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Extraction and Determination of Phenolic Compounds in the Berries of *Sorbus americana* Marsh and *Lonicera oblongifolia* (Goldie) Hook

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Abstract Phenolic compound content in *Sorbus americana* Marsh and *Lonicera oblongifolia* (Goldie) Hook berries was determined for the first time. An improved solid-liquid microextraction (SLME) method combining with high-performance liquid chromatography (HPLC)-diode array detector (DAD)-mass spectrometry (MS) has been developed to determine the phenolic compounds present in these berries, reducing the amount of sample, reagents, and time consumed. The major phenolic compound identified and quantified was 3-*O*-caffeoylquinic acid (3-CQA) in both berries. To a lesser extent, 5-*O*-caffeoylquinic acid (5-CQA) and quercetin-3-*O*-glucoside (QG) were also determined. The existence of these phenolic compounds and the great abundance of these fruits in the northeast of North America make *S. americana* Marsh and *L. oblongifolia* Hook berries a new and excellent source of natural phenolic compounds (antioxidants), which can be very useful in biotechnological exploitation.

Keywords *Sorbus americana* Marsh · *Lonicera oblongifolia* (Goldie) Hook · Phenolic compounds · Solid-liquid microextraction · HPLC-DAD-MS

Introduction

Phenolic compounds are reported to have multiple biological effects. Phenols are potent antioxidants (PhOH) due to their high capacity to scavenge free radicals (R•) (Perona et al. 2006). They prevent food degradation and some human diseases like arthritis, artherosclerosis (Belay and Gholap 2009), several types of cancer, and coronary heart disease (Trichopoulou and Lagiou 1997) and provide protection against inflammation (Camuesco et al. 2004; Yu et al. 2011).

In this regard, numerous plant species have been analyzed for their phenolic content and antioxidant capacity, berries being among the best sources (Halvorsen et al. 2001; Kähkönen et al. 2001). Chlorogenic acids (CGAs) and its derivatives (3-O-caffeoylquinic (3-CQA) and 5-caffeoylquinic acid (5-CQA)) are the main compounds in rowanberries and in coffee (Johnston et al. 2003; Hukkanen et al. 2006). Several reports have shown a connection between a diet rich in CGA compounds and the prevention of the oxidative stress associated with cancer, aging, cardiovascular and neurodegenerative diseases (Belay and Gholap 2009), reduction in the risk of type 2 diabetes, and inflammation reactions, among many others (Camuesco et al. 2004; Yu et al. 2011; López-Giraldo et al. 2009).

Food and medical industries are increasingly interested in natural substances with high contents of bioactive compounds as a source of biologically active non-nutrient compounds. The extraction and purification of these molecules from natural sources are encouraged to

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use them as bioactive compounds in the preparation of dietary supplements, nutraceuticals, pharmaceuticals, and cosmetic products.

Sample heterogeneity makes difficult to find an appropriate extraction method. Indeed, many analytical procedures for the extraction of phenolic compounds from berries have been proposed within the last decade (Mikulic-Petkovsek et al. 2012; Nuri Seo et al. 2012; Pizarro et al. 2013). Recently, some interesting approaches were presented to deal with the extraction of phenolic compounds from olive oil using liquid-liquid microextraction (Becerra-Herrera et al. 2014; Moerman 1998), demonstrating the feasibility of this methodology working with fewer volumes of sample and reagent. In this work, an efficient and environmentally friendly solid-liquid microextraction (SLME) process has been developed. After obtaining the desired extract, a high-performance liquid chromatography (HPLC) with a diode array detector (DAD) coupled to a mass spectrometer (MS) can be used to identify and quantify individual phenolic compounds. Following this procedure, the determination of phenolic compounds in two different varieties of berries (*Sorbus americana* Marsh and *Lonicera oblongifolia* Hook) was successfully achieved.

S. americana Marsh is commonly known as the American Mountain-ash. It is particularly common in mountainous regions and along the coast (typically in the northeast of North America). The flowers are attractive to bees. The fruit is bright red, berry-like pome, and globular about 6.3 mm in diameter. These remain on the tree late into the winter and are sometimes used as an astringent in medicine.

L. oblongifolia Hook is a shrub that grows up to 1.5 m high with upward pointing branches covered with small hairs and opposite oval leaves 2–5 cm in length. The flowers, borne in pairs, are yellow, two-lipped, and narrow. The fleshy red berries also occur in pairs. Figure 1 shows a map of the geographical distribution and images of both berries.

Plants comprise an important part of the Native American people's diet, and their uses are well documented in ethnobotanical literature. The Ojibwa used the fruits of *S. americana* Marsh as a food source (Muñoz Acuña et al. 2002; Oszmiański et al. 2011).

The aims of this study are to extract, identify, and quantify the phenolic compounds in the berries of these plants, because since these berries are present in great abundance in North America, it makes them good candidates as natural sources of antioxidants and of the extraction of their phenolic compounds to be employed as antioxidant agents by food, pharmaceutical, and cosmetic industries.

Material and Methods

Samples and Chemicals

The wild berries of *S. americana* Marsh and *L. oblongifolia* Hook were hand-harvested in eastern North America during March 2012. Both of them were lyophilized and stored at 4 °C in the dark.

The phenolic compound gallic acid (95 %) was purchased from Sigma-Aldrich (Steinheim, Germany). To prepare the stock standard solution of gallic acid (with concentration of 1000 μg/mL), the appropriate amount of the solid reagent was weighed and dissolved in 10 mL of Milli-Q water. This solution was stored at 4 °C in the dark and it was passed through a 0.22-μm nylon filter before injection into the HPLC system.

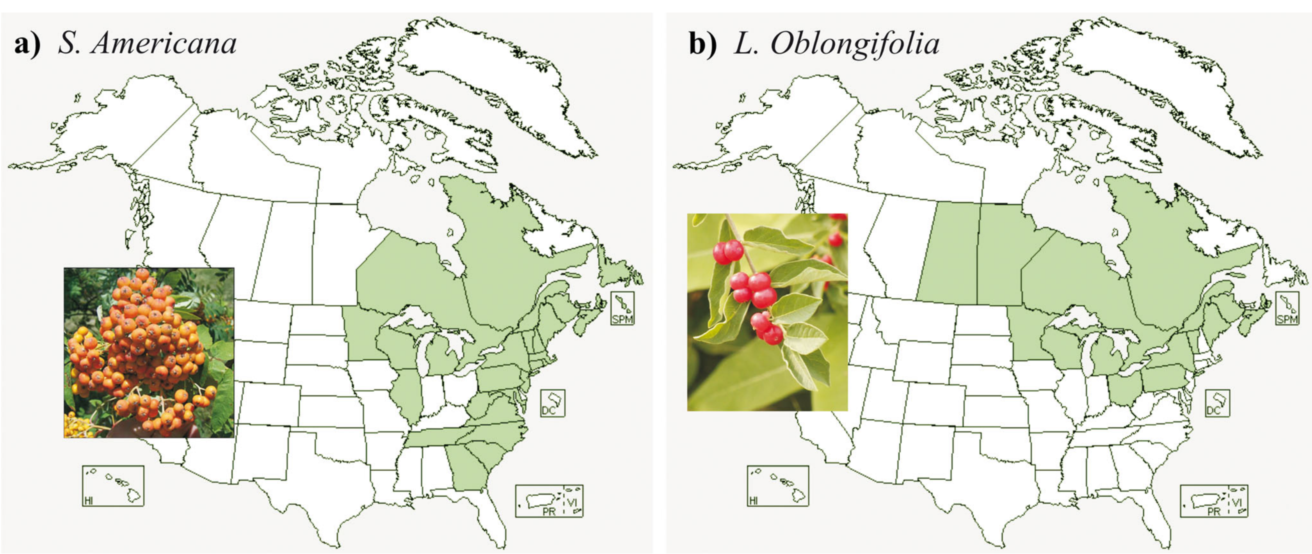

Fig. 1 Maps of the geographical distribution and some images of *Sorbus americana* Marsh (**a**) and *Lonicera oblongifolia* Hook (**b**) berries

Table 1 Spectral data of the phenolic compounds in berries

Phenolic compounds	λ_{max} (nm)	$[M - H]^+$ (*m/z*)	$[MS/MS]^+$ (*m/z*)	Retention time (min)
5-Caffeoylquinic acid	310	355	163.1	11.9
3-Caffeoylquinic acid	310	355	163.1	17.1
Quercetin-3-*O*-galactoside	360	465	303	31.9

HPLC-grade acetonitrile and glacial acetic acid were supplied by J.T. Baker (Deventer, Holland). The ultrapure water was obtained from an in-house Milli-Q® Type I purification system (18.2 MΩ cm and TOC <10 ppb).

Solid-Liquid Microextraction of Phenolic Compounds

The extraction procedure was performed according to the method described in Qi et al. (2009) with slight modification. In order to improve this methodology and reduce reagent consumption, different volumes of methanol were tested to extract the phenolic compounds. The lyophilized berry material (500 mg) was crushed and placed into polyethylene centrifuge tube (50 mL, conical bottom) and extracted with 5, 10, and 20 mL of methanol. After shaking for 40 min using an ultrasonic bath, the sample was centrifuged (Thermo Electron Corp.®, ALC Multipeed Centrifuge PK121) at 8000 rpm for 20 min at room temperature. The upper methanolic phase was collected.

Finally, methanolic phase was filtered through a 0.22-μm pore size and 13-mm-diameter nylon filter and analyzed using an HPLC-DAD-MS.

HPLC-DAD-MS Analysis

The HPLC analysis was performed using an Agilent 1100 Series LC-MSD system with a diode array detector (DAD, model G1315A) coupled to a mass spectrometer (MS, model G1946D) equipped with an electrospray ionization source (ESI).

Chromatographic separation was carried out using a 150× 4.6 mm, 5-μm SS Wakosil C18 column with a 4×3 mm Phenomenex C18 guard cartridge both thermostated at 32 °C. The injection volume was 10 μL. The mobile phase was composed of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) at a flow rate of 1 mL/min. The following gradient was chosen: 0 min, 0 % B; 2 min, 5 % B; 10 min, 10 % B; 30 min, 20 % B; and 50 min, 30 % B. Absorbance spectra were recorded every 2 s, between 250 and 600 nm, with a bandwidth of 4 nm, and chromatograms were acquired at 280, 310, 340, and 360 nm. MS parameters were as follows: capillary voltage 4000 V, fragmentor voltage 160 V, drying gas temperature 350 °C,

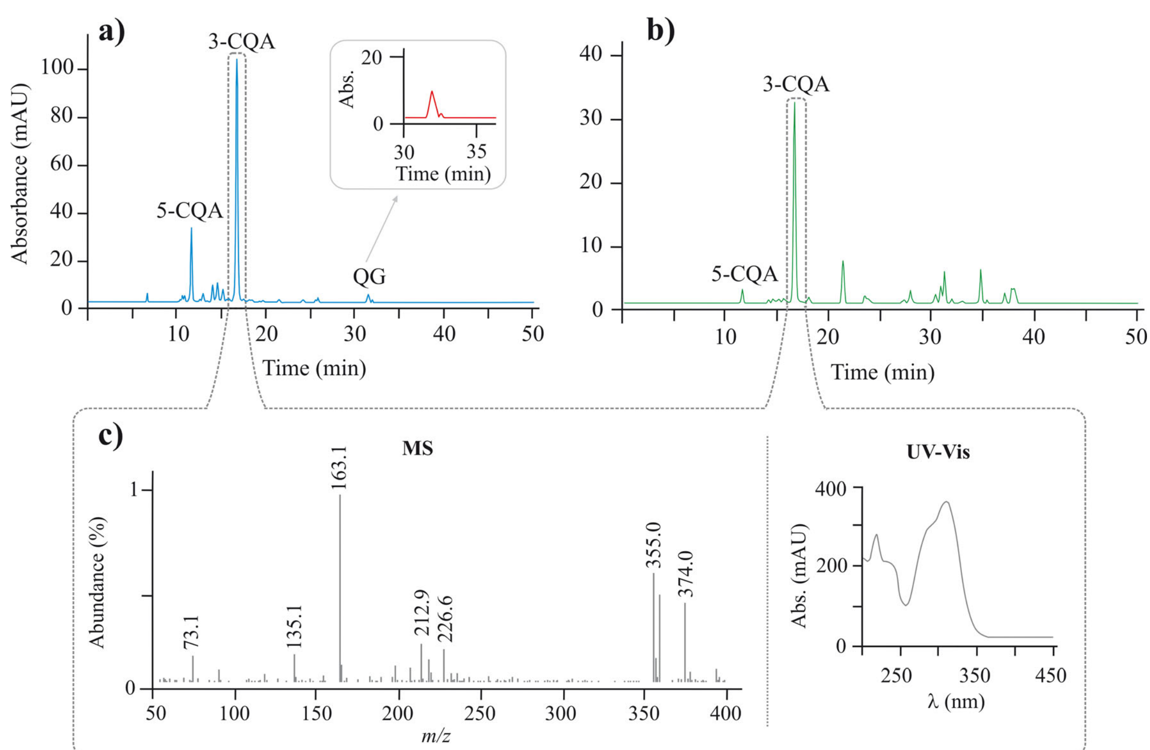

Fig. 2 Chromatograms from the HPLC-DAD-MS analysis of phenolic compounds in *Sorbus americana* Marsh (**a**) and *Lonicera oblongifolia* Hook (**b**). Peak designation is *5-CQA* for 5-caffeoylquinic acid, *3-CQA* for 3-caffeoylquinic acid, and *QG* for quercetin-3-*O*-glucoside. MS and UV-Vis spectra of 3-CQA (**c**) are also shown

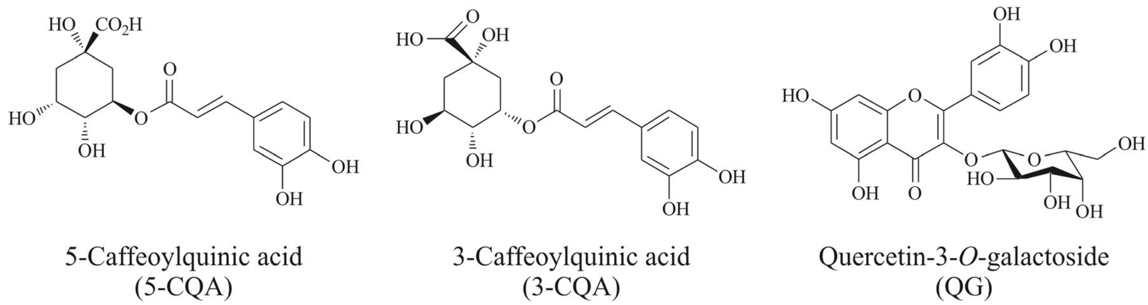

Fig. 3 Phenolic compound (3-caffeoylquinic acid, 5-caffeoylquinic acid, and quercetin-3-*O*-glucoside) structures

gas flow (N_2) rate 10 L/min, and nebulizer pressure 50 psi. The instrument was operated in positive ion mode scanning from *m*/*z* 100 to *m*/*z* 800 at a scan rate of 1.43 s/cycle.

Identification and quantification wavelengths were 310 and 360 nm for chlorogenic acids and quercetin-3-*O*-glucoside, respectively. Taking into account that gallic acid has its maximum absorbance at 280 nm, it was determined at this wavelength. Thus, the external calibration curve was produced by the integration of absorption peaks generated from analysis of a dilution series of gallic acid at 280 nm. Results were expressed as gallic acid equivalent (mg/g)(Olszewska et al. 2012). All analyses were carried out in triplicate and the results were expressed as mean values.

Results and Discussion

Determination of Phenolic Compounds in Berries

The phenolic composition of *S. americana* Marsh and *L. oblongifolia* Hook berries was determined by means of HPLC-DAD-MS analysis. The identification of the phenolic compounds was carried out by comparison of HPLC retention times, elution order, photodiode array UV-Vis spectroscopic and ESI-MS spectrometric data (Table 1) with our phenolic library, and published data (Nuri Seo et al.2012; Oszmiański et al. 2011).

HPLC chromatograms of the phenolic extracts at 310 nm evidenced good resolution, as depicted in Fig. 2. The major phenolic compound corresponding to 3-*O*-caffeoylquinic acid (3-CQA) represented about 75 and 63 % of the total peak area in *S. americana* Marsh (Fig. 2a) and *L. oblongifolia* Hook (Fig. 2b), respectively. Another peak was detected at 310 nm. It was identified as 5-*O*-caffeoylquinic acid (5-CQA) in both berries; besides, quercetin-3-*O*-glucoside (QG) was identified at 360 nm in *S. americana* Marsh. Figure 2c shows MS and UV-Vis spectra of 3-CQA in order to confirm the identification of this phenolic compound. The chemical structures of 3-CQA, 5-CQA and QG are shown in Fig. 3.

With the purpose for working in the most suitable conditions, %RSD values obtained using 5, 10, and 20 mL of methanol were compared (Table 2). The results showed that 5 or 10 mL were the most appropriate volumes exhibiting both %RSD values <12 %. When standard deviations of samples were compared, the achieved values using 5 mL were lowest. Regarding this, and for the sake of reducing the amount of reagents and concentrating the extract, 5 mL was selected. An example of that is the quantification of 5-CQA in *L. oblongifolia*

Table 2 Phenolic compound concentration (mg/g) and %RSD values in berries when different volumes of methanol (5, 10, and 20 mL) were used

Berries	Phenolic compounds	Methanol volume (mL)					
		5		10		20	
		Concentration	%RSD	Concentration	%RSD	Concentration	%RSD
Sorbus americana Marsh	5-CQA	0.662±0.04	6.91	0.714±0.05	7.61	0.905±0.07	8.65
	3-CQA	2.837±0.14	5.19	3.599±0.22	6.17	4.417±0.22	5.20
	QG	0.101±0.01	12.04	0.119±0.01	9.69	0.066±0.01	19.24
	Total	3.599±0.2	5.70	4.432±0.28	6.49	5.388±0.3	5.61
Lonicera oblongifolia Hook	5-CQA	0.79±0.06	8.28	0.88±0.06	9.76	0.654±0.07	9.86
	3-CQA	0.005±0.0001	12.44	n.q.	–	n.q.	–
	Total	0.795±0.06	8.23	0.88±0.06	9.76	0.654±0.07	9.86

n.q not quantified

Hook, which was only possible when 5 mL of methanol was used (Table 2).

Identification and Quantification of Phenolic Compounds in Berries

After separation and optimization of UV-Vis and MS conditions, the quality parameters of the chromatographic method were studied with the purpose of establishing its performance characteristics, assuring the suitable identification and quantification of the studied compounds.

The gallic acid calibration curve was linear throughout the range of study, and the coefficient of correlation (r^2) was higher than 0.999. Method precision expressed in terms of relative standard deviation (%RSD) was assessed for each phenolic compound in real samples. Table 2 shows that acceptable %RSDs were achieved, with values ranging from 5.2 to 12.4, except for quercetin-3-*O*-glucoside. It is important to take into account that the heterogeneity of berry samples makes difficult the extraction process as well as the identification and quantification of their phenolic compounds.

The quantitative results of phenolic compounds in the berries analyzed are shown in Table 2. As can be observed, the concentrations obtained using 5, 10, and 20 mL of methanol showed some differences. Several explanations can be given as follows: the heterogeneity of samples, and the error resulting when large reagent volumes were used.

Comparing the collected results when 5 mL of methanol was used, it is worth noting the main content of 3-CQA in both berry extracts, having concentrations of 2.837 and 0.790 mg/g in *S. americana* Marsh and *L. oblongifolia* Hook, respectively. In addition, the compound 5-CQA was quantified showing a higher concentration (0.662 mg/g) in *S. americana* Marsh, and QG was just determined in *S. americana* Marsh. The obtained results (Table 2) were in accordance with the values shown by similar berries previously studied (Kylli et al. 2010; Kusznierewicz et al. 2012).

Conclusions

This is the first report on the phenolic composition of *S. americana* Marsh and *L. oblongifolia* Hook berries. Furthermore, according to the literature, this study opens the discussion to investigate the phenolic compounds present in leaves and flowers of these plants, because they were previously studied in other plants and high concentrations of polyphenols were detected (Nuri Seo et al. 2012; Olszewska et al. 2012). Besides, the fact that two molecular forms of chlorogenic acid are produced (3-CQA and 5-CQA) and the great abundance of these plants in the northeast of North America make *S. americana* Marsh and *L. oblongifolia* Hook berries a new and good source of natural antioxidants, representing an interesting aspect for biotechnological exploitation. A clear example of the aforementioned potential is the manuscript recently published in collaboration with several researchers from University of Salento, Italy (Bloise et al. 2014). In this work, *S. americana* Marsh was used in the environmentally friendly preparation of "green nanocarriers" using natural renewable materials.

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Conflict of Interest Mercedes Becerra-Herrera declares that she has no conflict of interest. Maria Rosaria Lazzoi declares that she has no conflict of interest. Ana Sayago declares that she has no conflict of interest. Rafael Beltrán declares that he has no conflict of interest. Roberta del Sole declares that she has no conflict of interest. Giuseppe Vasapollo declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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