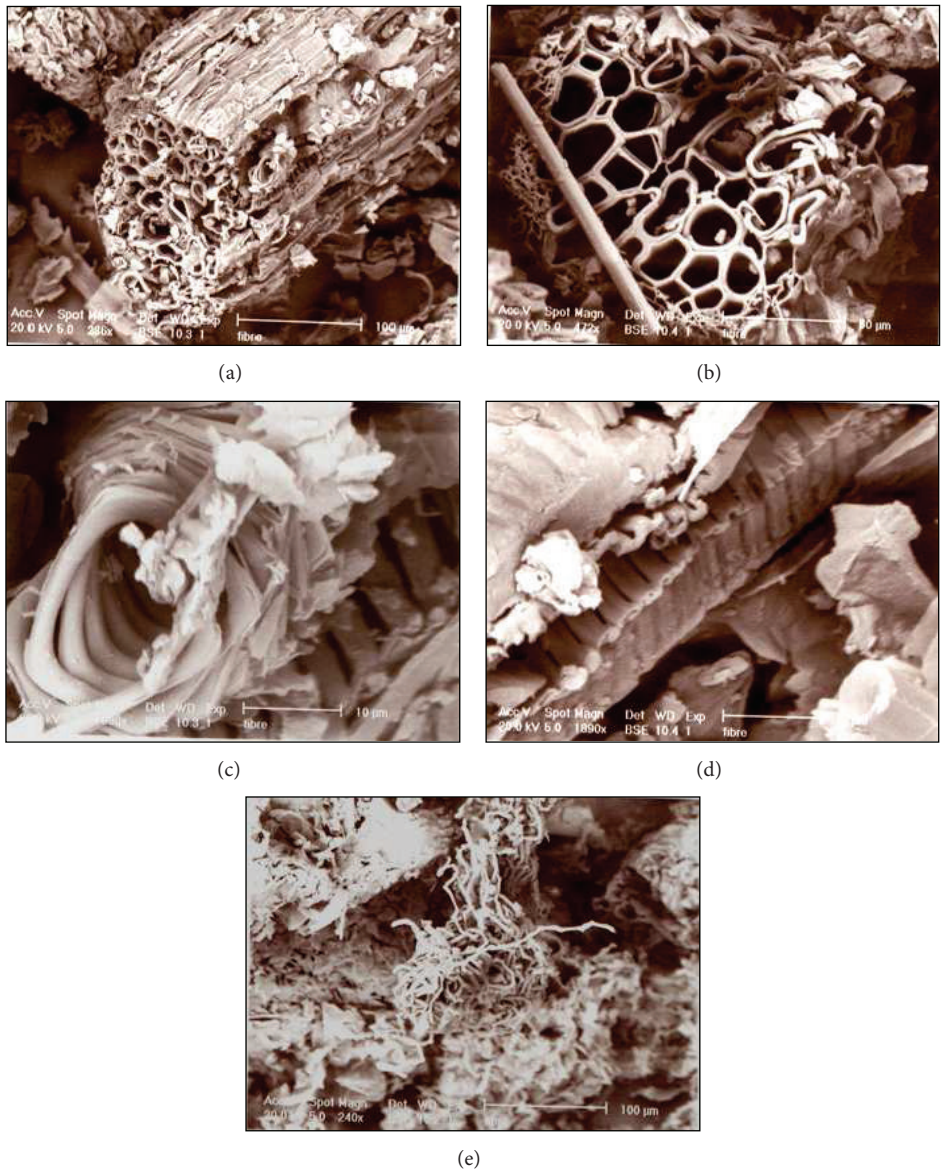

Figure 5: Scanning electron microscopy images of insoluble fibres concentrate from *Agave americana* L.; scale bar for (a) and (e), 100 μm; scale bar for (b), 50 μm; scale bar for (c) and (d), 10 μm.

Izydorczyk and Dexter [35] revealed similar structures for barley fibres and found a high WHC when they incorporate this product in bread formulation.

Agave fructans morphology was also visualized using scanning electron microscopy and compared to commercial chicory inulin in order to have additional information concerning impact of botanic source and extraction conditions on fructans structures (Figure 6). Micrographs of Figures 6(a), 6(b), and 6(c) show irregular structure of commercial product. Nonuniform particles sized from 50 to 70 μm are squeezed with small spherical particles sized under 10 μm. However, micrographs of Figures 6(d), 6(e), and 6(f) show a heterogenic structure illustrating pronounced morphological and dimensional variability. Indeed, commercial product is finely milled and microfluidized; this fact explains the observed homogeneity of structure. This result is in accordance with the discussion of other works which illustrated that microfluidized inulin particles have a uniform shape and were interspaced by voids when nonmicrofluidized inulin was arranged as a superposition of lamella or on amorphous shape [23–36].

4. Conclusion

This work has revealed that after screening step, the response surface methodology was a useful tool to determine the optimal experimental conditions of soluble and insoluble fibres extraction from *Agave americana* leaves. The extraction yield of insoluble fibres increased significantly with increasing temperature, *Agave* powder to water ratio, and agitation

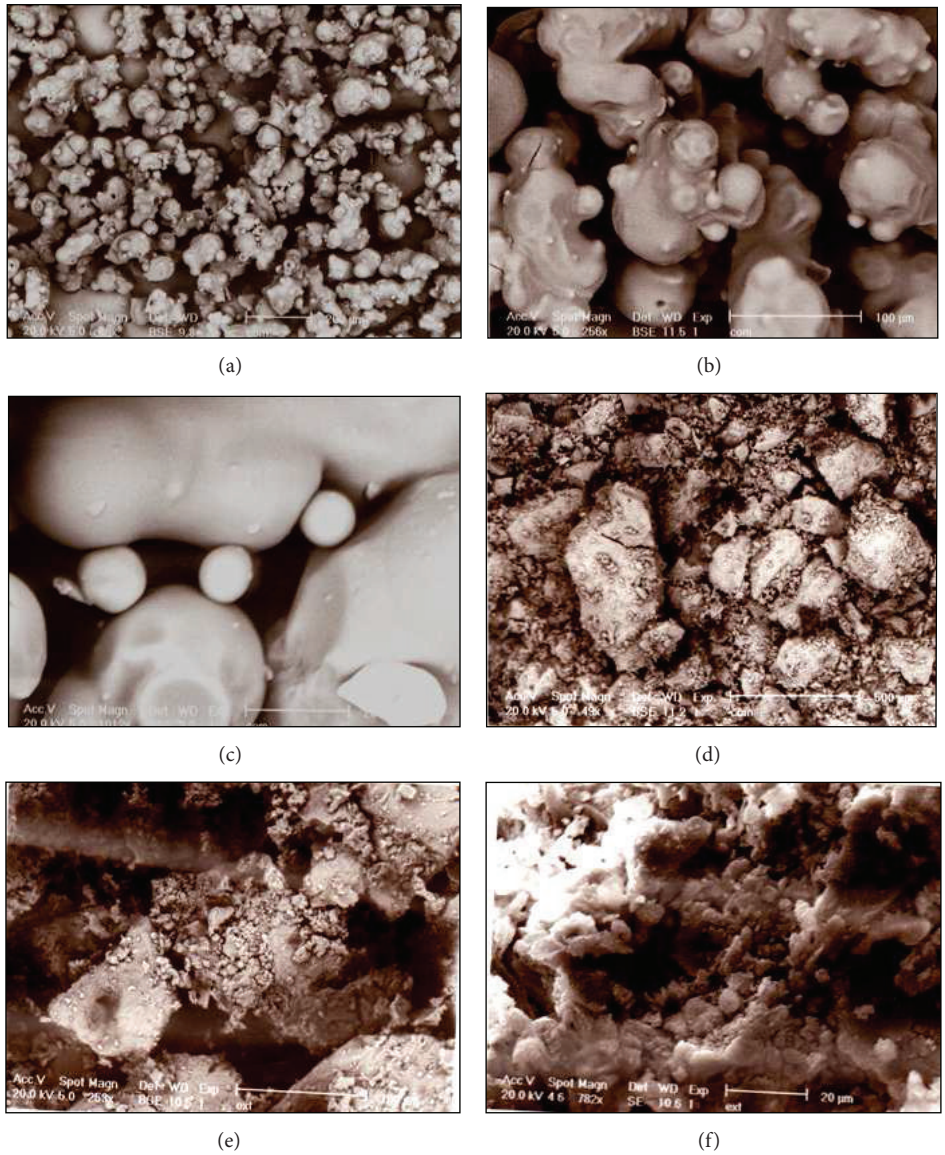

Figure 6: Scanning electron microscopy images of commercial inulin (a, b, and c) and soluble fibres concentrate of *Agave Americana* L. (d, e, and f); scale bar for (d), 500 μm; scale bar for (a) and (e), 200 μm; scale bar for (b), 100 μm; scale bar for (c) and (f), 20 μm.

speed, while ionic strength has no effect on the studied response. However, fructans extraction yield depended on all investigated factors. Under selected optimal conditions (temperature: 90°C; P/W ratio: 0.1625; agitation speed: 400 rpm; and ionic strength: 1.5 g/L), insoluble and soluble DF extraction yields reached 92.56 ± 2.43% and 81.53 ± 2.55%, respectively. These concentrates showed a high purity and excellent functional properties. Thus, it is promising to focus on the possibility to incorporate these fibres concentrates in food applications. In a future study, the extraction and the characterisation of protein should be studied in order to add value to this unexploited plant.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Cocoa Polyphenols: Can We Consider Cocoa and Chocolate as Potential Functional Food?

Djurdjica Ackar,[1] Kristina Valek Lendić,[2] Marina Valek,[2] Drago Šubarić,[1] Borislav Miličević,[1] Jurislav Babić,[1] and Ilija Nedić[3]

[1] *Faculty of Food Technology Osijek, Franje Kuhača 20, 31000 Osijek, Croatia*
[2] *Institute of Public Health of the Osijek-Baranja County, F. Krežme 1, 31000 Osijek, Croatia*
[3] *Sugar Factory Osijek, Frankopanska 99, 31000 Osijek, Croatia*

Correspondence should be addressed to Djurdjica Ackar; dackar@ptfos.hr

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Chocolate has been consumed as confection, aphrodisiac, and folk medicine for many years before science proved its potential health benefiting effects. Main compounds of cocoa and chocolate which contribute to human health are polyphenols that act as antioxidants and have potential anti-inflammatory, cardioprotective, antihepatotoxic, antibacterial, antiviral, antiallergenic, and anticarcinogenic properties. This paper gives a short overview of scientific literature regarding cocoa polyphenols and influence of cocoa and chocolate on human health. Although research on health benefits of dark chocolate and cocoa is quite extensive nowadays and shows potentially beneficial effects of dark chocolate and cocoa, there are still lots of unknowns and some controversies. This is obviously an area that needs more research in order to determine factual influence of chocolate on health.

1. Introduction

Cocoa and chocolate are consumed by humans for thousands of years. To Mayan people, cocoa pods were symbols of fertility and life and food of gods. Aztecs believed that consumption of cocoa gave wisdom and power and used cocoa as currency. Aztecs and Mayas made dark, unsweetened drink based on cocoa, which was called *xocoatl*. They seasoned it with chili peppers and added corn meal, but sugar was unknown to them. In 1492 Columbus brought cocoa beans from America to Europe, but at that time they were not interesting to Europeans [1].

Hernan Cortez, in 1528, brought cocoa to Spain along with secret of making *Chocolatl*. In Spain, sugar, vanilla, nutmeg, cloves, allspice, and cinnamon were added to the original recipe and aphrodisiac shortly made breakthrough in Europe [1].

However, chocolate bars were not produced until the 18th century, when mechanical mills for squeezing cocoa butter from cocoa mass were produced, and milk chocolate was first produced in the 19th century by Daniel Peter and Henry Nestle. Rodolphe Lindt invented a process called conching, which enabled formation of smooth chocolate aroma and Milton Hershey was a pioneer of mass production of affordable chocolate bars.

For many years, chocolate was consumed purely for pleasure, but in the last 20 years researches have shown that dark chocolate and cocoa could have beneficial effect on human health due to high content of polyphenols.

Polyphenols are large and heterogeneous group of biologically active secondary metabolites in plants, where they act as cell wall support materials, colourful attractants for birds and insects, and defence mechanisms under different environmental stress conditions (wounding, infection, excessive light, or UV irradiation) [2]. Based on a number of phenolic rings and of the structural elements that link these rings, they are divided into four groups: phenolic acids, lignans (recognized as phytoestrogens; flaxseed and flaxseed oil are the main source), flavonoids (the most abundant polyphenols in human diets), and stilbenes (resveratrol is under investigation for its anticarcinogenic properties). Flavonoid group is subdivided into: anthocyanins, flavonols, flavanols (catechins

in tea, red wine, and chocolate), flavanones (citrus fruit are the main source), flavones, and isoflavones (main source is soya) [3] (Figure 1).

2. Cocoa Polyphenols

Three main groups of polyphenols in unfermented cocoa bean are flavan-3-ols or catechins, anthocyanins, and proanthocyanidins, with average content of 120–180 g/kg [4, 5]. Main polyphenol compound in fresh cocoa bean is (−)-epicatechin (Figure 2), with average content of 21–43 mg/g of defatted sample, followed by (+)-catechin, and dimers and trimers of these compounds [6]. Complex alteration products of catechin and tannin give brown and purple colour to cocoa bean, and leucoanthocyanins are present as glycosides [6].

Research of Counet et al. (adopted from [7]) showed that genetic characterization influences polyphenol content in cocoa. Namely, they found that *Criollo* cultivars contained higher levels of procyanidins than *Forastero* and *Trinitario* beans. In addition, crop season and country of origin have impact on polyphenols in cocoa beans [7].

Cocoa bean processing highly affects polyphenol content. During fermentation, polyphenols diffuse with cell liquid from storage cells and are subjected to oxidation (both nonenzymatic and polyphenol-oxidase-catalyzed), polymerisation, and reactions with proteins [4, 8]. Anthocyanins are hydrolysed to anthocyanidins and sugar component, leucocyanidins are dimerised [5], and (−)-epicatechin and soluble polyphenol content are reduced to 10–20% [8]. Hurst et al. [9] studied levels of flavan-3-ol monomers during fermentation, drying, and roasting cacao bean. They reported that unripe and ripe cacao pods contain solely (−)-epicatechin and (+)-catechin. During fermentation, levels of both of these compounds were reduced, but (−)-catechin was formed due to heat-induced epimerization.

During drying, additional loss of polyphenol occurs, mainly due to nonenzymatic browning reactions [4, 5].

Roasting results in significant loss of polyphenols due to thermolabile flavanols [10] and oxidation of epicatechin and catechin to quinones which complex with amino acids and proteins and polymerize with other polyphenols [11]. According to research of Hurst et al. [9], in this processing step loss of (−)-epicatechin and (+)-catechin is partly attributed to heat-induced epimerization to (−)-catechin.

All these processes are needed to develop characteristic cocoa aroma. Polyphenols give astringent and bitter aroma to cocoa and contribute to reduced perception of "cocoa flavour" by sensory panel [5]. However, nowadays processes are conducted in such manner to preserve as much polyphenol as possible with maintaining satisfactory aroma.

Research of Crozier et al. [12] showed that cocoa powder had significantly higher content of polyphenols and higher antioxidant activity compared to pomegranate and blueberry powder. However, alkalization has been shown to destroy polyphenols and significantly reduce antioxidant activity of cocoa powder.

During process of chocolate making, composition and content of polyphenols are furtherly altered, mainly due to rather high temperatures and presence of oxygen [8]. Therefore, dark chocolate had similar antioxidant activity to pomegranate juice, despite higher content of polyphenols [12].

3. Bioavailability of Cocoa Polyphenols

Generally, bioavailability of polyphenols is affected by chemical structure of polyphenols, food matrix, factors related to food processing, and interactions with other constituents in diet, as well as with some host related factors (genetic aspects of individuals, gender and age, disorders and physiological condition, and microbiota metabolism and enzyme activity in the colon) [3, 13]. The most important food sources of polyphenols are vegetables and fruits, green and black tea, red wine, coffee, chocolate, olives, and some herbs and spices, as well as nuts and algae [14]. Besides, some polyphenols are specific to particular food and some are found in all plant products, so that, generally, food is considered to contain complex mixtures of polyphenols [13].

Isoflavones and phenolic acids have highest absorption, followed by catechins, flavanones, and quercetin glucosides, whereas proanthocyanidins, anthocyanidins, and galloylated tea catechins are poorly absorbed [15].

Once absorbed, polyphenols are conjugated to glucuronide, sulphate, and methyl groups in the gut mucosa and inner tissues, where epicatechin and epigallocatechin are mostly present as the glucuronide and sulfate conjugates. Absorption of epicatechin and catechin in the intestine averages between 22% and 55%, while dimers and trimers are poorly absorbed (less than 0.5%). Procyanidins cross intestinal barrier and are transported to liver, where they undergo methylation, glucuronidation, and sulfation which result in antioxidant capacity [15].

Polyphenols that reach colon are fermented by microflora to phenolic acids of low molecular weight [15].

Epicatechin from chocolate is rapidly absorbed by humans, with plasma levels detected after 30 min of oral digestion, peaking after 2-3 h and returning to baseline after 6–8 h. In addition, cumulative effect in high daily doses was recorded [10].

Generally, it can be stated that the smaller the polyphenol, the higher the concentration in blood and the higher the chance it will reach its target organ in the body [16]. Chirality might also influence bioavailability of polyphenols—(+)-form of catechin is almost 10 times more absorbed than (−)-form [16].

Presence of sugars and oils generally increases bioavailability of polyphenols, while proteins, on the other hand, decrease it [3]. Research of Neilson et al. [17] showed that milk proteins and sucrose modulate metabolism, plasma pharmacokinetics, and bioavailability of catechins from chocolate confections. They found that milk proteins reduce bioavailability of epicatechin in chocolate confectionary. Serafini et al. [18] reported inhibition of *in vivo* antioxidant activity of chocolate by addition of milk either during manufacturing process or during ingestion. However, this effect was not observed in chocolate beverages [17]. Study of interactions

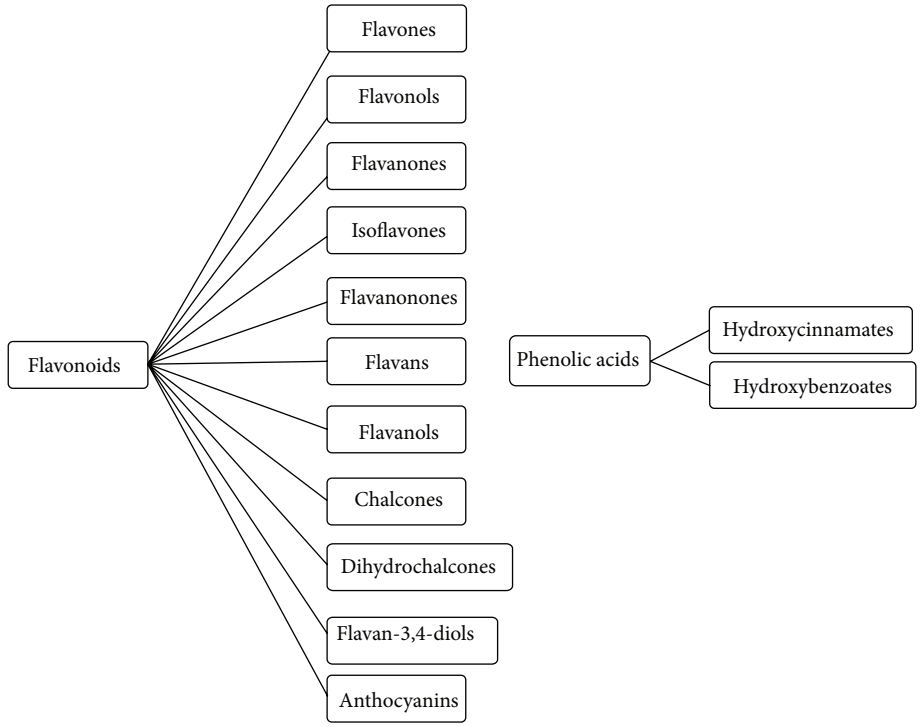

Figure 1: Basic classification of flavonoids and phenolic acids [59].

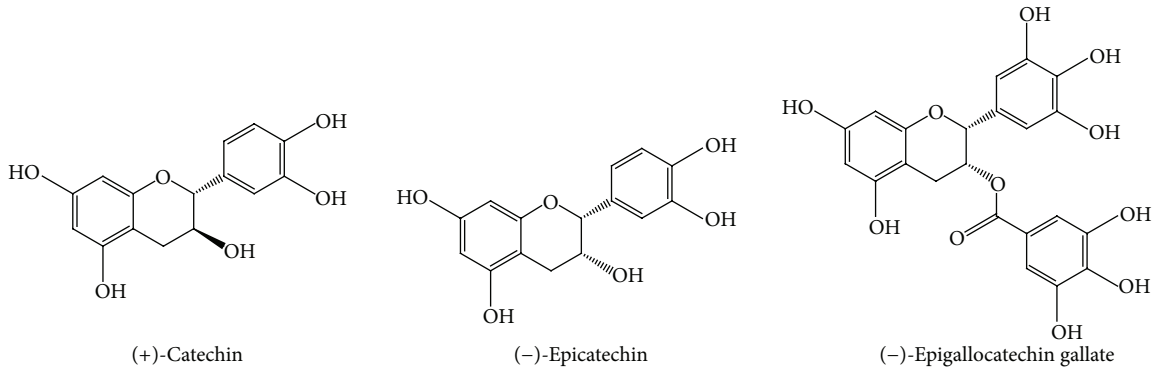

Figure 2: Structure of main cocoa polyphenols [60].

of cocoa polyphenols with milk proteins by proteomic techniques demonstrated that protein-polyphenol complex formation involves covalent binding of free SH-group of the free cysteine residue of protein. This was supported by the fact that alkylated form of peptide did not react with flavanols, while lactosylation did not prevent polyphenol binding. Since only small portion of protein interacts with polyphenol, bioavailability of polyphenols themselves is not significantly influenced [19]. This is supported by researches of Roura et al. [20] and Keogh et al. [21] who reported that milk does not affect bioavailability of cocoa powder flavonoids in healthy adults.

Sucrose increased bioavailability of polyphenols, but formulation also influenced the extent of sucrose impact. Schramm et al. [22] observed enhanced uptake of aglycone flavanols when they were consumed immediately after carbohydrate-rich meal. Peters et al. [23] concluded that sucrose addition to green tea resulted in delay of catechin absorption, partly due to viscosity increase, but it also improved catechin uptake by intestine.

4. Influence of Cocoa Polyphenols on Health

Unlike vitamins, polyphenols are not essential components of human diet. Nevertheless, they are consumed on daily basis due to their ubiquitous presence in fruits and vegetables. Many researches have shown that polyphenols and/or polyphenol-rich foods have an important role in health preservation due to antioxidant properties [15, 16, 24]. The antioxidant activity of cocoa and chocolate was shown to be correlated with their catechin and procyanidin contents [25].

Antioxidant properties of polyphenols highly depend on the arrangement of functional groups around the nuclear structure. Free radical scavenging capacity is primarily attributed to hydroxyl groups, and aglycones are more potent antioxidant than their responding glycosides [26].

Polyphenols can act as proton donor-scavenging radicals [27], inhibitors of enzymes that increase oxidative stress, chelate metals, bind carbohydrates, and proteins [26]. These properties enable them to act as anticarcinogenic, anti-inflammatory, antihepatotoxic, antibacterial, antiviral, and antiallergenic compounds [27–30].

This is supported by research of Hollenberg et al. [31], who established relationship between high consumption of cocoa beverages and very low blood pressure levels, reduced frequency of myocardial infarction, stroke, diabetes mellitus, and cancer in Kuna Indians residing in archipelago on the Caribbean Coast of Panama, unlike Kuna Indians residing on Mainland. Another study, conducted on elderly men free of chronic diseases in Zutphen, Netherlands, showed that consumption of cocoa reduced blood pressure and decreased risk of cardiovascular and all-cause death by 45–50% [32].

Grassi et al. observed decrease of blood pressure by short-term administration of dark chocolate in healthy [33] and glucose-intolerant, hypertensive subjects [34]. However, they investigated only 15 subjects per research and these findings should be taken with reserve. Djoussé et al. [35, 36] associated frequent consumption of dark chocolate with lower prevalence of cardiovascular diseases in men and women independently of traditional risk factors estimated based on health questionnaire. This association was perceived both in smokers and nonsmokers, as well as in subjects under and above 60 years of age. The research included large number of examinees, but data about consumption of chocolate were self-reported and there was no differentiation between dark and milk chocolate.

On the other hand, Ried et al. [37] did not find a blood pressure reducing effect of dark chocolate in 36 prehypertensive healthy adult volunteers with daily consumption of 50 g of dark chocolate.

Almoosawi et al. [38] investigated influence of 2-week consumption of polyphenol-rich dark chocolate on blood pressure of 14 obese and overweight individuals. Results of their research showed that both systolic and diastolic pressure decreased, along with reduction of fasting blood glucose levels and urinary free cortisone levels.

In addition to lowering blood pressure levels, cocoa polyphenols might be involved in cholesterol control. Waterhouse et al. (1996) reported polyphenols from chocolate inhibited LDL oxidation by 75%, compared to 37–65% of red wine (adopted from [16]). In addition, Vinson et al. [39] reported that dark chocolate had higher quality of phenol antioxidants expressed as IC_{50} for LDL + VLDL oxidation compared to red wine and black tea, with high lipoprotein bound antioxidant activity, which is very important in prevention of heart diseases. A survey implemented by a group of experts showed that in the case of similar absorption, about 50 g of dark chocolate should be eaten to provide equivalent flavonoids to about 200 mL of red wine, which has been shown to reduce heart attack risk for an average adult [40].

Mursu et al. [41] reported increase of HDL cholesterol after 3-week consumption of dark and polyphenol-rich dark chocolate. Total and LDL cholesterol were decreased after 15-day consumption of polyphenol-rich dark chocolate by 6.5% and 7.5%, respectively [33, 34]. Hamed et al. [42] reported 6% decrease of LDL cholesterol and 9% increase of HDL after 7-day consumption of regular dark chocolate.

Hot cocoa beverage was proven to successfully reduce LDL cholesterol, increase HDL cholesterol, and suppress LDL oxidation in research of Baba et al. [43]. Atherosclerotic cholesterol profile (cholesterol : HDL ratio) in patients with diabetes was improved after 8-week chocolate consumption without affecting weight, inflammatory markers, insulin control, or glycaemic control [44].

On the other hand, Kurlandsky and Stote [45] reported no significant difference in HDL and LDL cholesterol levels between "chocolate consuming" and control group. However, large age difference between control and "chocolate consuming" group may have influenced these results. Almoosawi et al. [38] also observed no significant change in total cholesterol level after consumption of dark chocolate.

Glucose blood levels could be reduced by consumption of dark chocolate; however, treatment duration and dark chocolate dose seem to significantly influence the effectiveness of treatment. Namely, as opposed to short-term treatments of Stote et al. [46, 47] and low-dose treatment of Taubert et al. [48], long-term high-dose treatment of Almoosawi et al. [38] proved to be effective. Researches of Grassi et al. showed that short-term administration of dark chocolate increases insulin sensitivity both in healthy [33] and in glucose-intolerant hypertensive people [34]. Davison et al. [49] reported significant insulinaemia and differential plasma glucose response after consumption of dark chocolate 2 h prior to exercise compared to fasting, but no significant effect on oxidative stress reduction.

Insulin response and blood pressure could be linked with the regulation of nitric oxide production by dark chocolate flavanols. Increased generation of nitric oxide (NO) and reactive oxygen species (ROS) in the vessel wall in response to dietary isoflavones enhances the activity of antioxidant defense enzymes in endothelial and smooth muscle cells (probably owing to estrogenic properties of isoflavones) by activation signaling pathways that increase NO bioavailability and regulate phase II and antioxidant enzyme expression via the redox sensitive transcription factor Nrf2 [50, 51]. Investigations carried out by Ried and coworkers showed that flavonol-rich chocolate may have a small but statistically significant effect on lowering blood pressure by 2-3 mm Hg in the short term [52, 53].

Flavanol-rich cocoa increases blood flow to key areas of brain increasing blood oxygenation level-dependent response to cognitive task switching paradigm in healthy young people [54] and could be useful in treatment of cerebrovascular flow (CBF) dementia [55], Alzheimer's disease [56], and stroke [57]. Chandranayagam et al. [58] reported that tannin-rich chocolate can be considered as functional food which effectively antagonizes adverse effects of arsenic intoxication. However, this research was conducted

on Sprague Dawley rats and should yet be confirmed by research on humans.

5. Conclusion

Recent researches have shown that cocoa and dark chocolate could have beneficial impact on our health, mainly on cardiovascular system. However, part of the researches could be arguable, since either a small number of examinees were included or information about type of chocolate and consumption was scarce and/or ambiguous. In addition, since chocolate is a rich source of sugar and saturated fat, it is questionable whether chocolate consumption can be recommended in vascular health promotion because of its contribution to total calorie intake and impact on weight.

More systematic approaches should be applied in human studies to reduce possible misinterpretation of data—more examinees, longer test periods, and larger age differences should be involved in addition to controlled chocolate administration with specified polyphenol content and composition. Individual nutritive preferences which could have great impact on results should also be taken into consideration.

Chocolate and cocoa contain not only polyphenols but also methylxanthines which could additionally contribute to the health impact of these foods. There is need for additional researches that would elucidate the extent of polyphenols and methylxanthines health impact and possible synergy of these compounds in chocolate, with respect to energy contribution.

Obviously, elucidation of cacao and chocolate impact on human health is rather a complex problem and should be addressed as such data should not be lightly interpreted but closely examined and reassessed before withdrawing conclusions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Wild Edible Plants as Potential Antioxidants in Vegetables Oils

Ana Romojaro,[1] Paloma Sanchez-Bel,[2] Maria Serrano,[1] and M. Teresa Pretel[1]

[1] *Departamento Biología Aplicada, Escuela Politécnica Superior de Orihuela, Universidad Miguel Hernández, Ctra Beniel-Orihuela, km 3.3, 03312 Alicante, Spain*
[2] *Departamento Biología del Estrés y Patología Vegetal, Centro de Edafología y Biología Aplicada del Segura, CSIC, Espinardo, Murcia, Spain*

Correspondence should be addressed to M. Teresa Pretel; mteresa.pretel@umh.es

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The effect of the addition of fruits of *Rosa canina* and *Quercus ballota* and leaves of *Sanguisorba minor* to sunflower, seed, and olive oils on lipid oxidation during the frying process was analyzed. The three underutilised edible plants increased the oxidative stability of the sunflower oil, since the malondialdehyde (MDA) content was significantly decreased with respect to values of the control sunflower oil after the heating process. However, in olive oil, the effect of these edible plants on decreasing the lipid peroxidation was only evident for the highest concentration, while in seed oil the addition of the edible plant showed a prooxidant effect. Thus, these wild edible plants could be used to enrich vegetable oils with low content of natural antioxidant, such as sunflower oil, and avoid or decrease the use of synthetic antioxidant.

1. Introduction

Edible vegetable oils such as sunflower oil, seed oil, and olive oil have an important place in human diet, since their use in culinary processes is a constant. However, during frying, a gradual deterioration of oil quality occurs due to lipid oxidative decomposition reactions leading to lowering quality and nutritional value of foods, with consequences for the health of the consumer, causing aging, membrane damage, and numerous diseases, such as chronic inflammation, neurodegenerative diseases, adult respiratory distress syndrome, atherogenesis, diabetes, and different types of cancer [1]. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butyl hydroquinone (TBHQ), are widely used in the food industry to avoid oxidation [2] due to their low cost and good performance, although their safety has been questioned due to carcinogenic effect [3]. According to the European Bulletin (order of 24 August 2007) [4] the maximum dose that allowed for these artificial additives in frying oils is 200 mg kg^{-1} of oil.

Under these circumstances, research on development of safe natural antioxidants is therefore essential. Recently it has been shown that *Nigella sativa* seed extract could be an interesting alternative to the use of synthetic antioxidants, since the addition of *Nigella* seed extract to sunflower oil improved their thermal stability and shelf-life [5]. Accordingly, Cordeiro et al. [6] have shown that extract of *Rosmarinus officinalis* added to sunflower, corn, and soybean oils displayed a more effective antioxidative action than TBHQ and it was stable at the frying temperature of the oils, unlike the majority of the antioxidants commonly used. Moreover, even in olive oils which contain high concentration of natural antioxidants, the addition of oregano essential oils leads to a protective effect on primary lipid oxidation [7].

Recent phytochemical studies have revealed that fruits from *Rosa canina* L. contain high amounts of vitamin C, carotenoids, and polyphenols making them a suitable source of antioxidants to be used commercially to retard rancidity in fatty materials in food manufacturing [8, 9]. Accordingly, acorns from *Quercus* spp. also possess biologically active substances, such as α- and γ-tocopherol and phenolics compounds, specially tannins, with proved antioxidant and free-radical-scavenging capacities [10, 11]. Finally, leaves and shoot from *Sanguisorba minor* Scop. (Sm) have also high

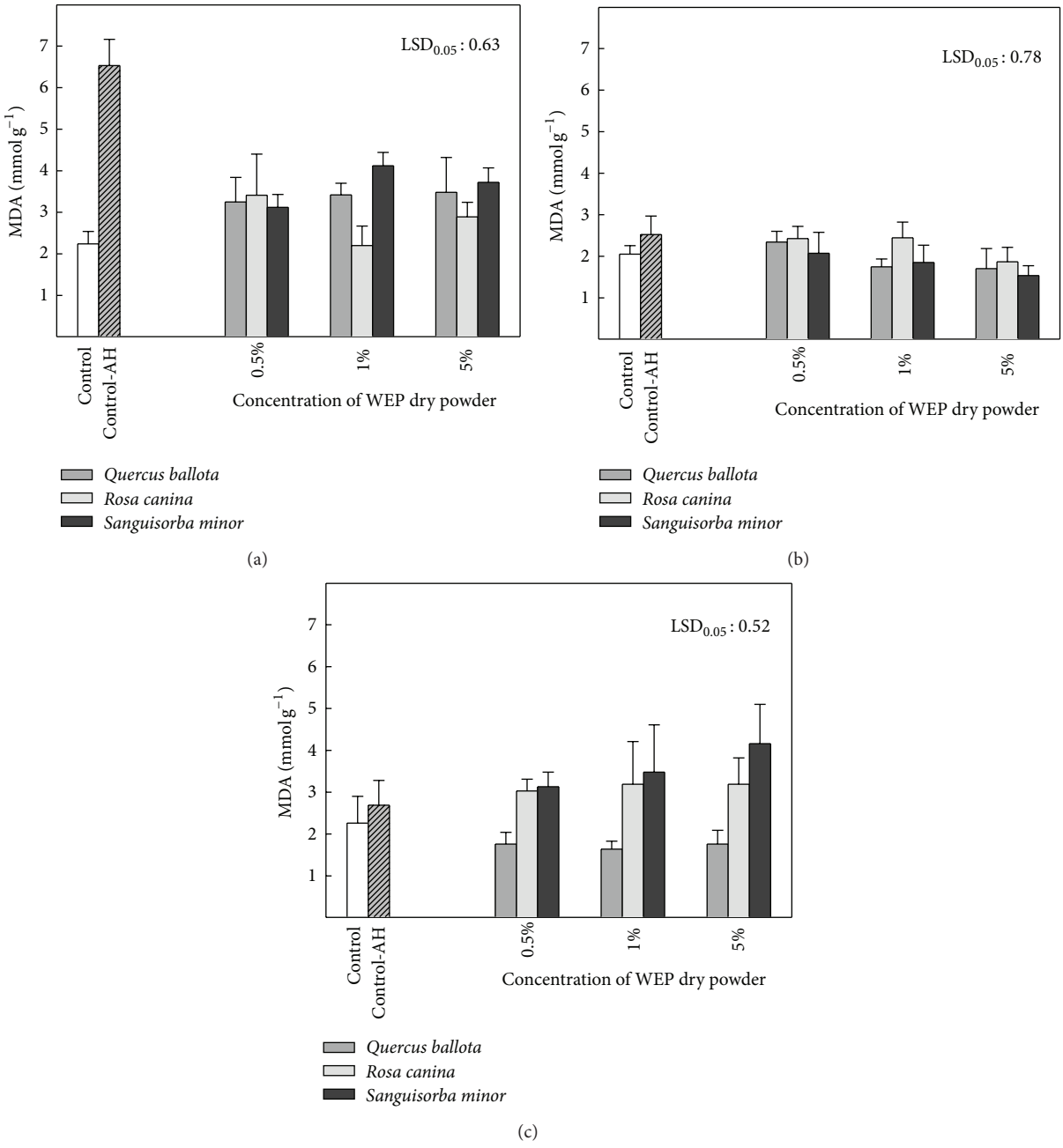

Figure 1: Lipid peroxidation value, expressed as malondialdehyde (MAD) concentration, in vegetable oils before the heating process (control) and after heating (AH) and after the heating process of these edible oils with the addition of several concentrations of the wild edible plants (WEP). For each vegetable oil ((a) sunflower, (b) olive, and (c) seed oil) data are the mean ± SE of two determinations performed in three replicates and LSD values are shown.

concentrations of phenolics compounds, the major being derivatives of caffeic acid, apigenin, quercetin, and kampherol [12].

Thus, the aim of this work was to evaluate the effect of the addition of dry powder from three underutilised edible plants, fruits of *Rosa canina*, corn of *Quercus ballota,* and shoots and leaves of *Sanguisorba minor* to sunflower, seed, and olive oils on lipid oxidation during the frying process.

2. Material and Methods

2.1. Wild Edible Plants (WEP) and Vegetable Oils. Fruits of *Rosa canina* L., acorns of *Quercus ballota* Desf., and leaves of *Sanguisorba minor* Scop. were harvested at their optimum stage for consumption according to traditional practices and transferred to laboratory. Then, 3 homogeneous samples (50 g) were randomly performed for each species,

which were frozen in liquid N_2, lyophilized, and ground to be used as additives to vegetable oils. The vegetable oils used were virgin olive oil from oil press of Jaen (Spain), sunflower oil (Savena-Ibérica, Sevilla, Spain), and special seed oil for frying with increased oleic acid content and E-900 antifoam (Hacendado, Mercadona, Spain). Samples of lyophilized WEP were added to the vegetable oils at 0.5, 1, and 5% (w : v) and the vegetable oils were heated at 100°C for 12 h. Experiments were performed in triplicate.

2.2. Lipid Peroxidation Measurement. Malondialdehyde (MDA) was quantified in duplicate in vegetable oils after the heating process as index of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS) assay [13]. Reaction mixture contained 1.5 mL 0.5% thiobarbituric acid (TBA) in 20% TCA and 0.5 mL of vegetable oil. The mixture was incubated at 90°C in a shaking water bath for 20 min, and the reaction was stopped by placing the reaction tubes in an ice-water bath. The samples were then centrifuged at 1000 g for 5 min, and the absorbance of supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient 155 mM cm^{-1} and expressed as mmol MDA g^{-1} of vegetable oil. The data are the means ± SE of quantifications made in duplicate in three replicates.

2.3. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance (ANOVA). The results were calculated using the statistical software (SPSS, version 14.0, SPSS Inc., Chicago, USA). Least significant difference values (LSD; $P \leq 0.05$) were calculated to find significant differences among treatments.

3. Results and Discussion

Peroxides and hydroperoxides are products that represent the beginning of lipoxidation, a step in which the chain-breaker antioxidants can interrupt the process and restore the fatty acid chain [14]. Then, hydroperoxides are spontaneously decomposed to MDA, which can be used as an indicator of lipid peroxidation damage [15]. MDA concentration increased in vegetable oils after the heating process, with important differences among the vegetable oil, and the addition of WEP showed different effect depending on plant species and vegetable oils (Figure 1). Thus, in sunflower oil the concentration of MDA increased from initial values of 2.24 ± 0.30 mmol g^{-1} to 6.53 ± 1.63 mmol g^{-1} after the heating process, this increase being significantly reduced by the addition of the three WEP. Moreover, this effect was similar to the three plant species and the three used concentrations (Figure 1(a)) and could be attributed to the high phenolics content of these plants [8–11]. Accordingly, the addition of *Nigella* seed extract to this vegetable oil increased its oxidative stability [5]. Similar effects have been reported in soybean oil enriched with white tea and olive leaves extract, in which the oxidative extension during microwave heating was reduced [16].

However, in olive and seed oils the heating process had no significant effect on MDA content (Figures 1(a) and 1(b)), probably due to the high natural antioxidant compounds present in virgin olive oil, the most important being phenolic compounds (phenolic alcohols and acids, flavonoids, lignans, and secoiridoids), carotenes, and tocopherols [17, 18], and to the synthetic antioxidant added to the seed oil special for frying. Moreover, the addition of these WEP powders to olive oil was effective in reducing lipid peroxidation when added at 5%, while at doses of 0.5 and 1% no significant effect was observed. Accordingly, the incorporation of rosemary, thyme, and lemon dried plants to olive oil just relatively helped to improve its thermal resistance [19]. On the contrary, in seed oil, the addition of WEP leads to different effect depending on plant species. Thus, MDA content decreased after the heating process with the addition of *Quercus ballota* corn, while the addition of increased concentration of *Rosa canina* fruit and leaves and shoots of *Sanguisorba minor* led to increases in MDA concentration, showing a prooxidant effect (Figure 1(c)). Other antioxidants have also different effects when added to different oils. Thus, TBHQ showed antioxidant effect in sunflower and soybean oils, while it displayed a prooxidant effect in corn oil [6].

Results show that the addition of dry powder of wild edible plants with high concentration of natural antioxidants could be an innovative and safety tool to increase thermal stability of vegetable oils such as sunflower, soybean, or corn and avoid or decrease the use of synthetic antioxidant.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Ana Romojaro and Paloma Sanchez-Bel contributed equally to this work.

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Research Article

Effect of Milk Fat Substitution of Rennet Milk Induced Coagulation on Physico-Chemical Properties

Imène Felfoul,[1,2] Salwa Bornaz,[1] Wiem Belhadj Hmida,[1] Ali Sahli,[3] and Hamadi Attia[2]

[1] *Tunisian High School of Food Industries, University of Carthage, 58 Avenue Alain Savary, 1003 Tunis, Tunisia*
[2] *Alimentary Analysis Laboratory, National Engineering School of Sfax, University of Sfax, BPW, 3038 Sfax, Tunisia*
[3] *National Agronomic Institute of Tunisia, University of Carthage, 43 Avenue Charles Nicolle, 1082 Tunis, Tunisia*

Correspondence should be addressed to Imène Felfoul; imenef@gmail.com

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The objective of this paper was to study the effect of milk fat substitution by ($W_1/O/W_2$) multiple emulsions based on olive oil in comparison with full and low-fat milks on milk behavior during rennet coagulation. Therefore, based on the turbidimetric and conductivimetric methods, a follow up of enzymatic coagulation is realized. Drainage of renneted gels was followed by syneresis study and cheese yield. The comparison between the coagulation aptitude of low fat milk and milk-olive oil emulsion showed that the hydrolysis phase extended up to 35 minutes for full fat milk and up to 38 minutes for milk-olive oil emulsion. The transition phase solid/gel was shorter in the case of the whole milk. The reticulation phase was shorter in the case of milk-olive oil emulsion. The milk conductivity depended on the milk richness in fat content. Milk-olive oil emulsion showed the lowest cheese-making yield compared to its full and low-fat counterpart.

1. Introduction

During the last decade, the consumption of low-fat food products has become more than just a trend. In view of the general consensus that the amount and type of fat consumed is important to the aetiology of many chronic diseases (e.g., cardiovascular diseases, cancer, and obesity), there is no surprise that consumers easier adhere to dietary guidelines regarding fat consumption. In fact, to better fit with the relationships between health and diet, a significant change is observed in consumer's behavior to reduced fat variants with sensory and physical characteristics that are close to those of the full-fat standard products.

Due to the crucial role of fat in flavor, texture, and appearance of food, it became clear that the development of reduced-fat products with matching quality of their full-fat counterparts is a difficult task when fat is replaced with alternative ingredients. The substitution of milk fat by emulsified vegetable oils in milk is an option to obtain cheese with healthier saturated/unsaturated fat balance [1]. However, incorporation of emulsified vegetable oils could alter the type, content, and distribution of the fat droplets in the protein network, causing modifications in cheese textural and microstructure behavior [2, 3]. Many factors are involved during dairy fat replacement by vegetable fat. As an example, the nature of oil used [4], the size of the emulsion [4], and the nature of emulsifiers employed.

All these parameters could have an effect on physico-chemical, textural, rheological, and sensory qualities of the gel obtained also called "reduced fat gel-like product".

Previous studies were focused on milk fat substitution by canola oil [1–4]. Few data were available in literature concerning milk fat substitution by olive oil emulsion. The aim of this paper was, therefore, to study the effect of milk fat substitution by an emulsified olive oil in comparison with full and low-fat milks on milk behavior during rennet coagulation and to compare the curd drainage of these different milk samples.

2. Materials and Methods

2.1. Materials. Olive oil (OO) (Chaâl Oil, SOCOHUILE, Sfax., Tunisia) was used as the oil phase of the $W_1/O/W_2$

emulsion. The hydrophilic emulsifier (WE) (P4780, SIGMA-ALDRICH, St Louis, USA, esters of monoglycerides and diglycerides of diacetyltartaric acid) and hydrophobic emulsifier (OE) (85548, SIGMA-ALDRICH, Chemie Gmbh, CH-9471 Buchs, Spain, esters of polyglycerol and polyricinoleate fatty acids) were purchased from SIGMA-ALDRICH France. The biopolymers used were gellan gum (GG) (P8169, SIGMA-ALDRICH, St Louis, USA) carboxymethylcellulose (CMC) (GA 20529, SIGMA-ALDRICH, CH-9471 Buchs, Steinheim, the Netherlands). The water used in all of the experiments was double-distilled.

2.2. Formulation and Preparation of the $W_1/O/W_2$ Emulsions. $W_1/O/W_2$ emulsions were prepared at room temperature using a two-stage emulsification procedure [5, 6]. In the first stage, a W_1/O emulsion was made having a 20% (w/w) dispersed aqueous phase, a GG concentration of 0.1% (w/w) and a total emulsifiers concentration of 8% (w/w) (one part of WE to four parts of OE). In all the cases the aqueous inner phase (W_1) (distilled water + WE + GG) was added drop-wise to the oil phase (O) (OO + OE) with an Ultra-Turrax homogenizer (Ultra-Turrax H 500 SLT, Service Trade Laboratory Equipment, Germany) at 5800 rpm for 5 min. In the second stage the W_1/O primary emulsion was reemulsified in the biopolymer aqueous solution (0.5% w/w CMC), at 5200 rpm for 10 min using the Ultra-Turrax homogenizer, yielding the following $W_1/O/W_2$ emulsion. The $W_1/O/W_2$emulsion had a dispersed phase fraction of 0.2.

2.3. Milk Samples. Cow milk used in this study derived from the same breeding spot in northern Tunisia (Holstein breed, BICHE farm, Nabeul region). Once at the laboratory at 4°C, pH was measured; 10 L batches of milk were added with 0.06% (w/v) potassium dichromate against all bacterial growth and immediately frozen at −10°C for further utilizations. 10 L batches of milk were divided into three equal volumes.

(a) Control full-fat milk.

(b) Low-fat milk which is the result of blending skimmed milk, prepared from full-fat milk centrifugation at 3600 g/15 min, with whole milk (v/v).

(c) Milk-olive oil emulsion obtained by mixing skimmed milk with X amount of an emulsified olive oil. X is equal to fat content in control full-fat milk.

2.4. Milk Composition. Total solid and ash contents were determined according to AFNOR methods (1993). The milk pH was measured using a pH meter (Model pH 315i /SET, WTW Inc., Weilheim, Germany) according to N.F. V 04–281 [7]. Different nitrogen fractions of milk: total nitrogen (TN), nonprotein nitrogen (NPN), and noncasein nitrogen (NCN) were extracted using the procedure of Rowland [8] and determined by the Kjeldhal method [9]. Fat, calcium and phosphorous contents were measured using, respectively, Gerber method and atomic absorption spectrophotometer [10].

2.5. Rennet Coagulation. Full, low-fat milks and milk-olive oil emulsion were heated at 36 ± 0.5°C for 30 min and added with 0.35 mL L^{-1} of microbial rennet (*M. miehei*, strength 1 : 10000, Laboratories ARRAZI, PARACHIMIC, Sfax., Tunisia) at the normal pH of the milk.

2.6. Turbidimetric Study and Conductivity Measurement. The turbidity and the conductivity of different milk samples were measured during coagulation using a turbidimeter (Model Analite NEP 160, Turbidity Meter. Mc Van Instruments PTY-LTD, Mulgrave, Australia) and a conductivity meter (Model 18.38 pH/EC meters, EijKelkamp Agrisearch Equipment, Giesbeek, The Netherlands), respectively. Milk samples were initially controlled every 10 seconds for 30 minutes, since turbidity and conductivity change very fast at the beginning, and then every 30 seconds until 1 hour of coagulation and finally every one minute until 5 hours. Turbidity and conductivity profiles have been obtained by plotting these parameters as a function of time. These profiles were used to define the different steps of milk coagulation.

2.7. Syneresis Kinetics. Skim, low fat milk and milk-olive oil samples prewarmed to 35°C were coagulated for 1 h. According to Hocine et al. [11], milky curd was cut into equal portion (1 cm) and placed in a pierced mould at room temperature (20°C). The weight of expulsed whey was collected each 30 min for 4 h and a half. The mass of serum collected after this period was named syneresis capacity.

2.8. Cheese Yield Evaluation. Cheese yield (g/L) was expressed as the quantity of cheese obtained from a given mass of milk (g/L) after 4 h of drainage.

2.9. Statistical Analysis. Analysis of variance (ANOVA) was carried out using the SPSS statistics 19 software. Significant differences ($P < 0.05$) among treatments were detected using Duncan's multiple range tests. Values expressed are means ± standard deviation of triplicate measurements.

3. Results and Discussion

3.1. Milk Composition. The average composition and physicochemical properties of full, low-fat milk, and milk-olive oil emulsion samples are given in Table 1.

It was observed that the difference in pH of the various milks was not significant ($P < 0.05$) (Table 1). Therefore, it was concluded that fat substitution by emulsified olive oil had no effect on pH. This result confirmed those of Romeih et al. [12] and Kavas et al. [13] who noticed that the combined action of fat reduction and fat replacers addition had no effect on pH.

Table 1 showed that milk-olive oil emulsion contained ≈ 15 g/L of vegetable fat; this value was almost similar to that of low fat milk.

Milk-olive oil emulsion had a lower dry matter content compared to that of the whole milk sample since it was poorer in proteins but essentially in milk fat (Table 1). Milk-olive oil

Table 1: Physicochemical characteristics of milk samples.

	pH	Fat*	TS*	TN*	NPN*	CN*	PR*	Ash*	Ca*	P*
Full-fat milk	6.55 ± 0.01^{a}	32.13 ± 1.26^{b}	118.55 ± 5.41^{b}	33.39 ± 0.05^{b}	1.44 ± 0.05^{a}	23.39 ± 0.05^{a}	31.95 ± 0.05^{b}	7.26 ± 0.37^{a}	1.19 ± 0.02^{a}	0.738 ± 0.07^{a}
Low-fat milk	6.53 ± 0.06^{a}	15.22 ± 1.76^{a}	101.08 ± 1.30^{a}	31.66 ± 1.76^{a}	1.60 ± 0.00^{b}	21.95 ± 1.78^{a}	30.06 ± 0.00^{a}	6.92 ± 0.48^{a}	1.24 ± 0.06^{b}	0.823 ± 0.18^{a}
Milk-olive oil emulsion	6.56 ± 0.01^{a}	15.65 ± 0.23^{a}	95.03 ± 1.44^{a}	32.72 ± 0.09^{ab}	1.63 ± 0.05^{b}	22.85 ± 0.04^{a}	31.09 ± 0.05^{ab}	8.35 ± 0.29^{b}	1.09 ± 0.02^{c}	0.795 ± 0.04^{a}

*(g/L). TS: total solids. TN: total nitrogen. NPN: nonprotein nitrogen. CN: casein. PR: protein. Ca: calcium. P: phosphorous.
Means ± standard deviation (SD) of three separate determinations. Values sharing same small letter (a-b-c) within a column are not significantly different by Duncan's multiple-range test ($P < 0.05$).

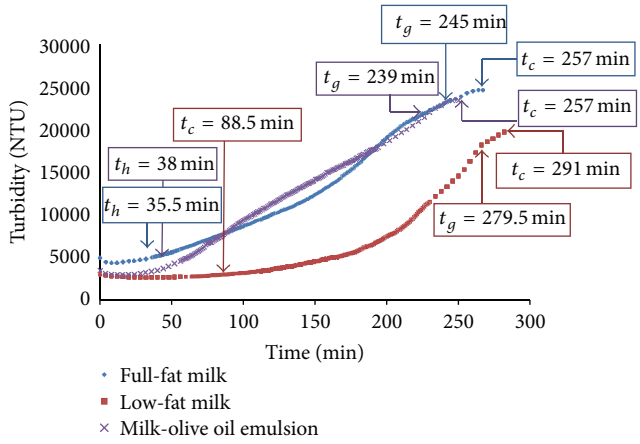

FIGURE 1: Turbidity evolution versus time of various renneted milk samples.

emulsion presented lower total nitrogen content than that of the control whole milk.

The high content in ash of milk-olive oil emulsion was probably attributed to the addition of emulsifiers and stabilizers during the preparation of this sample (Table 1).

3.2. Renneting Properties

3.2.1. Milk Turbidity. The enzymatic coagulation mechanisms were common for full, low-fat milk, and milk-olive oil emulsion. In fact, the turbidity data (Tr) obtained during rennet coagulation of these milks showed a sigmoid profile (Figure 1). Based on subsequent works, three phases could be identified. Phase one of latency through which a low variation of turbidity was observed. This phase corresponds to a step of enzymatic hydrolysis. It is characterized by specific proteolysis of casein-κ by rennet. The enzyme cleaves the peptidic bond Phe_{105}-Met_{106} of casein-κ and the protein is divided into two peptids, caseino-macro-peptide and paracasein-κ. It is situated between "t_0" and "t_h", hydrolysis time. The "t_h" value is taken as the time corresponding to turbidity variation of 0 NTU.

There was an initial decrease in the turbidity due to the decrease in micelles size presumed to be due to removal of the hairy layer by the rennet.

This observation was in agreement with those of Martin et al. [14] who studied coagulation kinetics of skim and reconstituted milks while following the evolution of the relative viscosity of renneted milks. These authors have measured the viscosity of milk samples as an indicator of the kinetics of the initial stages of coagulation. They explained the first reduction in relative viscosity by the size reduction of casein micelles due to the departure of the glycomacropeptide in the serum.

The second phase corresponds to a micelles aggregation step. During this phase, destabilized micelles were associated to form hydrophobic bonds. This is due to the decrease of surface charges of the micelles when the glycomacropeptide is released in the serum which enabled the association of micelles attacked by the enzyme, facilitating their aggregation.

This phase corresponds to a high increase of turbidity. Since the hairy layer was removed, intermicelle interactions became evident as an increase in turbidity. Insofar, as the cleaved part of the casein is responsible for the stability of the particles, it follows a micellar aggregation resulting in an increase in the turbidimetric signal.

This step is characterized by the presence of an inflexion point of the turbidimetric profile (t_i, Tr_i). Time t_i, sol-gel transition time, is determined from the derivative of the output signal and materialized by the time at which a peak in speed curve occurs.

This second phase is situated between "t_h" and "t_g". The gelation point (t_g, Tr_g) is represented in the first derivative curve of turbidity profile over time, either by a change in the slope, or by the presence of a second peak.

In the third phase, starting at time "t_g", a constant turbidity level is reached when the gel is made. It is a gel restructuration phase, gel formation, and implementation of interactions in the three-dimensional network.

In the case of the milk-olive oil emulsion, obtained by substituting milk fat by emulsified olive oil, the evolution of turbidity was very similar to its counterparts (Figure 1).

The initial values of turbidity recorded for the three samples were 4978.0 NTU, 3044.0 NTU, and 3451.0 NTU for full, low fat milks and milk-olive oil emulsion, respectively (Figure 1). These values differed significantly ($P < 0.01$). It was clear that the measurement of the initial milk turbidity was in relationship with dry matter content and particles size. Sadar [15] and McClements [16] affirmed that the dispersion of the light was as intense as the concentration in particles was important.

The shape of the turbidity curves was the same for all the milk samples. The experimental values of the characteristic points of turbidimetric patterns obtained from different milk samples were illustrated in Table 2. These results showed that there was an important variability between the samples. This high variability in the behavior of different milks has been observed in the registrations of hydrolysis time "t_h", inflexion time "t_i", gelation time "t_g", and coagulation time "t_c".

Table 2 shows that the hydrolysis step characterized by "t_h" has extended up to 35 min for the full-fat milk and up to 38 min for the milk-olive oil emulsion. The caseins hydrolysis was faster in the case of the whole milk. This difference could be due to the accessibility of the enzyme to casein-κ. This accessibility is dependent of the membrane nature of the fatty globules as well as of the emulsion size which might modify the possible interactions that could exist between casein and milk fat.

From Tables 1 and 2, it was shown that dry matter content of full-fat milk was statistically higher than that of milk-olive oil emulsion ($P < 0.05$). However, there was no statistical difference in hydrolysis phase duration between these two milk samples ($P < 0.05$). This result confirmed those found by Bornaz et al. [17] and Remeuf et al. [18] who showed that the composition of cow milk had no influence on the duration of hydrolysis phase. Furthermore, Lomholt and Qvist [19]

Table 2: Experimental values of characteristic points of the enzymatic coagulation of the milk samples (min).

	t_h	t_i	t_g	t_c	$t_g - t_h$	$t_c - t_g$	$t_g - t_i$
Full-Fat milk	35.5 ± 0.71[a]	176.5 ± 2.12[a]	245.0 ± 5.66[a]	257.0 ± 4.24[a]	209.5	12.0	68.5
Low-Fat milk	88.5 ± 3.54[b]	271.0 ± 8.49[c]	279.5 ± 13.44[b]	291.0 ± 7.07[b]	191.0	11.5	8.5
Milk-olive oil emulsion	38.0 ± 1.41[a]	232.0 ± 11.31[b]	239.0 ± 11.31[a]	247.0 ± 11.31[a]	201.0	8.0	7.0

t_h: hydrolysis time; t_i: time at the point of inflection; t_g: gelationtime; t_c: coagulation time. $(t_g - t_h)$: phase II duration; $(t_c - t_g)$: phase III duration; $(t_g - t_i)$: gel solidification phase duration.
Means ± standard deviation (SD) of three separate determinations. Values sharing same small letter (a-b-c) within a column are not significantly different by Duncan's multiple-range test ($P < 0.05$).

showed that enzymatic hydrolysis is a first-order reaction tightly linked to the amount of casein-κ.

Moreover, Calvo [20], studying the effect of temperature and fat content on coagulation, noticed that fat content could prevent either the action of the chymosin or the first stages of the destabilized micelles aggregation. Furthermore, this could explain the difference between full fat and low fat milks in hydrolysis step duration where "t_h" was lower in the case of full fat milk than for low fat milk.

There was a significant difference ($P < 0.05$) between full fat milks and milk-olive oil emulsion regarding the characteristic point "t_i" of the transition step solid/gel. This time "t_i" was shorter in the case of the whole milk with a difference of 55.5 minutes compared to milk-olive oil emulsion and of 94.5 minutes in comparison with low fat milk.

The release of casein aggregation at a significant rate was associated with a proteolysis degree situated between 60% and 80% depending on renneting conditions [21, 22].

The second step, which corresponded to the aggregation of casein micelles beforehand hydrolyzed, proceeded in a particular way in milk-olive oil emulsion. The paracaseins-κ association in the case of milk-olive oil emulsion was slower than that of the whole milk. However, the duration of the second step was as important as the milk was rich in fat. This confirmed the results obtained by Larcher [23] which showed the existence of a negative behavior of the milk fat during coagulation and explained this by the big size of the fatty globules blocking the connection between casein micelles.

The comparison between the coagulation aptitude of low fat milks and milk-olive oil emulsion showed that the nature of the fatty globules had a strong influence over the hydrolysis time but a less marked effect over the duration of the coagulation step $(t_g - t_h)$. Conversely of what was noticed by Eck and Gillis [24], this study did not show any proportionality between the aggregation speed of destabilized micelles and the progress of the enzymatic reaction.

The last step, corresponding to the reticulation phase, characterized by "$t_c - t_g$", was shorter in the case of the milk-olive oil emulsion. This could be explained by higher ash content in curd resulting from the milk-olive oil emulsion (Table 2), probably indicating a significant number of phosphocalcic links established in this case and thus facilitating the phase of gel reorganization.

However, the role of the ionic calcium which influenced the coagulation time t_c and the gel firmness could be also explained by the masking of the charged groupings and

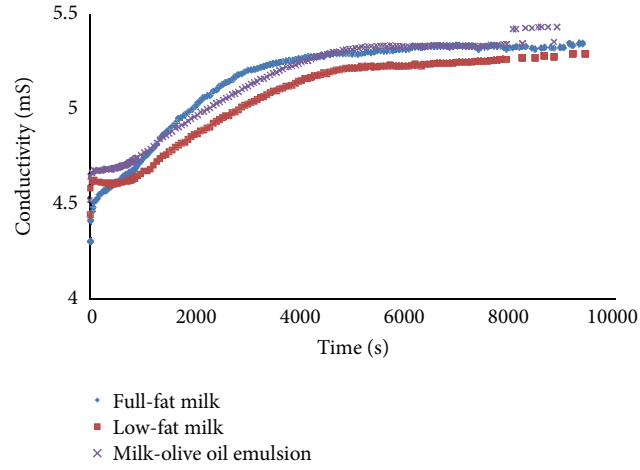

Figure 2: Conductivity evolution versus time of various renneted milk samples.

the increase in hydrophobicity [25]. Indeed, it was established that ionic calcium and the availability of micellar calcium played a prevalent role in the reticulation phenomenon [26].

The duration of the reticulation phase was as important as the Ca/P ratio increased. According to Remeuf et al. [18] and Eck and Gillis [25], phosphorus could probably intervene in the creation of links between casein micelles. It conferred a real structure to the coagulum whose consistency was firmer as the colloidal calcium phosphate content in milk was higher.

3.2.2. Milk Conductivity. The evolution of the conductivity of milk coagulated by the enzyme showed an exponential pattern (Figure 2).

The curves corresponding to the three milk samples had the same profile. At the beginning of coagulation, an abrupt increase at the first ten seconds was observed then a progressive increase in the conductivimetric signal followed by a prolonged stage were recorded, it is the reorganization phase (Figure 2).

The brutal increase in the conductivity of the various milk samples, just after renneting, was probably due to the release of caseinomacropeptides after the casein-κ hydrolysis in the micellar envelope and reflects the high mobility of ions [27].

The establishment of the phosphocalcic bridges and the solubilization of some other colloidal salts during the organization and the reorganization of the gel could explain the progressive increase of the milk conductivity during

coagulation. The end of coagulation was thus defined by a stabilization of the curd conductivity.

Milk had conductive properties because of the existence of charged compounds such as salts. The distribution of the salt fractions between the colloidal phase and the soluble phase had a significant effect on milk conductivity.

Figure 2 shows that the initial low conductivimetric signal corresponded to the whole milk sample (4.31 mS) followed by low fat milk (4.45 mS). The conductivity of the milk-olive oil emulsion was about 4.53 mS. The milk conductivity was tributary not only of its richness in ashes but also of fat content. Indeed, Norberg [27] and Mabrook and Petty [28] reported that fat addition resulted in a reduction in the milk conductance due to the presence of a fine non-conducting membrane which covers the fatty globules.

The evolution of the milk-olive oil emulsion conductivity was similar to that of full fat milk with a light predominance of coagulation during the first period. Then, an inversion of the phenomenon at the 21st minute of coagulation was observed (Figure 2).

At the end of coagulation, measurements of curds conductivity resulting from whole milk reached 5.35 mS; that is, an increase of about 18.13% compared to the initial state. The low fat milk presented a conductivimetric signal of about 5.29 mS with an increase of 19.01%. Concerning the milk-olive oil emulsion sample, the curds conductivimetric signal was equal to 5.36 mS with an average improvement of 18.40% compared to the initial value.

3.3. Renneted Gels Drainage

3.3.1. Syneresis Study. It was suggested that syneresis resulted from two different properties of the milky gel: (a) a contraction capacity of the protein framework formed by casein micelles during coagulation expressed by a compaction of the gel and (b) an ability of the gel to evacuate interstitial whey which is a function of permeability and porosity. These characteristics were strictly correlated to the particular conditions under which the gel was formed as well as the physicochemical and chemical composition of the milk.

It was clear from Figure 3 that the serum exudation was more important in the case of the milk-olive oil emulsion (84.13%). In the case of the whole milk, the serum exudation was approximately 77.89% and 81.97% for low fat milk.

It has been suggested that water could bind directly to fat replacers and fat replacers could interfere with the shrinkage of the casein matrix. Therefore, this lowered the driving force involved in expelling water from curd particles [29].

Relatively large spaces existed in the network where the fat globules were present and acted to interrupt it. When the curd is cut, whey is expelled, and the mesh-like structure shrinks around the fat globules [30]. Milk fat globules in cheeses reduced the degree of shrinking and expelling of whey carrying water-soluble components including proteins and peptides [31]. This might explain that the more the cheese was rich in milk fat, the less they would be expulsed, which was confirmed in this present study (Figure 3).

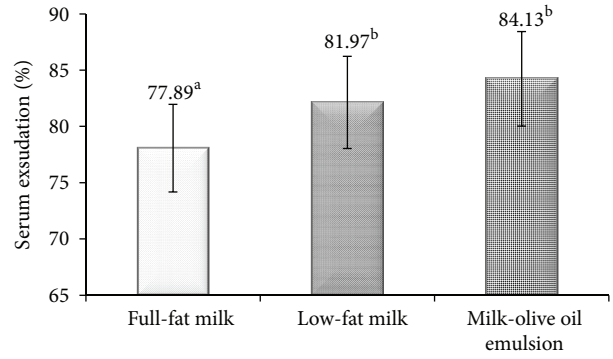

Figure 3: Serum exudation rate evolution of various renneted milk samples. Vertical bars indicate standards errors of the means. Identical letters above the bars indicate no significant differences by Duncan's multiple-range test ($P < 0.05$).

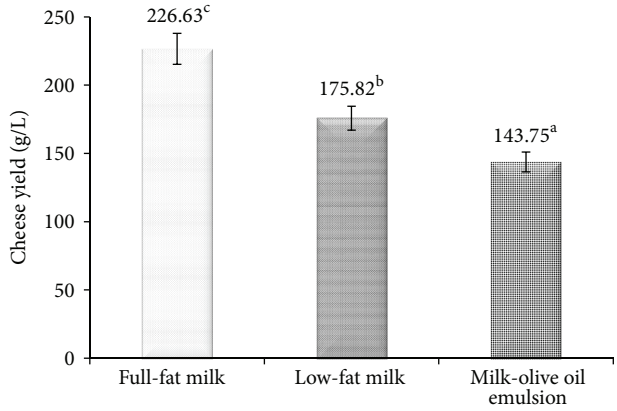

Figure 4: Cheese yields of various renneted milk samples (g/L). Vertical bars indicate standards errors of the means. Identical letters above the bars indicate no significant differences by Duncan's multiple-range test ($P < 0.05$).

Lobato-Calleros et al. [32] noticed that emulsifiers blend contributed to higher emulsified canola oil retention in the reduced fat cheese-like product; therefore, the syneresis phenomenon was inhibited and less whey proteins were expelled from the cheese network. This is not the case in this study where the phenomenon of syneresis was the most intense for the reduced fat cheese-like product. This could be due to the lower water retention capacity of the emulsified olive oil than that of the animal milk fat.

3.3.2. Cheese Yield. Figure 4 represents cheese yields of the various milk samples. It was noticed that low fat milk yielded less cheese than the whole milk which confirmed the result previously found in syneresis section. Thus, it can be concluded that cheeses containing higher milk fat contents exhibited higher yields. This confirmed the results found elsewhere [33, 34].

The lower yield of reduced fat cheese-like products, when compared with low-fat cheeses, might be attributed to their higher moisture contents and/or their higher protein contents [29].

For the same initial concentration of fat content in milk (Figure 4), the cheese yield was more significant in the case of the curd resulting from low fat milk (175.82 g/L) than that of the curd resulting from the milk-olive oil emulsion (143.75 g/L). In other words, the quality of milk fat incorporated in milk for the manufacture of reduced fat cheese-like products affected in a significant way its cheese yield. This finding was in agreement with the results of Drake et al. [35] for Cheddar.

Thus, in complex systems, such as the ones under study, a whole range of interactions could take place between the low molecular weight emulsifier molecules, containing lipid and polar fractions and the molecules of the rest of the food components, including proteins, both at the interface as in the network [36].

According to Romeih et al. [12], the yield was also related to the fat content of milk. An overall reduction in yield was inevitable in the production of cheese from low fat milk, since casein and fat contents of the milk, which are the principle components determining cheese yield, were reduced.

4. Conclusion

Milk-olive oil emulsion was prepared from the total substitution of milk fat by an emulsified olive oil.

The examination of the turbidimetric signal obtained during enzymatic coagulation showed that the whole milk had a hydrolysis step shorter than that of low-fat milk and milk-olive oil emulsion. The association of paracaseins-κ was later than that of the whole milk. The milk conductivity was dependant not only of the milk richness in ashes but also of milk fat content. The drainage aptitude, expressed in terms of expulsed whey weight, was reduced by important dry matter content.

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Research Article

Phenolic Contents and Antioxidant Potential of *Crataegus* Fruits Grown in Tunisia as Determined by DPPH, FRAP, and β-Carotene/Linoleic Acid Assay

Farouk Mraihi,[1] Mohamed Journi,[1] Jamila Kalthoum Chérif,[1,2] Munevver Sokmen,[3] Atalay Sokmen,[4] and Malika Trabelsi-Ayadi[1]

[1] *Laboratoire d'Application de la Chimie aux Ressources et Substances et Substances Naturelles et à l'Environnement (LACReSNE), Faculté des Sciences de Bizerte, 7021 Zarzouna, Tunisia*
[2] *Institut Préparatoire aux Etudes d'Ingénieurs de Tunis (IPEIT), 1008 Tunis, Tunisia*
[3] *Department of Chemistry, Faculty of Science and Literature, Karadeniz Technical University, 61080 Trabzon, Turkey*
[4] *Department of Biology, Faculty of Science and Literature, Karadeniz Technical University, 61080 Trabzon, Turkey*

Correspondence should be addressed to Farouk Mraihi; mraihi_farouk@yahoo.fr

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Crataegus fruit is one of most important fruits in Tunisian flora. Some fruits of this genus are edible. This study was undertaken in order to examine the benefits of these fruits in human health and their composition of antioxidants including total polyphenol, flavonoids, proanthocyanidins content, and total anthocyanins. The antioxidative properties of the ultrasonic methanolic extract were assessed by different in vitro methods such as the FRAP, DPPH, and β-carotene/linoleic acid assay. We concluded that peel fraction of red fruits possessed relatively high antioxidant activity and might be a rich source of natural antioxidants in comparison with the pulp and seed fruit extract. The results also showed that hawthorn yellow fruit presents lower amounts of phenolic content, absence of anthocyanins, and less antioxidant capacity. Most of peel and seed fractions were stronger than the pulp fractions in antioxidant activity based on their DPPH IC_{50}, FRAP values, and results of β-carotene/linoleic acid. The total phenolic compounds contents were also highly correlated with the DPPH method and the FRAP assay.

1. Introduction

Dietary phenolic compounds have received much attention during the recent years due to their antioxidant and other biological properties imparting possible benefits to human health [1, 2]. Crude extracts of fruits, herbs, and vegetables are rich sources of polyphenols. These compounds include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), flavonoids (flavonols, flavones, flavanols, flavanones, isoflavones, anthocyanins, and proanthocyanidins), vitamins, and carotenoids. These bioactive molecules can delay or inhibit the oxidation of lipids and other molecules by inhibiting the initiation or propagation of oxidative chain reactions [3].

Antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals [4]. In order to receive a reliable picture of antioxidants content in *Crataegus monogyna* and *Crataegus azarolus* fruits extract, total polyphenols, total flavonoids, proantnocyanidins content, and total anthocyanins content were determined quantitatively using spectrophotometer methods. It was also shown that the measure of antioxidant capacity in natural products by only one assay is often not reliable; therefore, in this investigation, we used three complementary assays such as DPPH radical scavenging assay, ferric-reducing/antioxidant power (FRAP) and β-carotene linoleic acid assay to check the antioxidant activity of these fruits.

2. Materials and Methods

2.1. Plant Material. Two series of *Crataegus azarolus* and *Crataegus monogyna* fruits (2 kg) were collected from Jendouba, north of Tunisia, in September 2011. Fruits were immediately transported to the Department of Chemistry of the Faculty of Sciences in Bizerte. Each fruit was separated into three parts such as peel, pulp, and seed. They were lyophilized and kept in desiccators, and the fresh fruit was stored at −4°C until analysis.

2.2. Chemical Reagents. The chemical reagent Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Linoleic acid, BHT, free radical DPPH, and FRAP reagent were from Sigma Co. (St. Louis, MO, USA). Folin-Ciocalteu's reagent and all standard antioxidants were from Fluka Chemie (Buchs, Switzerland). Tween 40 is from Merck. All other chemical reagents used for extract were obtained from Sigma Co. (St. Louis, MO).

2.3. Ultrasound-Assisted Procedures Extraction. The peel, pulp, and seed of red and yellow fruit of *Crataegus* were processed separately; approximately 2 g of lyophilized fruit parts was extracted 3 times by 15 mL of methanol/acidified water HCl 1.5 N (80/20 v/v) during 30 min in an ultrasonic bath (FALC Instruments, Italy) [5]. The extracts were then washed with hexane to remove chlorophyll and other low molecular weight compounds. The extracts were centrifuged, the solvent was evaporated under reduced pressure, and the residue was dissolved in ultrapure water and lyophilized. The crude extract was kept to quantify the total antioxidant contents.

3. Determination of Total Antioxidant Compounds

3.1. Total Phenolic Content. Total phenolic content was quantified using the modified Folin-Ciocalteu's method [6]. Folin-Ciocalteu's reagent/water (750 μL, 1 : 14) mixture was added to a 50 μL sample and the reaction was stopped exactly 3 min after adding 200 μL of 20% Na_2CO_3. The solution was homogenized, vortexed and heated at 100°C for 2 min, and kept in the dark room for 30 min for incubation. Absorbance was read at 760 nm using a UV-Vis spectrophotometer T60U. All assays were carried out at least in duplicate and MeOH was used as blank (50 μL instead of the extract). Methanolic dilutions of gallic acid were used as standard; results were expressed as gallic acid equivalent per gram of lyophilized sample.

3.2. Total Flavonoid Content. Total flavonoid content was measured using the modified colorimetric method of Zhishen et al. [7]. In sealed tubes, 1.5 mL of a 2% methanol solution of $AlCl_3{\cdot}6H_2O$ was added to 0.5 mL of sample and then kept in dark for 10 min. Absorbance was read at 430 nm, methanolic $AlCl_3$ was used as blank, and each measure was made in triplicate. A series of methanolic dilutions of rutin were prepared and assayed; flavonoid amounts in extract were expressed in mg rutin equivalent flavonoid/100 g dry matter [8].

3.3. Proanthocyanidin Content. In sealed tubes, 0.5 mL sample was added to a solution of 0.5 mL MeOH, 6 mL of n-BuOH/concentrated HCl (95 : 5 v/v), and 0.2 mL of a 2% $NH_4Fe\,(SO_4)_2{\cdot}12H_2O$ solution in 2 M HCl. Absorbance was read at 550 nm before and after heating for 40 min at 95°C (each measure in triplicate, blank n-BuOH/HCl mixture). A series of dilutions of cyanidin chloride in n-BuOH/HCl were assayed; proanthocyanidin amounts in extracts were expressed in mg cyanidin/100 g dry matter and were calculated from the following equation [9]:

$$\mathrm{PC} = \left[\frac{\left(A_{550}\mathrm{sample} - A_{550}\mathrm{control}\right)}{(\varepsilon * L)}\right] * \mathrm{MW} * \mathrm{DF} * 1000, \tag{1}$$

where PC is proanthocyanidin contents expressed in mg cyanidin/100 g dry matter, A_{550}sample is the absorbance of the sample at 550 nm, A_{550}control is the absorbance of the control at 550 nm, ε = 17,360 $L^{-1}\,M^{-1}\,cm^{-1}$ molar absorptivity coefficiency of cyanidin, L is the cell path length (1 cm), MW = molecular weight of cyaniding (287 g mol^{-1}), DF is the dilution factor (g L^{-1}), and 1000 is the factor for conversion from g to mg.

3.4. Total Monomeric Anthocyanins (TMA). Total anthocyanins were quantified using the pH differential method described by Giusti and Wrolstad [10]. This method was based on reversible structural transformations of anthocyanin pigments in different pH solutions using a UV-Vis spectrophotometer (model T60U, PG Instruments). 960 μL of pH 1 (25 mL of 1.49% KCl + 67 mL of 1.7% HCl, pH corrected with HCl) and pH 4.5 (1.64% AcONa, pH corrected with AcOH) buffer solutions were each added to 40 μL of extract. Absorbance was read at 700 and 510 nm against water as blank. Each measure was made in triplicate. The results were expressed in mg cyanidin-3 glucoside/100 g dry matter:

$$\begin{aligned}\mathrm{TACY} &= \left[\frac{\Delta A \times \mathrm{MW} \times \mathrm{DF} \times 1000}{\varepsilon}\right] \times 0.1,\\ \Delta A &= \left[A_{512\,\mathrm{nm}} - A_{700\,\mathrm{nm}}\right]_{\mathrm{pH\ 1.0}}\\ &\quad - \left[A_{512\,\mathrm{nm}} - A_{700\,\mathrm{nm}}\right]_{\mathrm{pH\ 4.5}},\end{aligned} \tag{2}$$

where TACY is the total anthocyanins expressed as mg cyanidin-3-glucoside/100 g DF fruit, MW is the molecular weight of cyaniding-3-glucoside (449.2 g mol^{-1}), DF is the dilution factor, ε is the molar absorbance coefficient of cyaniding-3-glucoside (26,900 $M^{-1}\,cm^{-1}$), and 0.1 is the conversion factor per 1000 g to 100 g basis.

4. Determination of Antioxidant Activity

4.1. DPPH Radical Scavenging Assay. Radical scavenging activity of *Crataegus* fruits extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined spectrophotometrically. The method first introduced by Blois [11], developed by Brand-Williams et al. [12], and criticized by Molyneux [13] was employed. The principle of the assay is based on the color change of the DPPH solution from purple to yellow as the radical is quenched by the antioxidant. Briefly, 100 μL of methanol hawthorn fruits extract was added to 1.9 mL of 410–3 mM of DPPH in methanol up to completing 2 mL. The free radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm every 2 min until the reaction reached its state. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve. The inhibition activity I(%) was calculated as follows:

$$I\,(\%) = 100 \times \frac{(A_0 - A_1)}{A_0}, \tag{3}$$

where A_0 is the absorbance of the control sample and A_1 is the absorbance of the test compound.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration.

4.2. Ferric-Reducing/Antioxidant Power (FRAP). The procedure of FRAP assay was according to Benzie and Strain [14]. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form in presence of antioxidants. Briefly, the FRAP reagent contained 2.5 mL of 10 mmol L^{-1} TPTZ (2,4,6-tripyridyl-*s*-triazine, Sigma) solution in 40 mmol L^{-1} HCl plus 2.5 mL of 20 mmol L^{-1} $FeCl_3$ and 25 mL of 0.3 mol L^{-1} acetate buffer, pH 3.6, and was prepared freshly and warmed at 37°C. Aliquots of 40 μL sample supernatant were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min in a microplate (PowerWave XS, BioTek). The 1 mmol L^{-1} $FeSO_4$ was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol L^{-1} $FeSO_4$. Adequate dilution was needed if the FRAP value measured was over the linear range of standard curve.

4.3. β-Carotene/Linoleic Acid Assay. The antioxidant activity of extracts was evaluated using β-carotene/linoleic acid system according to the modified literature procedure [15, 16]. A stock solution of β-carotene/linoleic acid (Sigma-Aldrich) was prepared as follows: first, 0.5 mg of β-carotene was dissolved in 1 mL of chloroform (HPLC grade); then 25 μL of linoleic acid and 200 mg of Tween 40 (Merck) were added. The chloroform was subsequently evaporated using a vacuum evaporator (Buchi, Flawil, Switzerland). Then, 100 mL of distilled water saturated with oxygen (30 min at 100 mL/min) was added with vigorous shaking. Aliquots (2.5 mL) of this reaction mixture were transferred to test tubes, and 350 μL portions of the extracts (2 g/L in ethanol) were added before incubating for 48 h at room temperature. The same procedure was repeated with BHT at the same concentration and a blank containing only 350 μL of ethanol. After the incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidant capacities of the samples were compared with those of BHT and of the blank.

5. Results and Discussion

5.1. Total Phenolic Contents. The level of phenolic compounds in methanolic aqua acidified extracts measured according to the Folin-Ciocalteu method of peel, pulp, and seed of *Crataegus* varieties is presented in Table 1. All parts of red and yellow *Crataegus* varieties were a significant source of polyphenols; however, the total amount varied significantly between 45.7 and 123.35 mg gallic acid/100 g DW in the red variety and from 36.3 to 71.24 mg gallic acid/100 g DW in yellow fruit. The red peel had the highest amount of total polyphenols (123.35 mg gallic acid/100 g DW) whereas seed of yellow fruit had the lowest amount. Referring to Table 1, in both varieties, total flavonoid contents in peel extract were more than in pulp, followed by seed. Comparing the varieties, it was found that all different parts of *Crataegus monogyna* had higher contents of flavonoids (198.53 mg eq. rutin/100 g DW) in peel followed by (160.35 mg eq. rutin/100 g DW) in pulp and (96.01 mg eq. rutin/100 g DW) in seed; however, peel, pulp, and seed of *Crataegus azarolus* had the lowest TF (155.40, 60.45, and 14.71 mg eq. rutin/100 g DW, resp.). Findings also established the main source of total phenolics and total flavonoids in *Crataegus monogyna* to be the peel, which were about 123.35 mg gallic acid/100 g DW and 198.53 mg eq. rutin/100 g DW. The results of the present study indicated consistently the lowest values of total anthocyanins contents in the both varieties when compared to the other bioactive compounds. The red fruit contained higher amounts (5.85 mg eq. cyanidin/100 g DW) in peel and (0.31 mg eq. cyanidin/100 g DW) in fruit; however, yellow fruits are poured in anthocyanins. Proanthocyanidins or condensed tannins are ubiquitous and present as the second most abundant natural phenolics. The proanthocyanidins have been suggested to contribute to the phenomenon called health promoting effects, such as antioxidant, anticarcinogenic, and anti-inflammatory effects [17, 18]. According to Table 1, flavon-3-ols are the most abundant compounds present in *Crataegus* fruits; they represent more than half of the other compounds. Total proanthocyanidins content is most abundant in the red peel (873.58 mg eq. cyanidin/100 g DW), and a lower level was found in the yellow fruits (97.06 mg eq. cyanidin/100 g DW). We show that total antioxidants contents are influenced by the species of *Crataegus* and the different parts of fruit in the same variety, both peels were the rich source of antioxidant compounds.

5.2. Antioxidant Activities. In the present study, the antioxidant activities of *Crataegus* determined by free radical scavenging activity (DPPH) assay method indicated a steady

Table 1: Phenolics contents in different crude part extracts of red and yellow *Crataegus*.

		Source of extract		
	Variety	Peel	Pulp	Seed
Total phenolics[a]	*C. monogyna*	123.35 ± 0.02	122.26 ± 0.16	45.72 ± 0.04
	C. azarolus	71.24 ± 0.01	60.89 ± 0.04	36.03 ± 0.02
Total flavonoids[b]	*C. monogyna*	198.53 ± 0.11	160.35 ± 0.1	96.01 ± 0.01
	C. azarolus	155.40 ± 0.23	60.45 ± 0.06	14.71 ± 0.02
Proanthocyanidins[c]	*C. monogyna*	873.58 ± 0.33	507.31 ± 0.32	399.68 ± 0.29
	C. azarolus	352.91 ± 0.29	291.16 ± 0.19	97.06 ± 0.21
Total anthocyanins[d]	*C. monogyna*	5.58 ± 0.05	0.31 ± 0.03	0
	C. azarolus	0	0	0

[a]Total phenol: Folin-Ciocalteu, in mg eq. gallic acid/100 g DW.
[b]Flavonoids: $AlCl_3$ method, in mg eq. rutin/100 g DW.
[c]Anthocyanins: direct colorimetry in mg eq. cyanidin-3-O-glucoside/100 g DW.
[d]Procyanidins: butanol-HCl methods, in mg eq. cyanidins/100 g DW.

Table 2: Free radical scavenging capacity, FRAP assay, and standard antioxidants of *Crataegus* fruit extract.

		DPPH IC_{50} (μg/mL)	FRAP (mM Trolox)	FRAP (mM AA)
C. monogyna	Peel	750	8.88	9.12
	Pulp	720	5.44	5.68
	Seed	540	5.71	5.95
C. azarolus	Peel	780	6.89	7.13
	Pulp	560	4.40	4.64
	Seed	240	6.15	6.39
Control	BHT	820	—	—

increase in the scavenging activity of free radicals in all extracts and standard range between 240 and 800 μg/mL (Table 2). It was observed that the ability of test materials (pure antioxidants and fruits extracts) to scavenge DPPH was assessed on the bases of their IC_{50} values, defined above as the concentration of test material to decrease the absorbance at 515 nm (or concentration) of DPPH solution to half of its initial value. These IC_{50} values of *Crataegus* fruit extract are given in Table 2. It can be seen that seed from yellow variety shows higher IC_{50} value (780 μg/mL) than red peel (750 μg/mL). This result can be attributed to the higher phenolic content of the peel and pulp. The higher DPPH radical scavenging activity is associated with a lower IC_{50}. FRAP values of peel, pulp, and seed fractions of *Crataegus* fruits are also summarized in Table 2. The reducing ability of different parts of red *Crataegus* fruits extracts is expressed, respectively, in mM Trolox equivalent and mM ascorbic acid equivalent: they ranged from 5.44–8.88 mM Trolox/100 g DW to 5.68–9.12 mM ascorbic acid/100 g DW. These values are more important than the reducing ability of yellow fruits; however, they ranged from 4.64 to 7.13 mM Trolox/100 g DW and, 4.4 to 6.89 mM A A/100 g DW. The decreasing order efficiency in FRAP system is as follows: peel > pulp > seed in both varieties of *Crataegus* fruits extract. These results agreed with the DPPH values. The basis of β-carotene/linoleic acid assay is discoloration of β-carotene in reaction with linoleic acid free radical. That radical is formed at elevated temperature upon removal of hydrogen atom located between two double bonds of linoleic acid [16].

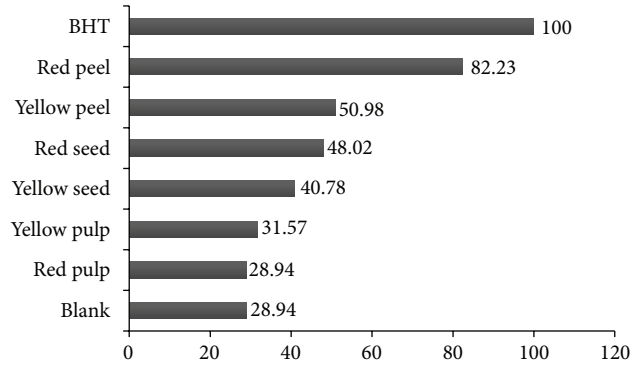

Figure 1: Relative antioxidant activity of *Crataegus* extracts and positive control (BHT) in β-carotene/linoleic acid assay.

The consequence is the loss of conjugation and, accordingly, a decrease in absorbance at 470 nm. Antioxidants present in solution can prevent the degradation of β-carotene by reacting with the linoleate free radical or any other radical formed in the solution.

The reduction in absorbance of β-carotene-linoleate emulsion in presence of the extracts is shown in Figure 1. Relative antioxidant activity of *Crataegus* extracts increased with the species and the parts of fruits. In the β-carotene/linoleic acid model system, we could conclude that results were consistent with the data obtained from DPPH test and FRAP assay. Peel extract of red fruit showed markedly relative

Table 3: Linear correlation of Trolox equivalent antioxidant capacity (TEAC) versus the total phenolic content of *crataegus* fruit extract.

Variety	Phenolic contents	Correlation coefficients	
		$TEAC_{DPPH}$	$TEAC_{FRAP}$
C. monogyna	TP	$y = 2.5328x + 424.06$ $R^2 = 0.9856$	$y = 0.0245x + 4.2986$ $R^2 = 0.3249$
	TF	$y = 2.1267x + 347.53$ $R^2 = 0.9412$	$y = 0.0305x + 2.054$ $R^2 = 0.6819$
	PC	$y = 0.3507x + 461.84$ $R^2 = 0.5885$	$y = 0.0076x + 2.1564$ $R^2 = 0.9783$
C. azarolus	TP	$y = 14.884x - 307.64$ $R^2 = 0.983$	$y = 0.0707x + 1.8527$ $R^2 = 1$
	TF	$y = 3.6082x + 249.36$ $R^2 = 0.9094$	$y = 0.0162x + 4.5682$ $R^2 = 0.8267$
	PC	$y = 1.9996x + 32.668$ $R^2 = 0.9666$	$y = 0.0096x + 3.4515$ $R^2 = 0.9964$

TP: Total polyphenols.
TF: Total flavonoids.
PC: Proanthocyanidin content.

antioxidant activity (82.23%), as did the peel extract of yellow fruit (50.98%). These results implied that the potential antioxidant capabilities in *Crataegus monogyna* were attributed to the phenolic compounds in this species compared with *Crataegus azarolus* species. Pulp of yellow fruit showed the weakest activity potential in this test system (28.24%) (Table 3).

5.3. Relationships amongst Different Antioxidants. Free radical scavenging of phenolic compounds is an important property underlying their various biological and pharmacological activities. Recently, Awika et al. [19] found positive correlations between the determinations of phenolic antioxidant using the oxygen radical absorbance capacitys (ORAC), ABTS, and DPPH assays. Results revealed that polyphenols from *Crataegus* fruit extract had high antioxidant activities (Table 1). The total phenolic compounds contents were also highly correlated with the antioxidant activities with the DPPH method and the FRAP assay. Data of the correlations (R^2) summarized in Table 2 with total polyphenols content in red fruits were 0.98, 0.94, and 0.58, respectively. DPPH was also highly correlated with TP, TF, and PC of red fruit; the data show 0.98, 0.90, and 0.96, respectively. The correlation between Trolox equivalent antioxidant capacity (TEAC) (Y) and total phenolic contents (X) of *Crataegus* monogyna had a coefficient (R^2) varied from 0.32 to 0.97 while the correlation coefficient of *Crataegus* azarolus are 1, 0.99 and 0.82 (Table 1). This result suggests that higher percentage of the antioxidant capacity of Tunisia *Crataegus* accessions results from the contribution of phenolic compounds. Also, it can be concluded that antioxidant activity of plant extracts is not limited to phenolics content but also comes from the presence of other antioxidant secondary metabolites, such as flavonoids, proanthocyanidins, and anthocyanins. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

They may also have a metal chelating potential [20]. Besides flavoring purposes, spices and herbs have also been used for their medical or antiseptic properties because of their richness in bioactive molecules and consequently their benefits for human health [21].

6. Conclusion

Comparison of phenolic contents and antioxidant activities of methanol extracts of *Crataegus azarolus* and *Crataegus monogyna* fruits cultivated in Tunisia shows the presence of total phenols, proanthocyanidins, and flavonoids with some difference. Anthocyanins are present only in red fruit. This richness in antioxidants contributes to the antioxidative effect. A linear correlation of Trolox equivalent antioxidant capacity (TEAC) versus the total phenolic content of *Crataegus* was established. The richest composition in antioxidant compounds and the higher antioxidant capacity activity of Crataegus can improve the use of these fruits in various fields such as agroalimentary and pharmaceutical industry.

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Research Article

Angiotensin-I-Converting Enzyme Inhibitory and Antioxidant Activities of Protein Hydrolysate from Muscle of Barbel (*Barbus callensis*)

Assaad Sila,[1] Anissa Haddar,[1] Oscar Martinez-Alvarez,[2] and Ali Bougatef[3]

[1] *Laboratory Enzyme and Bioconversion, National School of Engineering, PB 1173, 3038 Sfax, Tunisia*
[2] *Institute of Food Science, Technology and Nutrition (ICTAN, CSIC), C/José Antonio Novais 10, 28040 Madrid, Spain*
[3] *Higher Institute of Biotechnology of Sfax, PB 1175, 3038 Sfax, Tunisia*

Correspondence should be addressed to Ali Bougatef; ali.bougatef79@gmail.com

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The present study investigated angiotensin-I-converting enzyme (ACE) inhibitory and antioxidant activities of barbel muscle protein hydrolysate prepared with Alcalase. The barbel muscle protein hydrolysate displayed a high ACE inhibitory activity (CI_{50} = 0.92 mg/mL). The antioxidant activities of protein hydrolysate at different concentrations were evaluated using various *in vitro* antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method and reducing power assay. The barbel muscle protein hydrolysate exhibited an important radical scavenging effect and reducing power. These results obtained by *in vitro* systems obviously established the antioxidant potency of barbel hydrolysate to donate electron or hydrogen atom to reduce the free radical. Furthermore, these bioactive substances can be exploited into functional foods or used as source of nutraceuticals.

1. Introduction

Hypertension is related to the incidence of coronary heart disease and its treatment is effective in reducing the risk of the disease [1]. The angiotensin-I-converting enzyme (EC 3.4.15.1; ACE) plays an important physiological role in the regulation of blood pressure [2]. The ACE can increase blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II. Therefore, inhibition of ACE activity is considered to be a useful therapeutic approach in the treatment of high blood pressure. Several effective oral ACE inhibitors have been developed, namely, captopril, enalapril, and lisinopril and all are currently used as clinical antihypertensive drugs [3]. Although synthetic ACE inhibitors are effective as antihypertensive drugs, they cause adverse side effects such as coughing, allergic reactions, and skin rashes. Therefore, research and development to find safer, innovative, and economical ACE inhibitors is necessary for the prevention and remedy of hypertension. Several reports have been published on the ACE inhibitory activity of peptides from food proteins, like casein [4], mushroom [5], whey protein [6], soybean [7], and fish proteins [8].

The excessive production of free radicals and the unbalanced mechanisms of antioxidant protection result in the onset of numerous chronic disorders: cancer, cardiovascular disease, diabetes, and other ageing-related diseases [9]. In the last decades, the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxy-toluene (BHT) in stabilization of foods in agro-alimentary industry is suspected to have negative effects on consumer's health [10]. In recent years, hydrolysed proteins from many animal and plant sources such as milk casein [11], canola [12], and egg-yolk protein [13] have been found to possess antioxidant activity. In addition, aquatic products and by-products such as smooth hound protein [8], hoki frame protein [14], yellow stripe trevally [15], and shrimp carotenoprotein hydrolysate [16] have also proven to be good sources of antioxidant peptides.

The new fisheries management constraint is how to adopt new development strategies coproducts. Currently, they are mostly converted into the meal and oil but it is a low value-added processing. Improved economic performance for a better application of the coproducts is therefore necessary [17]. Enzymatic hydrolysis applied to marine products in order to produce bioactive peptides is one of the possible ways to effectively use these resources. This process allows recovery of proteins that retain their content of essential amino acids and peptides which may have biological functions.

Within this context, the present work was carried out aiming to study angiotensin-I-converting enzyme inhibitory and antioxidant activities of barbel muscle protein hydrolysate obtained by enzymatic treatments.

2. Materials and Methods

2.1. Reagents. Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), angiotensin-I-converting enzyme from rabbit lung, and the ACE synthetic substrate hippuryl-l-histidyl-l-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals, namely, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium hydroxide, and other solvents, were of analytical grade.

2.2. Materials. The barbel (*Barbus callensis*) samples used in the present work were obtained from Barrage SIDI SAAD, Kairouan, Tunisia. The samples were packed in polyethylene bags, placed in ice (sample/ice ratio of about 1 : 3 (w/w)), and transported to the laboratory within 2 h after collection. The internal organs were separated and then stored in sealed plastic bags at −20°C.

2.3. Determination of Chemical Composition. The moisture and ash content were determined according to the AOAC standard methods as 930.15 and 942.05, respectively [18]. Total nitrogen content was determined by using the Dumas method. Samples were heated to 1050°C following AOAC 992.15 [18] in a LECO model FP-2000 protein/nitrogen analyser calibrated with EDTA. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples with hexane. All measurements were performed in triplicate.

2.4. Preparation of Barbel Muscle Protein Hydrolysate (BMPH). Barbel muscle (500 g), in 500 mL distilled water, was first minced using a grinder then cooked at 90°C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenised in a Moulinex blender for about 20 min. The samples were adjusted to pH 10.0 and 50°C. The substrate proteins were digested with Alcalase at a 3 : 1 enzyme/protein (U/mg) ratio for 5 h. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of NaOH (4 N) solutions. After the required digestion time, the reaction was stopped by heating the solutions for 20 min at 80°C to inactivate enzymes. Protein hydrolysate was then centrifuged at 5000 ×g for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions, referred to as protein hydrolysate, were freeze-dried using freeze-dryer at a temperature of −50°C and a pressure of about 121 mbar through a lyophilizer lab (CRIST, Alpha 1-2 LD plus, Germany) and then stored at −20°C for further use.

2.5. Determination of the Degree of Hydrolysis. The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the substrate studied (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis [19] according to the following equation:

$$\text{DH}\,(\%) = \frac{h}{h_{\text{tot}}} \times 100 = \frac{B \times \text{Nb}}{\text{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{\text{tot}}} \times 100, \quad (1)$$

where B is the amount of NaOH consumed (mL) to keep the pH constant during the proteolysis of the substrate. Nb is the normality of the base, MP is the mass (g) of the protein (N × 6.25), and α represents the average degree of dissociation of the α-NH_2 groups in the protein substrate expressed as

$$\alpha = \frac{10^{\text{pH-pK}}}{1 + 10^{\text{pH-pK}}}, \quad (2)$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the protein substrate was assumed to be 9.02 meq/g.

2.6. Determination of Color. Sample was placed between two steel dishes with a hole of 5.7 cm diameter. The color of hydrolysates was determined with a tristimulus colorimeter (HunterLab D25 A-9, Hunter Associates Laboratory Inc., Reston, VA, USA) using the CIE Lab scale (C/2°), where L^*, a^*, and b^* are the parameters that measure lightness, redness, and yellowness, respectively. A standard white plate with reflectance values of $L^* = 93.68$, $a^* = -0.69$, and $b^* = -0.88$ was used as a reference. The results were the average of five measurements taken at ambient temperature at different points on the samples.

2.7. Amino Acid Analysis. Barbel muscle protein hydrolysate was dissolved (1 mg/mL) in ultrapure water and hydrolysed in constant boiling with 6 N HCl containing 0.1% phenol and norleucine (Sigma-Aldrich, Inc., St. Louis, MO, USA) as internal standard. HCl was removed under vacuum after 24 h of hydrolysis 110°C. Dried samples were reconstituted in application buffer and injected onto a Biochrom 20 amino acid analyser (Pharmacia, Barcelona, Spain).

2.8. Determination of ACE Inhibition Activity. ACE-inhibitory activity was assayed as reported by Nakamura et al. [20]. A volume of 80 μL containing different concentrations of test sample was added to 200 μL of 5 mmol/L HHL and preincubated at 37°C for 3 min. Test sample and HHL were prepared in 100 mmol/L borate buffer (pH 8.3) containing 300 mmol/L NaCl. The reaction was then initiated by adding

20 μL of 0.1 U/mL ACE from rabbit lung prepared in the same buffer. After incubation at 37°C for 30 min the enzymatic reaction was stopped by adding 250 μL of 0.05 mol/L HCl. The liberated hippuric acid (HA) was extracted with ethyl acetate (1.7 mL) and then evaporated at 95°C for 10 min. The residue was dissolved in 1 mL of distilled water and the absorbance of the extract at 228 nm was determined using a UV-visible spectrophotometer. ACE-inhibitory activity was calculated using the equation

$$\text{ACE inhibition (\%)} = \left[\frac{B - A}{B - C}\right] \times 100, \quad (3)$$

where A is the absorbance of HA generated in the presence of ACE inhibitor component, B is the absorbance of HA generated without ACE inhibitors, and C is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). The IC_{50} value was defined as the concentration of inhibitor required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE). Method of Bradford [21] using bovine serum albumin as a standard was used.

2.9. Antioxidant Activity

2.9.1. DPPH Free Radical-Scavenging Activity. The DPPH free radical-scavenging activity of barbel muscle protein hydrolysate was determined as described by Bersuder et al. [22]. A volume of 500 μL of each sample at different concentrations (1 to 5 mg/mL) was added to 375 μL of 99% ethanol and 125 μL of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken then incubated for 60 min in darkness at room temperature. Scavenging capacity was measured spectrophotometrically by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control. DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \quad (4)$$

where A_{control} is the absorbance of the control reaction (containing all reagents except the sample) and A_{sample} is the absorbance of test sample (with the DPPH solution). The experiment was carried out in duplicate and the results are mean values.

2.9.2. Reducing Power Assay. The ability of BMPH to reduce iron(III) was determined according to the method of Yildirim et al. [23]. An aliquot of 1 mL sample of barbel hydrolysate at different concentrations (1 to 5 mg/mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide solution. The mixtures were incubated for 30 min at 50°C. After incubation, 2.5 mL of 10% (w/v) TCA was added and the reaction mixtures were then centrifuged for 10 min at 10000 rpm. Finally, 2.5 mL of the supernatant solution from each sample mixture was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% (w/v) ferric chloride. After a 10 min reaction time, the absorbance of the resulting solutions was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The control was conducted in the same manner, except that distilled water was used instead of sample. Values presented are the mean of triplicate analyses. BHA was used as reference antioxidant.

2.10. Statistical Analyses.

Statistical analyses were performed with Stratgraphics ver. 5.1, professional edition (Manugistics Corp., USA) using ANOVA analysis. Differences were considered significant at $P < 0.05$. All tests were carried out in triplicate.

3. Results and Discussion

3.1. Production of Barbel Muscle Protein Hydrolysate. It has been demonstrated that the biological activities of proteins can be increased through hydrolysis with certain enzymes [24]. Furthermore, the biological activity of protein hydrolysates depends on the protein substrate, the specificity of the enzyme, the conditions used during proteolysis, and the degree of hydrolysis. Since enzymes have specific cleavage positions on polypeptides chain, protein hydrolysates were prepared from barbell muscle by treatment with Alcalase to obtain peptides with different amino acids sequences and peptides length.

3.2. Chemical Composition of Barbel Muscle Protein Hydrolysate. The chemical composition of barbel muscle protein hydrolysate was determined. As shown in Table 1, the protein hydrolysate had high protein content (84.82%) and could be an essential source of proteins. The high protein content was a result of the solubilisation of proteins during hydrolysis, the removal of insoluble undigested nonprotein substances, and the partial removal of lipid after hydrolysis [25]. Barbel protein hydrolysate had relatively low lipid content (2.56%). Similar lipid levels (3.1%) were reported by Balti et al. [26] in the protein hydrolysate prepared from cuttlefish (*Sepia officinalis*) by-products. The ash content was 11.08%. These results are similar to those found in protein hydrolysate prepared from sardinella muscle (between 10% and 11.7%) [27].

3.3. Determination of Color. Color influences the overall acceptability of food products. The color values of the BMPH are also shown in Table 1. Indeed, barbel muscle protein hydrolysate was dark (L^* = 88.18) and yellow (b^* = 15.58). Enzymatic browning reactions are assumed to have contributed to the reduction in the luminosity. Sathivel et al. [28] reported that the color of whole herring and herring by-product hydrolysates, prepared using Alcalase, varied with substrates. Herring gonad hydrolysate was the darkest (L^* = 74.6) and most yellowish (b^* = 18), whereas whole herring

Table 1: Proximate composition of barbel muscle protein hydrolysate.

Composition	BMPH
Protein (%)	84.82 ± 7.4
Fat (%)	2.56 ± 0.12
Ash (%)	8.87 ± 0.20
Moisture (%)	11.08 ± 0.07
Color	
L^*	88.18 ± 0.06
a^*	0.07 ± 0.01
b^*	15.58 ± 0.09

Physicochemical composition was calculated based on the dry mater.

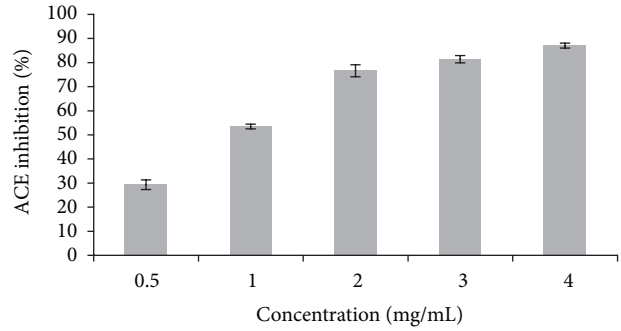

Figure 1: ACE-inhibitory activity of barbel muscle protein hydrolysate at different concentrations.

Table 2: Amino acid composition of barbel muscle protein hydrolysate (BMPH).

Amino acids	Number of residues/1000 BMPH
Asx (D + N)	104
Thr (T)	51
Ser (S)	58
Glx (E + Q)	154
Gly (G)	83
Ala (A)	94
Cys (C)	4
Val (V)	47
Met (M)	29
Ile (I)	40
Leu (L)	80
Tyr (Y)	21
Phe (F)	30
OHLys	1
His (H)	23
Lys (K)	86
Arg (R)	46
Pro (P)	41
OHPro	8
TEAA	407

Asx = Asp + Asn; Glx = Glu + Gln.
TEAA: total essential amino acids.

hydrolysate was the lightest ($L^* = 89.4$) and least yellowish ($b^* = 8.0$).

3.4. Amino Acid Composition. The amino acid composition of BMPH expressed as residues per 1000 total amino acid residues is shown in Table 2. Alanine is the most dominant amino acid in the barbel muscle hydrolysate (94 residues per 1000 residues). The BMPH has a high percentage of essential amino acids such as histidine, isoleucine, leucine, lysine, valine, and methionine (23, 40, 80, 86, 47, and 29 residues per 1000 residues, resp.). From the results, BMPH shows a high nutritional value, based on its amino acid profile, and could be a good dietary protein supplement.

Furthermore, amino acid composition analyses of BMPH help to study their biological effect and mechanism. Amino acids in the hydrolysate are possibly involved in antioxidative activity. Several amino acids, such as Tyr, Met, His, Lys, and Trp, may significantly contribute to the antioxidant activity of the hydrolysates [29].

3.5. ACE Inhibitory Activity of BMPH. The hydrolysate obtained with Alcalase was then assayed for ACE inhibitory activity. Result showed that the ACE inhibitory activities increase with the increase of hydrolysate concentration (Figure 1). Furthermore, the highest ACE inhibitory activity 87% was observed at a concentration of 4 mg/mL.

The IC_{50} values for ACE inhibition of BMPH was 0.92 mg/mL (Figure 1). The IC_{50} value of BMPH was lower than those of hydrolysates from oyster, scallop, codfish skin, and herring skin which presented an IC_{50} greater than 10 mg/mL [30], whereas it is higher than those from sardine ($IC_{50} = 0.62$ mg/mL) [31] and sardine ($IC_{50} = 0.082$ mg/mL) [32]. Proteolysis can operate either sequentially, releasing one peptide at a time, or through the formation of intermediates that are further hydrolyzed to smaller peptides as proteolysis progresses, which is often termed "the zipper mechanisms" [33].

3.6. Antioxidant Activity of BMPH

3.6.1. DPPH Radical-Scavenging Capacity. DPPH is a stable free radical which can be reduced by a proton-donating substrate such as an antioxidant, causing the decolorization of the DPPH and reducing the absorbance at 514 nm. The rate of the decrease in the color gives us an idea about the DPPH scavenging capacity.

DPPH radical scavenging capacities of BMPH and BHA (used as positive control) are shown in Figure 2(a). BMPH was a strong radical scavenger with an IC_{50} of 1.12 mg/mL. Similar results were reported by Guerard et al. [34] when they studied the free radical-scavenging activity in the hydrolysis of shrimp processing discards. BMPH showed a DPPH free radical-scavenging activity in the range of the concentrations tested. This activity was significantly lower than that of BHA at the same concentration.

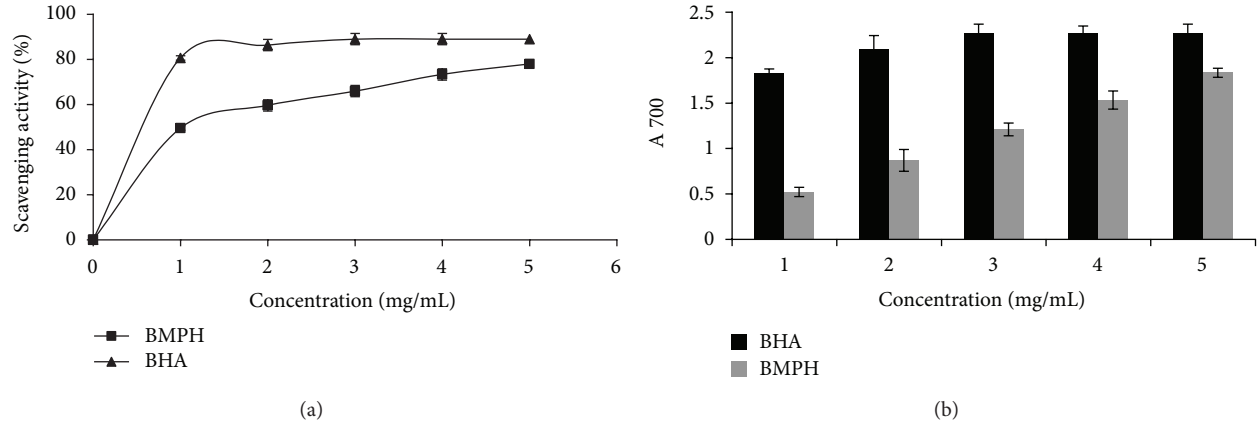

Figure 2: Antioxidant activities of barbel muscle protein hydrolysate at different concentrations. (a) DPPH free radical-scavenging activities, (b) reducing power.

3.6.2. Reducing Power. The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron or hydrogen. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain bioactive compounds [35]. In this assay, the ability of BMPH to reduce Fe^{3+} to Fe^{2+} was determined.

The presence of antioxidants in the protein hydrolysate results in reduction of the Fe^{3+}/ferric cyanide complex to the ferrous form. Therefore, the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 2(b) shows the reducing power (as indicated by the absorbance at 700 nm) of BMPH at different concentrations compared with that of BHA as the standard. BMPH showed some degree of electron donation capacity. At 4 and 5 mg/mL, the reducing power of BMPH was 1.532 and 1.833, respectively.

4. Conclusion

Fish protein hydrolysates in general are considered safe products and they are not subjected to restricted use in foods. Therefore, barbel muscle protein hydrolysate can be used in food systems such as meat products as a natural additive possessing antioxidative properties. Further works should be done to isolate and identify some specific peptides in barbel protein hydrolysate which are responsible for the overall biological activity.

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