

## Biological active compounds from native food sources for fermented dairy products

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### Abstract

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**Introduction.** The present research aims to identify and quantify valuable compounds from native products such as honey, walnut and sea buckthorn in order to produce fermented product from cow's milk.

**Materials and methods.** Honey bees of native production polyphora from the Drăgășani area (Romania) were used, and for the identification and quantification of the volatile compounds a GC-MS system was used. Qualitative and quantitative analyzes of the polyphenols were performed using the Agilent 1200 HPLC system consisting of a photodiode array (PDA) detector and an electrospray ionization mass detector.

**Results and discussion.** In polyflora honey bees, phenolic acids reach 79.284 mg/100 g sample, isoprenoids reach 127.449 mg/100 g sample, and flavonoids at 168.475 mg/100 g sample, and the results obtained by chromatographic analysis of honey. Bees have been found in this product a wide range of aroma compounds, namely: terpenic compounds, higher alcohols, aldehydes and ketones, esters, so that the aldehydes reach values of 7.889 mg/100g, ketones at 2.337 mg/100g, alcohols higher ones accumulate at amounts of 3.212 mg/100g, and the esters reach values of 8.993 mg/100g. Following the chromatograms obtained, the content of polyphenols in the walnut kernel was established at an amount of 786.553 mgGAE/100 g, and for the berries the content in polyphenols was 343.229 mgGAE/100 g. Also the highest concentration significant of tocopherols is found in the form of alpha tocopherol, with 33.245 mg/100g, followed by beta tocopherol with an amount of 12.723 mg/100 g of oil. Values lower than 4.553 mg/ 100g and 1.286 mg/100g respectively are visible in the case of the tocopherol gamma and the tocopherol delta.

**Conclusions.** The compounds identified and quantified from the three indigenous products such as polyflora honey, walnut and sea buckthorn will lead to the achievement of a harmonious aromatic profile and with certain taste qualities of the new fermented dairy product.

## 1. Introduction

The actual task is to analyze new perspectives and key areas for future research in the development of high-quality innovative dairy products as a function of valuable compounds from native food products and some new processing techniques. Previous studies [16–18] have shown that bee honey, walnuts and sea buckthorn have a number of positive effects in human nutrition and health, with beneficial action especially in the area of the nervous and vascular system, infectious diseases exerting a pronounced antibacterial action.

The current research has been done is to identify and quantify valuable compounds from honey, walnuts and sea buckthorn in order to produce an innovative fermented product from cow's milk.

## 2. Analyses of valuable compounds from native products used in obtaining innovative dairy products

### 2.1. Bee honey: characteristics and valuable components

Bee honey is a food naturally derived from the processing of floral nectar by bees [32]. It is sweet, so it contains many sugars, aromatic, of different consistencies, with a variable color palette, specific to its composition [8, 10]. The color palette starts from colorless, yellow, reddish, orange and can reach black [1, 2].

Honey is the result of the activity of the bees that collect the nectar with the help of the horn with which they are endowed, keep it in the gose, mix it with saliva and then a transfer to the bees left in the hive in a sugary form, which continues the processing until it reaches the known form [16, 33].

**2.1.1. Bee honey and its sensory characterization.** The appearance of honey and the color can be appreciated according to the degree of transparency examined in the direct sunlight [3, 7].

The taste and smell are appreciated by noting the intensity of the aroma and the sweet taste, or of the possible secondary shades:sour, bitter, fad, etc. conferred including polyphenols (Table 1) [4, 5].

Table 1

Sensory characteristics

No	Characteristics	Conditions of admissibility
1	Color	From colorless, bright yellow, to orange
2	Taste	Sweet, pleasant,
3	Appearance	Semifluid, viscous or crystallized
4	Flavor	Floral, characteristic of the type of honey

**2.1.2. Beehoneyandits chemical composition.** The chemical composition of honey bees depends to a large extent on the climatic factors and the vegetal nature of the collection area, the mode of exploitation of the product by the beekeepers, its properties being in direct correlation with them [6–10].

Bee honey contains protein substances, a number of microelements and vitamins (C, B1, B6, B2), small quantity organic acids (citric, lactic, malic, oxalic, succinic), dextrans, odorants and dyes, with clinical effects. Relevant, but also presents a microbiological load worth studying [11–15].

All honey is acidic and has a pH value generally between 3.5 and 5.5, due to the presence of organic acids that contribute to honey aroma and stability to microorganisms [11]. In honey, the main acid is gluconic acid, which is found together with the respective glucono-lactone in a variable equilibrium (Table 2) [9].

Table 2

Physico-chemical and microscopic characteristics

No	Parameter	Acacia honey	Forest honey	Other assortments
1	Water,% max	20	20	20
2	Acidity, ml NaOH 1N/ 100g, max	4	5	4
3	Reducing sugar, expressed in invert sugar,% min	70	60	70
4	Amylase index, min	6,5	13,9	10,9
5	Ash,% max	0,5	1,0	0,5
6	Hydroxymethylfurfural, mg/100g max	1,5	1,5	1,5
7	Colorimetric index, mm, max (on the Pfund scale)	12	65	-
8	Water insoluble substances, max	0,1	0,2	0,1
9	Lightly hydrolyzable sugar, expressed as sucrose,% max	5	10	5

**2.1.3. Microbiological indicators of honey.** Microbiological indicators of honey are presented in Table 3[12].

Table 3

Microbiological indicators of honey

No	Specification	NTM/g	Number of yeasts / g	Molds	Pathogenic microflora
1	Normal honey	<300	2-3	absent	Absence
2	Honey with limited conservabilite	<300	10-10 <sup>2</sup>	absent	Absence
3	Honey coming out of consumption	>300	10 <sup>4</sup>	present	Present

In order to detect forgeries, the physico-chemical control of honey is done by several methods: determination of acidity index, determination of sucrose (based on the identification of sucrose from honey forged with sugar syrup by reaction with silver nitrate), determination of the addition of flour or starch (based on the color reaction of starch with iodine), identification of honey nitrites [12, 13].

## 2.2. Walnuts: characteristics and valuable components

Walnuts (*Juglans regia* L.) belong to the family Juglandaceae. The native region of which they come is Central Asia, the western Himalayan chain, the region of Kyrgyzstan [19]. They arrived in Europe before the Roman era, and later spread to America and North Africa [21]. Walnuts and hazelnuts (*Corylus avellana* L.) make up about 60% of walnut production in Europe [20].

The results of clinical and epidemiological studies clearly indicate that regular consumption of nuts reduces the risk of cardiovascular disease, diabetes, cancer and inflammatory diseases [21]. According to many authors, these properties result from the chemical composition of walnuts. First, they are a rich source of fat (up to 70% dry mass), including unsaturated fatty acids, as well as phytochemicals such as phenols, tocopherols and sterols [19, 20]. In addition, walnuts contain polyunsaturated fatty acids (PUFAs) that lower LDL cholesterol and increase HDL cholesterol [22]. Moreover, nuts contain dietary fiber and essential micronutrients [23].

Nuts are also a rich source of complete proteins, which are abundant in exogenous amino acids, as well as minerals, including potassium and magnesium [24]. In walnut oil, there are a number of active phytochemicals that consist mainly of polyphenols [26]. In terms of polyphenol content, nuts dominate among all nuts; their average polyphenol content is about 1591.5 mg/100 g, and in peanuts it varies widely up to 900 mg/100 g [21–23]. Of all the polyphenols identified in walnuts, elagitannins are the most numerous group, followed by phenolic acids, flavanols and dihydroclacones [24–26].

## 2.3. Sea buckthorn (*Hippophae rhamnoides* L.): characteristics and valuable components

The sea buckthorn (*Hippophae rhamnoides* L.) presents a series of properties beneficial to the human body, through its rich content in minerals and vitamins. It presents a series of bactericidal and bacteriostatic effects, being rich in antioxidants that have the effect of neutralizing free radicals, is implicated by its composition in the elimination of toxins from the body [27].

Sea buckthorn contains over 200 bioactive components and volatile compounds, many vitamins (including A, P, C, B1, F, B2, E, K), carotenoids, tocopherols, sterols, flavonoids, phenolic compounds, lipids, ascorbic acid, citric acid and more than 15 microelements (including Fe, Mn, B, Al, K, F, Ti) [27]

Catenin reduces liver injury by antioxidant activity [28]. The sea buckthorn pulp is rich in carotenoids, tocopherols, sterols, lipids, ascorbic acid, flavonoids, triterpenes, etc. These compounds have biological activities and therapeutic properties such as antioxidants, antitumor and immunomodulators [29, 30]. In addition to medicinal use, the pulp is processed into different products, such as juice and jam [28].

Studies have shown the efficiency of dog consumption in the recovery of cancer patients, those with epidermal problems, being an optimal raw material including in the cosmetic industry [31].

### 3. Materials and methods

#### 3.1. Experimental samples

Polyflora honey, walnut kernel and sea buckthorn berries are valued as a source of natural compounds with antioxidant properties and pleasant sensory profile. In this study honey of polyflorous bees of local production in the Drăgășani area (Romania), walnut kernel arising from Station for Horticulture Research and Development (SCDP) (Romania), and sea buckthorn from Agricultural Cooperative from Biocătina (CAB) (Romania) were used.

#### 3.2. Determination of phenolic compounds in honey

For quantification and determination of phenolic compounds, 100 g of honey were dissolved in 500 ml of acidified water (pH = 2, HCl) [4]. The solution was then filtered with cotton to remove the solid particles. The obtained filtrate was passed through an Amberlite XAD-2 column, which has the ability to selectively retain phenolic compounds. To remove sugars and other polar compounds, a wash was carried out with the passage of acidified water to pH=2. Subsequently, the phenolic fraction was eluted with methanol and the extract was concentrated under reduced pressure at 40 °C. The final residue was then redissolved in 5 ml of distilled water and subjected to liquid/liquid extraction, using diethyl ether as the extraction solvent. The extracts were concentrated under reduced pressure, and the residue was dissolved in methanol.

The chromatographic system consisted of a quaternary pump, an automatic sampler maintained at 5 °C, a degasser, a photodiode detector and an automatic compartment for thermostatic columns. Chromatographic separation was performed with a C18 column of particles having a size of 1.8 μm and maintained at 30 °C. The mobile phase was composed of 0.1% formic acid diluted in water and 0.1% formic acid dissolved in acetonitrile, previously degassed and filtered using a nylon membrane filter with a porosity of 0.22 μm. The injection volume was 3 μl. Spectral data for all peaks were detected in the range 190-600 nm.

Each sample was filtered through a 0.2 μm nylon membrane (Whatman). The mass analysis was performed on a Varian 240 mass spectrometer in negative mode, equipped with an ESI electro-spray ionization source: spray voltage, 5 kV; capillary voltage, -20V; capillary tube voltage, -65V; capillary temperature, 325 °C; gas flow and auxiliary gases (N<sub>2</sub>), respectively 50 and 10 (arbitrary units). The mass spectra were obtained in the mass range 100-1000 m/z.

The collision energy used in the MS<sup>n</sup> experiments was 35 (arbitrary units). Data acquisition was performed using Xcalibur ® software (Thermo Scientific, CA, USA). The quantification was performed with calibration curves of the standard substances for caffeic acid, quercetin, caempferol, chrysin and pinocembrine. In the absence of standards the compounds were expressed by equivalents of the more structurally similar phenolic compound (method adopted and adapted after Silva Caveiro [31]).

#### 3.3. Determination of volatile compounds in honey

A GC-MS system was used to identify and quantify volatile compounds: GC-MS Varian 240 with column C18, helium carrier gas, the temperature being set upwards by 3 °C/min up to 170 °C, later with 25 °C/min up to 290 °C/min. The volatile compounds were identified by comparing the spectra obtained with those from the laboratory library [6, 25].

### 3.4. Determination of phenolic compounds in walnuts and sea buckthorn fruit

Qualitative and quantitative analyzes of the polyphenols were performed using the HPLC Agilent 1200 system consisting of a photodiode array (PDA) detector and an electrospray ionization mass detector. The polyphenolic compounds were separated on a C18 column using a linear gradient of 8 minutes. Solvents were used: solvent B containing 40% acetonitrile and 0.1% formic acid and solvent A: ultrapure water containing 0.1% formic acid at a flow rate of 0.30 mL / min. The injection volume of the samples was 5  $\mu$ L. The readings of the polyphenolic compounds were performed at the following wavelengths: hydroxycamenes at 320 nm and the ellagic tannins at 240 nm. Gallic acid was used as an equivalent [20, 23].

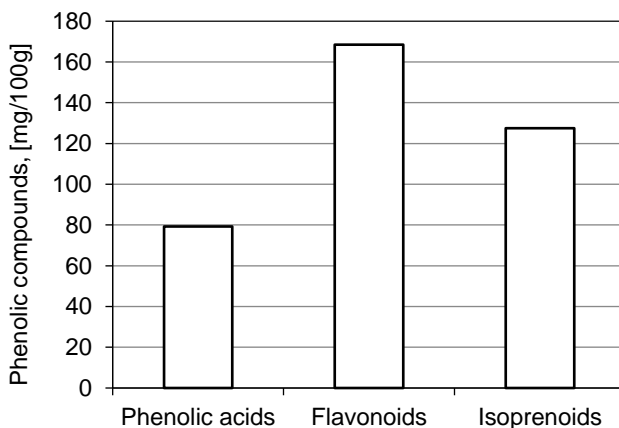
### 3.5. Determination of tocopherol compounds in walnuts and sea buckthorn fruit

To analyze the content of tocopherols, the oil extracted from nuts and sea buckthorn was used using the Soxhlet method. The tocopherol content ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) was measured by high performance liquid chromatography (HPLC) according to the method adapted by Pycia et al. The chromatography system consisted of an Agilent 1200 HPLC chromatograph, a C18 column. As a mobile phase, methanol with a flow rate of 0.8 mL/min and a Shimadzu fluorescence detector with the extinction wavelength set at 290 nm and the emission wavelength at 330 nm were used. The injection volume of a measured sample was 20  $\mu$ L. Tocopherols were quantified using standard curves calculated by linear regression analysis [19].

## 4. Results and discussions

### 4.1. Identification and quantification of phenolic compounds in honey

In order to approach as accurately as possible, the phenolic compounds in the honey of the bees subjected to the study, they were summed according to their chemical classification, namely: phenolic acids, flavonoids, isoprenoids (Figure 1).

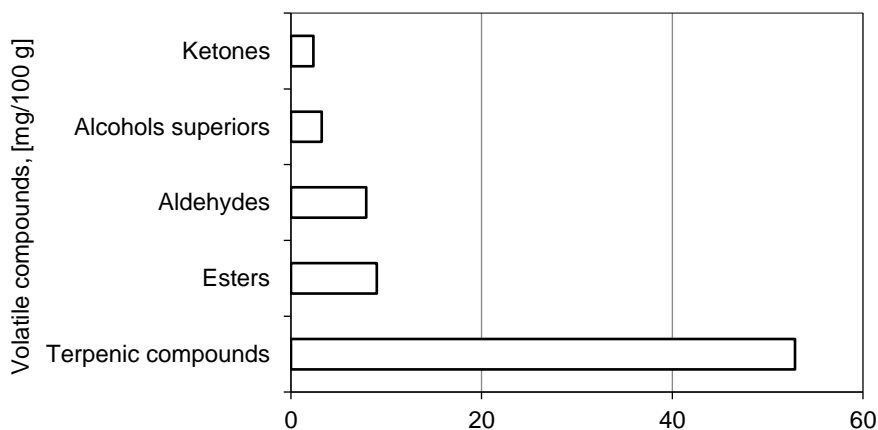


**Figure 1. Sum of the phenolic compounds identified and quantified in the honey of poliflorine bees from Drăgășani**

The amount of these compounds varies in the sample taken from 79.284 mg/100 g to 168.475 mg/100 g (Figure 1). Phenolic acids reach 79.284 mg/100 g sample, isoprenoids reach 127.449 mg/100 g, and flavonoids to 168.475 mg/100 g sample.

#### 4.2. Identification and quantification of volatile compounds in honey

The presence of volatile compounds in honey can provide information on its botanical origin, allowing it to verify whether it was produced by bees from the nectar of flowers or exudates secreted by plants or insects [3, 6]. Over 500 volatile compounds have been identified in honey as complex mixtures of different classes of compounds such as terpenes, isoprenoids, phenolic compounds, benzene derivatives, alcohols, ketones, aldehydes, esters, fatty acids, linear hydrocarbons and cyclical [33]. As a result of the results obtained by the chromatographic analysis of honey bee, it was found that in this product there is a wide range of aroma compounds, namely: terpenic compounds, higher alcohols, aldehydes and ketones, esters (Figure 2).

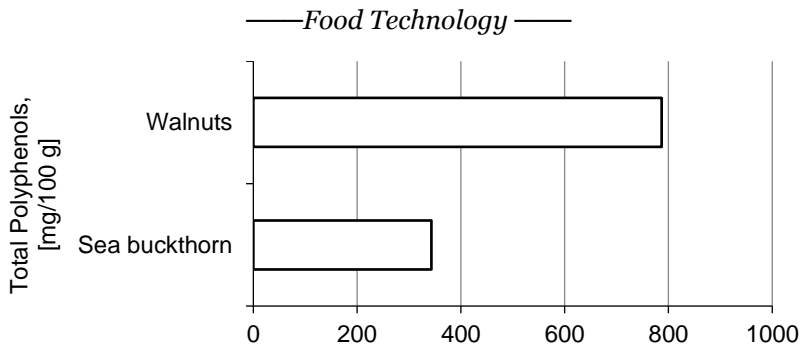


**Figure 2. Sum of the volatile compounds identified and quantified in the honey of polyflorous bees from Drăgășani**

We observe the determined quantities, noting especially the terpenic ones (Figure 2). From the results obtained the hotrienol is identified in remarkable amounts, the identified values being 47.335 mg/100g, and the sum of the terpenic compounds reaches 52.867 mg/100g. Aldehydes reach values of 7.889 mg/100g and ketones reach 2.337 mg/100g. Higher alcohols accumulate at 3.212 mg/100g, and esters reach 8.993 mg/100g.

#### 4.3. Identification and quantification of phenolic compounds in walnuts and fruits of sea buckthorn

Polyphenols are chemical compounds with multiple beneficial effects on the human body, generally acting as powerful antioxidant elements [20]. They are found in many fruits and vegetables, offering protection against free radicals [29, 30]. During the study it was determined the total polyphenol content in walnuts and sea buckthorn samples (Figure 3).

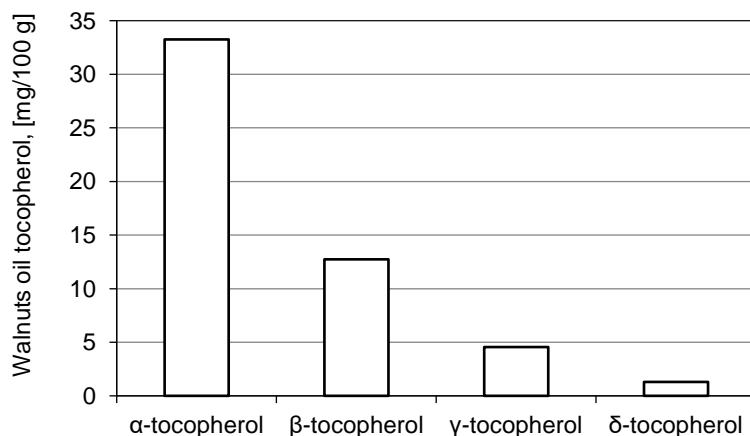


**Figure 3. Total polyphenol concentration identified in walnut kernel and sea buckthorn expressed in gallic acid equivalent**

Following the chromatograms obtained, the content of polyphenols in the walnut kernel was established, which is at an amount of 786.553 mgGAE/100 g according to Figure 3. For the berries the content in polyphenols is 343.229 mgGAE/100 g.

#### **4.4. Identification and quantification of tocopherols in walnuts and fruits of sea buckthorn**

Tocopherols or vitamin E is beneficial to the human body by its pronounced antioxidant character which leads to neutralization and elimination of free radicals [19]. The most active tocopherol is alpha, but it is found in walnut and buckwheat oil and as tocopherol beta, gamma or delta, or tocotrienol [24, 29]. During this study there were identified and determined several tocopherols: alpha, beta-, gamma and delta in the oil extracted from walnut (Figure 4).

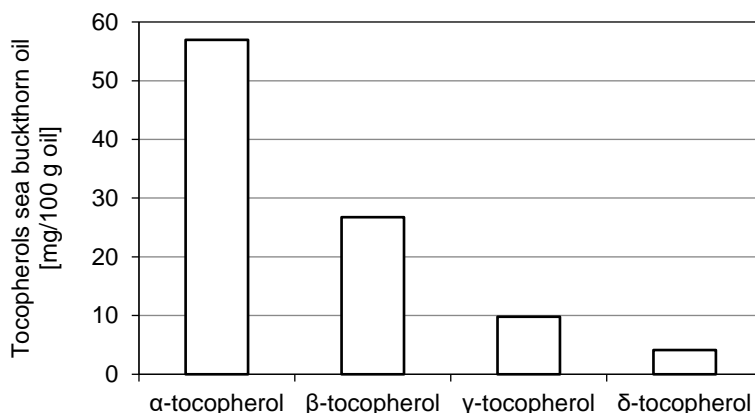


**Figure 4. The concentration of tocopherols identified and quantified in the oil extracted from walnut**



The most significant concentration of tocopherols (Figure 4) is found as alpha tocopherol, with 33.245 mg/100g, followed by beta tocopherol with an amount of 12.723 mg/100 g oil. Values lower than 4.553 mg/100g and 1.286 mg/100g respectively are visible in the case of the tocopherol gamma and the tocopherol delta.

Experimental results obtained regarding the tocopherols concentration in the oil extracted from the sea buckthorn are presented in Figure 5.



**Figure 5. The concentration of tocopherols identified and quantified in the oil extracted from the fruit of sea buckthorn**

A maximum concentration of alpha tocopherols in the amount of 56.973 mg/100g is observed, followed by 26.771 mg/100g beta tocopherols (Figure 5). Lower values of 9.774 mg/100g were identified in the case of tocopherol gamma and of 4.123 mg/100g in the case of the tocopherol delta. Compared with the values identified in walnut oil, the values found in sea buckthorn oil are more significant.

## Conclusions

Bee honey contains significant values of phenolic compounds, compounds that confer antioxidant qualities associated with nutritional ones.

The volatile compounds identified and quantified in honey bees lead to the achievement of a harmonious aromatic profile and with certain gustatory qualities, the share having terpenic compounds and esters.

The tocopherols identified and quantified in the oil extracted from the walnut core have significant values especially in the form of alpha tocopherols. Compared with the values identified in sea buckthorn oil, they are lower on average by 45%.

The ratio of alpha tocopherols to beta, gamma and delta tocopherols is on average 7:3, slight oscillations being visible in those identified and quantified in sea buckthorn oil.

The results of the present study suggest that bee honey, walnuts and sea buckthorn berries can be used as a valuable source of natural antioxidants and volatile compounds for obtaining innovative dairy milk products.

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