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Characterization of Canadian propolis fractions obtained from two-step sequential extraction

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ABSTRACT

The antioxidant capacity, as well as the concentration threshold for sensorial detection of Canadian propolis fractions in milk were determined. Propolis fractions were obtained using two-step sequential extraction with ethanol and water. Two processing pathways were carried out by interchanging ethanol and water extraction steps. Total polyphenol and flavonoid contents were quantified and ESI-MS fingerprints in negative mode were collected to determine the chemical composition of the propolis and propolis extracts. The ferric-reducing power assay and DPPH· methods were performed to determine antioxidant capacity. For both extraction pathways, the ethanolic extract showed higher polyphenol and flavonoid concentrations and higher antioxidant capacity as compared to commercial propolis and water extracts. The polyphenol composition of the extracts varied according to both, the extraction solvent and the extraction pathway as revealed by ESI-MS fingerprints. The sensory detection threshold concentration in milk was significantly higher in water extracts than in ethanol extracts. It was further increased when ethanol extraction was carried out as the first step and water extraction as the second step. The results of this study suggest that sequential extraction process can be used to produce propolis fractions with different polyphenol composition, antioxidant and sensory properties.

1. Introduction

The chemical composition of propolis, a resin produced by honey bees, is complex and changes with the vegetation visited by the bees as well as with the bee species (Lotti Fernandez, Piccinelli, Cuesta-Rubio, Hernandez & Rastrelli, 2010). Propolis can be purified by solvent extraction using mixture of water and ethanol. The chemical composition of propolis extracts and their antioxidant activity were shown to vary with the ethanol concentration in the extraction solvent (Cottica et al., 2011).

The antioxidant capacity of propolis has been evaluated by several methods, and mass spectrometry analyses have been used to examine the chemical composition of propolis extracts from different sources (Cottica et al., 2011; Kalogeropoulos, Konteles, Troullidou, Mourtzinos, & Karathanos, 2009; Kumazawa, Hamasaka, & Nakayama, 2004; Marcucci, Sawaya, Custodio, Paulino, & Eberlin, 2008; Sawaya et al., 2009). Chemical composition of Canadian propolis has been analyzed according to plant origin (Christov, Trusheva, Popova, Bankova, & Bertrand, 2005). However, the chemical characteristics, the antioxidant activity and sensory properties of Canadian propolis does not seem to have been fully investigated yet.

Owing to the antioxidant and antifungal activities of propolis, its use in different food formulations such as sausage, *turrón* and butter has been proposed (Ali, Kassem, & Atta-Alla, 2010; Narbona, García-García, Vázquez-Araújo, & Carbonell-Barrachina, 2010; Özcan & Ayar, 2003). The addition of propolis to food improves shelf-life, prevents lipid oxidation and also provides many health benefits to the consumer (Ali et al., 2010). However, the taste of propolis is quite intense (Narbona et al., 2010) and is not expected in foods with bland aroma profile, such as milks or dairy products. It is then a challenge to reduce of mask the taste of propolis in food formulations, while maintaining its beneficial characteristics for human health and food conservation.

The purpose of this study was to apply a two-step sequential extraction process to Canadian propolis and to compare the chemical profile and properties of the fractions. The extraction process consists of a water extraction step and an ethanol

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extraction step. Two extraction pathways were investigated by interchanging the order of the extraction steps. The ESI-MS profile, the antioxidant capacity and the determination of the concentration threshold for sensorial detection in milk were used to determine the effect of extraction conditions on propolis extracts.

2. Materials and methods

2.1. Chemicals

Gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH·), quercetin and Folin–Ciocalteu reagent were obtained from Sigma–Aldrich (Oakville, ON, Canada). All other chemicals were analytical-grade. Crude Canadian propolis powder (*Apis mellifera*) from Trophic (Richmond Hill, CA, USA, a good manufacturing practices [GMP] manufacturer) was purchased at a local natural product store.

2.2. Preparation of propolis extracts

Fractionation of commercial Canadian propolis was carried out using a two-step sequential extraction with ethanol and water according to two pathways. In the first extraction pathway (EW), the initial step corresponds to the extraction of commercial propolis with ethanol (EW-1) and the final step corresponds to the extraction of the residue with water (EW-2). For the second extraction pathway (WE), the initial step corresponds to the extraction of propolis with water (WE-1) and the final step corresponds to the extraction of the residue with ethanol (WE-2). Ethanol and water extractions were performed according to Laskar, Sk, Roy, and Begum (2010) with modifications.

For the EW processing pathway, 40 g of propolis were dispersed in 400 mL of 940 g/L ethanol and stirred for 24 h at 37 °C. The dispersion was then filtered through Whatman No. 42 filter paper and the filtrate was recovered (EW-1). Twenty grams of the filtration residue (dry basis) were dispersed in 200 mL of distilled water, refluxed for 3 h and filtered on Whatman No. 42 to eliminate non soluble material (EW-2). For WE pathway, 40 g of propolis were dispersed in 400 mL of distilled water under reflux for 3 h and then filtered through Whatman No. 42 (WE-1). Twenty grams of the filtration residue (dry basis) were dispersed in 200 mL of 940 g/L ethanol, stirred for 24 h at 37 °C and filtered on Whatman No. 42 to eliminate non soluble material (WE-2).

Water in EW-2 and WE-1 was removed by freeze-drying, and ethanol in EW-1 and WE-2 was evaporated under reduced pressure. All propolis extracts were protected from light and stored at −20 °C until analysis.

2.3. Total phenolic compound and flavonoid contents

Total polyphenol content (TPC) in crude propolis powder (P) and propolis extracts (PE) was analyzed by the modified Folin–Ciocalteu method using gallic acid as the standard (Singleton & Rossi, 1965). Methanol solutions of P and PE (2.5 mg mL^{-1}) were prepared, and 250-μL aliquots of these solutions or standard solutions were placed in test tubes. A pure methanol aliquot was used to prepare the blank. In each tube, 250 μL of Folin–Ciocalteu reagent (diluted in water 1:1), 500 μL of saturated Na_2CO_3 solution and 4 mL of distilled water were added and mixed. The solutions were incubated in the dark at room temperature for 25 min and then centrifuged at 3000 × *g* for 10 min. The sample absorbance was read against the blank at 725 nm using a spectrophotometer (Genesys, Thermo Scientific, Mississauga, Canada). The TPC was determined using a gallic acid calibration curve ($r^2 = 0.9955$) and reported as milligrams of gallic acid equivalent (GAE) per gram of P or PE on a total-solids basis.

Total flavonoids content (TFC) was determined by the $AlCl_3$ method (Woisky & Salatino, 1998) with modifications. Methanol solutions of P and PE (2.5 mg mL^{-1}) were prepared, and 500-μL aliquots were placed in test tubes. In each tube, 250 μL of $AlCl_3$ (50 g/L in methanol) and 4.25 mL of methanol were added. After 30 min, absorbance was read at 425 nm using a spectrophotometer (Genesys, Thermo Scientific, Mississauga, Canada) at room temperature. The TFC was determined using a quercetin calibration curve ($r^2 = 0.9946$) and reported as milligrams of quercetin equivalent (QE) per gram of P or PE on a total-solids basis.

2.4. Antioxidant capacity (DPPH· and ferric-reducing power)

Free-radical-scavenging capacity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH·) (Bondet, Brand-Williams, & Berset, 1997) with modifications. Briefly, different volumes of P and PE solutions (2.0 mg mL^{-1}) were added to 2.0 mL of DPPH· methanol solution (0.1192 mmol L^{-1}) and kept in the dark at room temperature for 30 min. Absorbance was measured at 515 nm (Genesys spectrophotometer, Thermo Scientific, Mississauga, Canada), and methanol was used as the control. Inhibition of DPPH· was calculated using Eq. (1) (where $Abs_{DPPH\cdot}$ is absorbance of DPPH· and Abs_{sample} is absorbance of the sample) and plotted as a function of P or PE concentration. Then, EC_{50}, which corresponds to the extract concentration (μg mL^{-1}) providing 50% inhibition, was obtained by linear regression. The antioxidant capacity is inversely proportional to the EC_{50} value.

$$\%\text{Inhibition DPPH}\cdot = \frac{\left(\text{Abs}_{\text{DPPH}\cdot} - \text{Abs}_{\text{sample}}\right)}{\text{Abs}_{\text{DPPH}\cdot}} \times 100 \quad (1)$$

To correct small concentration differences in the DPPH· solutions prepared on different days of analysis and to standardize the antioxidant capacity results obtained with this method, the antioxidant activity index (AAI) was calculated using Eq. (2) according to Scherer and Godoy (2009).

$$\text{AAI} = \frac{[\text{DPPH}\cdot]\,(\mu\text{g mL}^{-1})}{\text{EC}_{50}\,(\mu\text{g mL}^{-1})} \quad (2)$$

Ferric-reducing power (FRP) analysis was performed according to Zhu, Hackman, Ensunsa, Holt, and Keen (2002) with modifications. First, 1.0 mL of P or PE solution in methanol (1.0 mg mL^{-1}) was placed in test tubes, and then 2.5 mL of phosphate buffer (50 mmol L^{-1}, pH 7.0) and the same volume of 10 g/L potassium ferricyanide solution were added. After 20 min at 50 °C, 2.5 mL of 100 g/L trichloroacetic acid was added, and then the test tubes were centrifuged (4500 × *g*) for 10 min. The supernatant (2.5 mL) was diluted with 2.5 mL of distilled water, and 0.5 mL of 1 g/L $FeCl_3$ solution was added. Absorbance at 700 nm was immediately measured (Genesys spectrophotometer, Thermo Scientific, Mississauga, Canada). A calibration curve was obtained using gallic acid ($r^2 = 0.9957$), and the results are expressed as milligrams of GAE per gram of P or PE on a total-solids basis.

2.5. Electrospray ionization–mass spectrometry fingerprints

All electrospray (ES)–mass spectrometry (MS) experiments were performed on a Q-TOF SYNAPT mass spectrometer (Waters Corporation, Milford, MA, USA) with a nano-ESI source operating in negative ion mode. Mass Lynx software (v. 4.1, Waters Corporation, Milford, MA, USA) was used for data acquisition and analysis. Propolis extracts were first diluted 5 to 50 times in MeOH/NH_4OH solution at 5 g/L and then infused directly into the system at a flow rate of 0.4 μL min^{-1}. The experimental parameters were set as

follows: nano-ESI voltage, −2.8 kV; sampling cone, 40 V; extract cone, 4 V; source temperature, 100 °C; trap collision energy (CE), 6 V; transfer CE, 4 V; detector, 1650 V; mass range, 50–600 *m*/*z*.

2.6. Sensorial evaluation

The propolis fractions (EW-1, EW-2, WE-1 and WE-2) were tested by sensory analysis to determine their detectable concentrations (thresholds) in milk. Commercial propolis was not completely dispersible in milk and was not tested by the panel. To solubilize ethanolic extracts (EW-1 and WE-2), stock solutions, were prepared in ethanol (for human consumption) and added to milk in sterilized glass bottles. Final ethanol concentration was fixed to 1 g/L. Homogeneous dispersions were obtained by placing the capped bottles in an ultrasonic bath for 5 min. The water extracts (EW-2 and EW-1) were weighed, added directly to milk and dispersed in the ultrasonic bath for 5 min. Ethanol was also added to these beverages at 1 g/L. Beverages were prepared from pasteurized partly skimmed (20 g/L milk fat) milk purchased from a local grocery store. Beverages were prepared and tested at least two weeks before the expiration date printed on the package.

For each propolis extract, 10 beverages with various PE concentrations were prepared. A forced-choice task, the triangle test, was used. This method is designed to find the minimum concentration of each of the different extracts that is detected by 50% or more of the sample group. A panel of 30 subjects (10 men, 20 women) participated in the study (from 20 to 55 years old). The subjects were recruited according to their motivation and availability. Milk (20 mL) was placed in glass containers, coded with three-digit random numbers, and tempered at 4 °C for 1 h before tasting, which corresponded to the average consumption temperature of milk. The subjects were provided with water and plain crackers as palate cleansers between samples. Sensory analysis was conducted in a neutral environment, namely an air-conditioned room (20 °C) under white light in separate booths. The sensory measurements were performed 24 h after product preparation.

Scores were directly recorded on a computer system using FIZZ software (Biosystèmes, 1000, Couternon, France), and the group threshold concentrations were calculated according to Lawless and Heymann (2010).

2.7. Statistical analysis

Statistical analyses were performed using Statistical Analysis Systems (SAS) software, v. 2.0.3 (SAS Institute, Inc., Cary, NC, USA). All analyses were done in triplicate, and Tukey's test was run at 5% probability of error.

3. Results and discussion

3.1. Composition and antioxidant capacity

The composition and antioxidant capacity of the commercial propolis and propolis extracts are presented in Table 1. The yield of extraction of propolis solids with the EW pathway was 58.5%, with nearly twice the solids in the EW-1 fraction (39.5%) than in the EW-2 fraction (19.0%). Compared to commercial propolis, TPC concentration increased by a factor of ~2.5 in EW-1 fraction, while it is reduced by about 10% in the EW-2 fraction. TFC were also preferentially extracted in the EW-1 fraction with a concentration ~7 times higher than in EW-2. Similar results were observed by Miguel, Nunes, Dandlen, Cavaco, and Antunes (2010), who found higher TPC and TFC values in ethanol extracts of Portuguese propolis compared to water extracts. In contrast, Laskar et al. (2010) previously reported that the TPC in water extracts of Indian propolis was higher than in ethanol extracts. These results confirm the influence of the origin of the raw material on the composition and characteristics of the extracts. The mass balance reveals that the recovery of both TPC and TFC in the extracts is higher than 100%, which suggest that TPC and TFC in commercial propolis were not fully detected. Incomplete transfer of polyphenols/flavonoids from commercial propolis to the methanolic solution prior to the analyses is likely responsible for the overestimation or TPC and TFC recovery.

Table 1
Composition and antioxidant capacity of propolis and propolis extracts.

Parameter[a]	Sample[b]					SE[c]	CV[d] (%)
	P	EW-1	EW-2	WE-1	WE-2		
Yield	–	39.53	18.97	28.05	31.93	–	–
TS	926.5[b,c]	896.0[c,d]	954.9[a,b]	961.1[a]	867.9[d]	07.1	1.33
TPC	77.12[b]	199.35[a]	65.92[c]	68.79[c]	197.11[a]	1.63	2.32
TFC	41.66[c]	131.96[b]	1.85[e]	7.12[d]	137.06[a]	0.26	0.69
DPPH·	73.44[c]	27.11[d]	101.68[a]	91.78[b]	26.39[d]	0.53	1.43
AAI	0.69[b]	1.61[a]	0.43[c]	0.48[c]	1.65[a]	0.01	2.16
FRP	26.06[d]	63.03[b]	41.15[c]	41.07[c]	79.54[a]	1.03	3.55

[a] yield = (%, solids basis); TS = total solids (g/kg); TPC = total polyphenol content (mg GAE g^{-1} solids); TFC = flavonoid content (mg QE g^{-1} solids); DPPH· = EC_{50} (μg mL^{-1}); AAI = antioxidant activity index (Scherer & Godoy, 2009); FRP = ferric-reducing power (mg GAE g^{-1} solids).

[b] *P* = commercial propolis; EW-1 = ethanol extract from ethanol/water sequential extraction (first step); EW-2 = water extract from ethanol/water sequential extraction (second step); WE-1 = water extract from water/ethanol sequential extraction (first step); and WE-2 = ethanol extract from water/ethanol sequential extraction (second step).

[c] SE = standard error; ($n = 3$).

[d] CV = coefficient of variation. Different letters within a row mean different averages from Tukey's test ($P < 0.05$).

The yield of extraction of propolis solids with the WE pathway was 60%, which is similar to that of EW pathway. Unlike the EW pathway, the solids were almost equally distributed in the two fractions (28.1% in WE-1 fraction and 31.9% in WE-2 fraction). As for the EW pathway, both TPC and TFC were preferentially extracted in the ethanol fraction (WE-2). TPC concentrations in WE-1 and WE-2 extracts are similar to those in EW-2 and EW-1, indicating that interchanging ethanol and water extraction steps does not change TPC distribution in the extracts. As for EW pathway, TFC concentration in the water extract was low (7.1 mg QE·g^{-1} in WE-1), but interestingly, it was almost 4 times higher (1.8 mg QE·g^{-1} in EW-2). Some flavanoids compounds, which are soluble in both solvents, can be recovered in the water extract when water is used in the first extraction step (WE pathway).

The antioxidant capacity of commercial propolis and propolis extracts was measured by two different methods. The free-radical-scavenging capacity was measured by the DPPH· method, with the results expressed as an EC_{50} (μg mL^{-1}) value or as an AAI value according to Scherer and Godoy (2009). The FRP was measured by the potassium ferricyanide method. The ethanol extracts from both processing pathways (EW-1 and WE-2) presented higher antioxidant capacities than commercial propolis, correlating with higher TPC and TFC in these extracts. The water extracts (EW-2 and WE-1) showed lower antioxidant capacities than commercial using the DPPH· method. Because this method uses a non-polar free radical (Alamed, Chaiyasit, McClements, & Decker, 2009), higher antioxidant capacities are expected for extracts obtained with ethanol compared to water, since the polarity of water is higher than that of ethanol. This may have contributed to the higher antioxidant capacities measured in the ethanol extracts compared to the water extracts. Using the FRP method, however, higher antioxidant capacities were observed in the water extracts (EW-2 and WE-1) compared to commercial propolis, despite lower TPC and TFC values. This result indicates that the total concentration of polyphenols or flavonoids is not the only factor responsible for

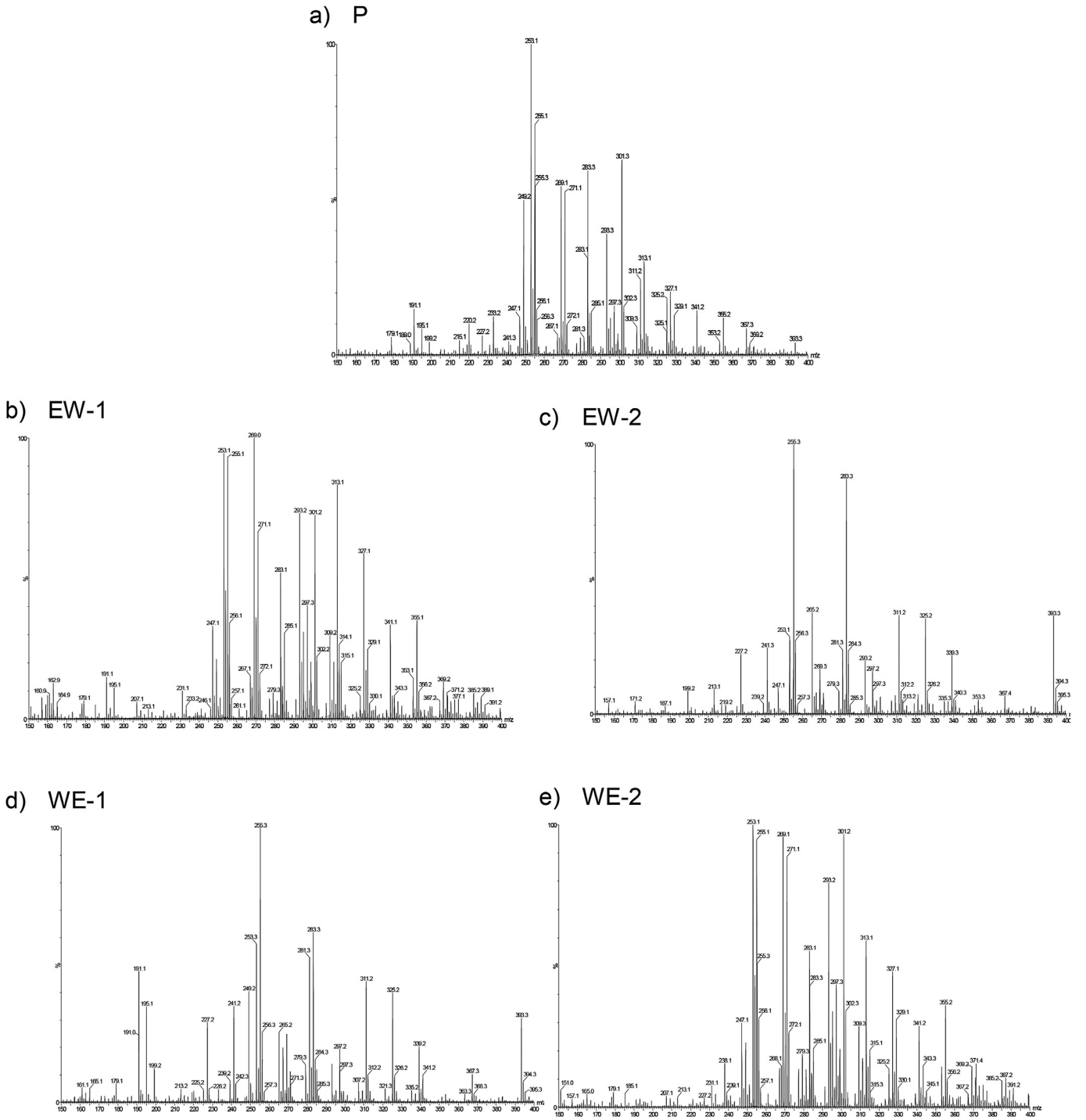

Fig. 1. Electrospray ionization (ESI)–mass spectrometry (MS) fingerprints in negative mode of commercial propolis and its extracts: a) commercial propolis (P); b) EW-1, ethanol extract from ethanol/water sequential extraction (first step); c) EW-2, water extract from ethanol/water sequential extraction (second step); d) WE-1, water extract from water/ethanol sequential extraction (first step); and e) WE-2, ethanol extract from water/ethanol sequential extraction (second step).

antioxidant properties. The chemical nature of polyphenols and perhaps the presence of other compounds contribute to the overall antioxidant capacity of the extracts.

According to Scherer and Godoy (2009), samples with AAI values lower than 0.5 have weak antioxidant activity, those with values between 0.5 and 1.0 have medium activity, and those with values between 1.0 and 2.0 have strong activity. Therefore, the antioxidant strength of the propolis and propolis extracts can be represented as follows: EW-1 = WE-2 > *P* > WE-1 = EW-2. Only the ethanol extracts can be considered as having strong antioxidant activity.

Other researchers have also studied the antioxidant capacity of propolis using various methods. Cottica et al. (2011) found DPPH· values (as EC_{50}) between 47 and 160 μg mL^{-1} for Brazilian hydroalcoholic propolis extracts, while Christov et al. (2005) found values between 65 and 79% DPPH inhibition for Canadian propolis ethanol extracts at 210 μg mL^{-1}. Laskar et al. (2010) found that water and ethanol extracts of Indian propolis had similar

antioxidant capacities using the FRP method at concentrations of 100 μg mL^{-1}.

3.2. Electrospray ionization–mass spectrometry fingerprints

The negative ESI-MS fingerprints of the propolis and propolis extracts (Fig. 1) were used for qualitative assessment. The commercial propolis fingerprint was close to the fingerprints of ethanol extracts (EW-1 and WE-2) but different from those of the water extracts (WE-1 and EW-2). The 10 most intense peaks on the negative ESI-MS fingerprints of the propolis and propolis extracts are reported in Table 2.

The peak with the highest intensity for commercial propolis occurred at *m/z* 253.1, which may correspond to chrysin (Sulaiman et al., 2011). Other important peaks with high intensity were found for commercial propolis, including those of *m/z* 255.1, 255.3, 269.1 and 301.3 (Table 2), which may correspond to pinocembrin, palmitic acid, benzyl caffeate or pinostrobin, and clerodane diterpenoid dehydrated, respectively (Sulaiman et al., 2011). All these peaks are markers of brown propolis, according to Sawaya et al. (2004). Pinocembrin and benzyl caffeate were also found in Canadian propolis from Vancouver Island and Rischmond (Christov et al., 2005).

Of the 10 most intense peaks observed on the commercial propolis fingerprint (Table 2), seven were also among the most intense peaks on the fingerprints of the ethanol extracts. Only two and three of these peaks were also observed among the most intense on the fingerprints of the water extracts WE-1 and EW-2, respectively. This indicates that the extraction with ethanol better maintained the original distribution of polyphenols found in commercial propolis compared to the extractions with water. Peaks of *m/z* 253.1, 255.1, 301.3, 269.1, 271.1, 283.1 and 293.3 were among the most intense in the ethanol extracts, whereas those with *m/z* 255.3 and 283.3 were among the most intense in the water extracts. The effect of extraction pathway (EW or WE) on the mass spectrometry fingerprint of the extracts was minor. One peak (*m/z* 253.1) is present in the water extract only when the water extraction step is carried on after the ethanol extraction step.

Table 2
Intensity (%) of major electrospray ionization (ESI)–mass spectrometry (MS) peaks for propolis and propolis extracts.

m/z	Sample[a] (%)					Compound
	P	EW-1	EW-2	WE-1	WE-2	
253.1	100	99	29	–	100	Chrysin[b]
255.1	75	98	–	–	97	Pinocembrin[b]
301.3	65	82	–	–	99	Clerodane diterpenoid dehydrated[b] or ellagic acid[c] or dihydrokaemferid or *E/Z* communic acid[d] or 3-prenyl-4-(2-methylproprionyloxy) cinnamic acid[e]
283.3	61	–	88	48	–	Unknown
269.1	56	100	–	–	97	Benzyl caffeate or pinostrobin[b]
255.3	55	–	100	100	–	Palmitic acid[b]
271.1	54	74	–	–	94	Naringenin or pinobanksin[b]
249.2	49	–	–	–	–	Isopentyl caffeate[b]
293.3	40	82	–	–	86	Unknown
283.1	32	61	–	–	68	Acacetin or caffeic acid phenethyl ester (CAPE)[b]

[a] Intensity values are relative to the most intense peak; *P* = commercial propolis; EW-1 = ethanol extract from ethanol/water sequential extraction (first step); EW-2 = water extract from ethanol/water sequential extraction (second step); WE-1 = water extract from water/ethanol sequential extraction (first step); and WE-2 = ethanol extract from water/ethanol sequential extraction (second step).
[b] Sulaiman et al., 2011.
[c] Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010.
[d] Cottica et al., 2011.
[e] Marcucci et al., 2008.

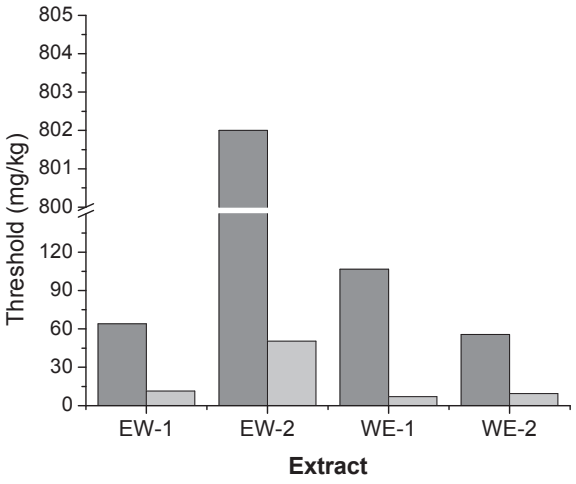

Fig. 2. Sensory threshold concentrations of propolis extracts in partly skimmed milk (20 g/L milk fat). Concentrations in milk of the extracts, ▬ and corresponding total polyphenol contents (TPC), ▭ are reported. The extracts were: EW-1, ethanol extract from ethanol/water sequential extraction (first step); EW-2, water extract from ethanol/water sequential extraction (second step); WE-1, water extract from water/ethanol sequential extraction (first step); and WE-2, ethanol extract from water/ethanol sequential extraction (second step).

Other researchers have also studied the chemical composition of propolis extracts obtained with different solvents. Buriol et al. (2009) found similar chemical compositions in oil and ethanol extracts. Cottica et al. (2011) found different chemical compositions in propolis extracts depending on the water/ethanol ratio used during hydro-alcoholic extraction.

3.3. Sensorial evaluation

Due to the peculiar odor of propolis, it was of great importance to determine its detection threshold in a bland-flavored food, such as milk. The panelists' group threshold was calculated by means of the best estimate of individual threshold, defined as the geometric mean of the first correct trial with all subsequent correct trials and the previous incorrect trial (Lawless & Heymann, 2010). The advantage of this method is that it makes it possible to eliminate all correct responses given by chance. The three accuracy values that were selected (α risk[1] = 0.01, β risk[2] = 0.10 and P_d[3] = 50%) were chosen based on the desired level of study sensitivity as well as time and space constraints. An α risk of 0.01 is strong evidence that a difference is detectable, a β risk of 0.10 is weak evidence that a difference is not detectable, and a P_d of 50% indicates a that a large proportion of subjects were able to detect a difference.

The threshold concentration for sensory detection of propolis extracts in milk is presented on Fig. 2. The threshold concentration for ethanol extracts (EW-1 and WE-2) was about 60 mg/kg, but water extracts (EW-2 and WE-1) showed significantly higher thresholds values (>100 mg/kg). Furthermore the use of EW pathway increases the threshold of the water extract by 7 times as compared to the WE pathway. Ethanol extraction in the first step of

[1] α risk = Probability of concluding that there is a perceptible difference where none exists.
[2] β risk = Probability of concluding that there is no perceptible difference where one exists.
[3] P_d = Proportion of evaluations in which a perceptible difference is detected between the two products.

the process is likely to eliminate aroma compounds responsible for the strong taste of propolis. Despite the lower TPC value in the EW-2 extract (Table 1), it could be used at a much higher concentration compared to any other extract in food formulations without affecting flavor. For comparison purposes, the threshold concentrations were also expressed on a TPC basis (Fig. 2). It appears that the EW-2 extract could be used to add polyphenols to milk at a rate of up to 50 mg/kg without detection. In comparison, the maximum polyphenol concentration before detection would be around 10 mg/kg with the other extracts.

Other researchers determined the sensory properties of propolis in different foods. Narbona et al. (2010) found that 50% of consumers were able to detect a significant difference in *turrón* with propolis compared to the control sample at a concentration of 550 mg/kg. Ali et al. (2010) determined that ethanol propolis extract at a concentration of 6000 mg/kg extended the shelf-life of oriental fresh sausage from 11 to 20 days because of its antibacterial and antifungal properties.

4. Conclusion

A two-step sequential extraction process with ethanol and water was used to produce extracts with different composition and properties. Two processing pathways were investigated by interchanging the order of extraction steps. Polyphenols and flavonoids were preferentially extracted by ethanol for the two pathways, resulting in improved antioxidant capacity. The Canadian sample of propolis analyzed in this study had similar ions to the brown type of Brazilian propolis according to the classification established by Sawaya et al. (2004). In addition, the ESI-MS fingerprints indicate that the original distribution of polyphenols was better maintained in ethanol extracts than in water extracts. The sensory threshold concentration of propolis extracts in milk was strongly affected by the extraction process. The water extract from the EW pathway showed the highest threshold. Compared to ethanol extracts, it would increase the fortification limit of milk with propolis polyphenols by a factor of 5 before being detected by the consumers.

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