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Analytical Methods

Major flavonoids of Argentinean honeys. Optimisation of the extraction method and analysis of their content in relationship to the geographical source of honeys

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ABSTRACT

In the present research we optimised an extraction procedure for the flavonoid aglycones: myricetin, quercetin and luteolin from honeys (as natural biological matrices), based on Amberlite XAD-4 resin followed by HPLC quantification. In addition, honeys from three geographical regions of Argentina were analysed with regard to the contents of these flavonoids. The extraction procedure was optimised for XAD-4 resin considering: resin/honey ratio, elution volume to desorb flavonoids and colour intensity of honeys. Differences in flavonoid recoveries were observed depending on the colour intensity. The flavonoid aglycones contents, in accordance with differences in geochemical characteristics and typical vegetation, varied with the geographical origin of honeys. The results obtained allowed us to consider these three flavonoids as chemical markers for the phytogeographical origin of honeys. In the case of monofloral honeys, the contribution of each one of the flavonoids was associated with the presence of a dominant pollen kind in these samples.

1. Introduction

Current tendency of consuming natural products with disease prevention properties beyond their nutritional roles (functional foods) is increasing the interest on honey. Argentinean honeys are well considered products in the international apicultural trade due to both their characteristic organoleptic properties and recognised quality. Around 92% of total honey production in Argentina has been exported in 2004. Nowadays, traceability systems applied to apicultural products allow producing honey of quality according to the most exigent requirements of importer countries such as Germany, United States, Japan, United Kingdom and Italy. In the last years, exportations of honey towards these countries approached a mean of 350,000 tons/year, with more than 20% coming from our country, occupying the second place as exporter country (Sabio & de los Santos, 2005).

Traditionally, honey has been considered to have therapeutic properties since ancient times (Molan, 1992). At present, great amount of research is focused on its antimicrobial activity (Allen, Molan, & Reid, 1991a, b; Alnaqdy, Al-Jabri, Al Mahrooqi, Nzeako, & Nsanze, 2005; Molan, 1992). It has been well established that the factors responsible for such activity are pH, sugar content, hydrogen peroxide levels and the presence of some phytochemicals, mainly phenolic compounds including phenolic acids and flavonoids (Weston, 2000). Individual or synergistic action of these factors has been proposed to play a role in the antimicrobial activity of honeys. It has been seen that the presence of flavonoids may contribute to antioxidant effects observed in some honeys (Aljadi & Kamaruddin, 2004; Küçük et al., 2007). Some studies have shown that flavonoids have the properties of inhibiting autoxidation reactions and the scavenging effect of free radicals by different mechanisms involving its phenolic structure (Burda & Oleszek, 2001; Cos et al., 1998).

Normally, the identification of honeys according to its botanical origin is performed through microscopical analysis of pollen. In recent years, the use of flavonoids as naturally occurring markers in foods has proved to be a useful complementary technique for this purpose (Crozier, Lean, Mc Donald, & Black, 1997; Gambelli & Santaroni, 2004; Tsanova-Savova & Ribarova, 2002). In this way, knowledge of the flavonoid profile would represent a valuable tool for a better characterisation of honey according to its botanical and/or geographical origin. In addition, the identification of bioactive compounds such as flavonoids would allow evaluating honey as a functional food.

Plant phenolics include phenol compounds, phenolic acids, coumarins, flavonoids, tannins, lignins, etc. Regarding the extraction methods used for analysing flavonoids compounds in honey, a liquid–liquid extraction using different solvents has been extensively used (Robards, 2003). The utilisation of non-ionic resins has the advantage of eliminating numerous polar and ionic interfering substances such as sugars and acids. Amberlite XAD type resins are polymeric adsorbents which have proved to be the best

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materials for extracting honey flavonoids with yields higher than 80% (Guyot- Declerck, Chevance, Lermusieau, & Collin, 2000). Amongst these resins, Amberlite XAD-2 has been the most widely used for this purpose. In the present work, we propose the use of Amberlite XAD-4 as a resin with a higher surface area compared with XAD-2 type and so a higher efficiency.

Furthermore, the procedure used for isolation of flavonoid compounds from honey matrix includes homogenisation with water acidified at a pH value that guarantees the hydrolysis of glycosylated forms. Aglycones are less polar compounds compared to the corresponding glycosylated species, and so, they result in more effective binding to a XAD type resin. In addition, the analysis of free flavonoids by the chromatography is more achievable due to availability of commercial standards (Robards, 2003).

It is interesting to mention that the study of the flavonoid aglycones proposed in this work represents a contribution to the knowledge of major flavonoids in Argentinean honeys. However, the study of these compounds has an additional significance because flavonoid aglycones might be potentially more active than its glycosylated forms in relation to its biological properties such as its antioxidant action.

The aim of the present work was to develop an efficient technique for the separation of flavonoid aglycones from honeys using an extraction system based on Amberlite XAD-4 resin followed by quantification by high performance liquid chromatography (HPLC). In addition, the characterisation of honeys that came from different regions of Argentina was made, in order to know its major flavonoid content and relate it to their floral origin. Studies made in other countries have focused on the chemical identification of phenolic compounds and flavonoids in honeys that came from other regions (Amiot, Aubert, Gonnet, & Tacchini, 1989; Gheldof, Wang, & Engeseth, 2002; Martos, Cossentini, Ferreres, & Tomás-Barberán, 1997; Martos, Ferreres, & Tomás-Barberán, 2000; Sabatier, Amiot, Tacchini, & Aubert, 1992; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001; Yao et al., 2004). However, to the best of our knowledge no data is available concerning the flavonoid contents in Argentinean honeys. Thus, taking into account both the results obtained in this research and the diversity of our honeys, this paper could be considered as provenance to study the composition of the major flavonoids such as myricetin, quercetin and luteolin in regional honeys.

2. Material and methods

2.1. Standards

Standards of flavonoids aglycones used were: quercetin dehydrate (minimum 98%) and luteolin (99%) from Sigma (USA) and myricetin ($\geqslant$95%) from Fluka (Switzerland). Standard stock solutions of each one was prepared with HPLC grade methanol at a concentration of 1 mg/ml and was conserved at –20 °C protected from light.

2.2. Honey samples

The honey samples used to evaluate the efficiency of the method proposed in this work were selected in relation to their phenolic compound contents determined according to the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Thus, considering the intention to work with a natural biological matrix, honey samples with the minor polyphenolic content (data not shown) were used as *control samples*. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100 g of honey.

In order to investigate the content of the major flavonoids: myricetin, quercetin and luteolin, the samples used in this study corresponded to honeys from different regions of Argentina that were harvested during the 2005–2006 flowering seasons (Table 1). Samples of each honey were extracted from comb by natural pouring (mature honey) and kept at −20 °C until its use. Honeys were clustered as light coloured honeys or dark coloured honeys reflecting in part the content of pigments such as carotenoids and flavonoids (Taormina, Niemira, & Beuchat, 2001).

2.3. Pollen analysis

The identity of honey samples was based on pollen analysis following the method suggested by Loveaux, Maurizio, and Vorwhol (1978). According to their frequency classes, pollen types were classified as: dominant pollen (D), >45%; secondary pollen (S), 45–16%; pollen of minor importance, 15–3%; pollen traces (T), <3%. Monofloral honeys were considered as such whenever the dominant pollen was found at over 45% of the total pollen. Monofloral honeys of *Lotus* sp. and *Eucalyptus* sp. (types under and over-represented in the total pollen content, respectively) were considered to be: (a) monofloral of *Lotus* sp. when the pollen of that plant was present at least in 20%, (b) monofloral of *Eucalyptus* sp. when the pollen content of these species was equal or higher than 70% (Sabatino, Iurlina, Eguaras, & Fritz, 2006).

2.4. Extraction method of flavonoids

2.4.1. Preparation of the resin

Amberlite XAD-4 was used as a adsorption resin for extracting honey flavonoids. XAD-4 resin 20–60 mesh, mean pore size 40 Å were supplied by Supelco (Bellefonte, USA). New resin was soaked in methanol for 15 min before use. Then, the solvent was decanted from the resin and replaced with distilled water. Thus, the mixture was stirred and allowed to stand for 10 min. The resin slurry was packed into a glass column (1.9 id × 30 cm) to give a bed height of 1.9 id × 13 cm. After that, the column was backwashed and allowed to settle before use.

2.4.2. Extraction method optimisation

Optimisation parameters for extraction were resin/honey (w/w) ratio (1:1, 1:2, 1:5), elution volume (150, 250 and 350 ml) and the colour intensity of honey. Analyses were performed in triplicate for each set of conditions tested. Flavonoids recovery was determined by spiking aqueous extracts of honeys with four concentration levels (10, 100, 500 and 1000 ppm) of each flavonoid investigated, and measuring losses during the extraction procedure by HPLC analyses.

2.4.3. Sample extraction procedure

The extraction method optimised was used on the honeys collected from different origins. Each sample was diluted with five parts of water acidified with HCl, pH 2. The homogenate was then filtered through a polyethylene filter, mesh 200 and was then put onto the Amberlite column (1.9 id × 13 cm). Polar compounds, like carbohydrates, were eluted with water acidified with HCl, pH 2, and subsequently with distilled water until no more sugars were detected (Mollisch́s Test). Hydrophobic compounds, like flavonoids, remained attached to the resin surface and were eluted with methanol. The volume of eluent was considered as optimum when UV absorbance at 340 nm was not detected in the eluate. The methanolic extract was rotary evaporated at 40 °C and the residue was redissolved with 5 ml of water. Three extractions with 5 ml of ethyl ether each were made and the organic pool was evaporated under a N_2 stream. The residue containing mainly phenolic compounds was resuspended in 1 ml methanol and membrane filtered

Table 1
Argentinean honey samples used in this study on the basis of date of harvest, geographical origin and colour intensity.

Sample code	Date of harvest	Place of production	Geographical origin	Colour intensity of honey
Control honey	2005	Buenos Aires province (37° 57′ S–57° 46′ W)	Sierras	Light coloured
M1	2006	Sierra de Los Padres, Buenos Aires province (37° 57′ S–57° 46′ W)	Sierras	Light coloured
M2	2005	Miramar, Buenos Aires province (38° 15′S–57° 50′W)	Plain	Light coloured
M3	2005	Coronel Vidal, Buenos Aires province (37° 27′ S–57° 44′ W)	Field	Light coloured
M4	2005	Sierra de Los Padres, Buenos Aires province (37° 57′ S–57° 46′ W)	Field	Light coloured
M5	2006	Miramar, Buenos Aires province (38° 15′S–57° 50′W)	Plain	Light coloured
M6	2005	Coronel Vidal, Buenos Aires province (37° 27′ S–57° 44′ W)	Field	Light coloured
M7	2005	Estacion Chapadmalal, Buenos Aires province (38° 01′ S–57° 43′ O)	Field	Light coloured
Control honey	2005	Santiago del Estero province (27° 46′ S–64° 16′ W)	Monte	Dark coloured
M8	2005	Mendoza province (32° 52′ S–68° 49′ W)	Monte desert	Dark coloured
MD9	2006	Mendoza province (32° 52′ S–68° 49′ W)	Monte desert	Dark coloured
MD10	2006	Mendoza province (32° 52′ S–68° 49′ W)	Monte desert	Dark coloured
MB11	2005	Santiago del Estero province (27° 46′ S–64° 16′ W)	Monte-Woodland	Dark coloured
MB12	2005	Santiago del Estero province (27° 46′ S–64° 16′ W)	Monte-Woodland	Dark coloured
MB13	2006	Santiago del Estero province (27° 46′ S–64° 16′ W)	Monte-Woodland	Dark coloured
M14	2005	El Sauzalito, Chaco province (25° 57′ S–60° 37′ W)	Monte	Dark coloured
MC15	2005	Chaco province (25° 57′ S–60° 37′ W)	Monte	Dark coloured

Table 2
Characterisation of the honey samples studied according to the botanical source.

		Predominant pollen	
Sample code	Botanical source	DP (>45%)	SP (45–16%)
M1	Mixed	Myrtaceae *Eucalyptus* sp.	Fabaceae *Lotus* sp. and *Trifolium* sp.
M2	Mixed	Myrtaceae *Eucalyptus* sp.	Fabaceae *Melilotus* sp.
M3	Mixed		Myrtaceae *Eucalyptus* spp.; Fabaceae *Lotus* sp. and *Melilotus* sp.
M4	Monofloral	Fabaceae *Lotus* sp., Myrtaceae *Eucalyptus* sp.	–
M5	Monofloral	Fabaceae *Lotus* sp., Myrtaceae *Eucalyptus* sp.	–
M6	Monofloral	Myrtaceae *Eucalyptus* sp.	Fabaceae *Lotus* sp.
M7	Monofloral	Myrtaceae *Eucalyptus* sp.	Fabaceae *Lotus* sp.
M8	Mixed	–	Vitaceae *Vitis* sp.; Leguminosae *Prosopis* sp.
M9	Mixed	–	Vitaceae *Vitis* sp.; Leguminosae *Prosopis* sp.
M10	Mixed	–	Vitaceae *Vitis* sp.; Leguminosae *Prosopis* sp.
M11	Mixed	–	Leguminosae *Prosopis* sp., Anacardiaceae *Schinus* sp. and *Schinopsis* sp.
M12	Mixed	–	Leguminosae *Prosopis* sp.; Anacardiaceae *Schinopsis* sp.
M13	Mixed	–	Leguminosae *Prosopis* sp.; Anacardiaceae *Schinopsis* sp.
M14	Mixed	–	Anacardiaceae *Schinopsis balansae* and *lorenzii*; Compositae *Tessaria* sp.
M15	Mixed	–	Anacardiaceae *Schinopsis balansae* and *lorenzii*; Compositae *Tessaria* sp.

DP, dominant pollen (>45%); SP, secondary pollen (45–16%).

Table 3
Influence of the resin/honey (w/w) ratio and elution volume on flavonoids recovery (%) from control light coloured honey.

Spiking level of flavonoid (ppm)	Retention index (min)	Elution volume (ml)								
		150 Resin/honey ratio (w/w)			250 Resin/honey ratio (w/w)			350 Resin/honey ratio (w/w)		
		1:1	1:2	1:3	1:1	1:2	1:3	1:1	1:2	1:3
Myricetin	13									
10		85.67	78.69	75.71	88.45	92.68	92.17	93.11	93.27	89.74
100		88.32	84.21	81.32	90.53	93.14	95.22	94.51	93.72	92.34
500		85.91	83	82.11	93.58	94.58	92.83	93.15	92.82	96.74
1000		92.14	84.47	75.64	90.61	95.31	94.53	94.21	93.00	94.52
Quercetin	23									
10		90.46	81.55	82.44	92.45	93.36	90.14	92.14	92.45	91.78
100		92.11	86.27	84.44	92.78	92.17	94.33	90.72	96.34	93.66
500		89.84	85.41	86.30	96.22	94.51	85.64	94.62	93.51	92.55
1000		86.77	89.68	84.70	96.90	96.44	90.16	95.61	93.00	94.61
Luteolin	31									
10		93.12	86.49	79.68	89.21	91.23	93.12	92.31	91.47	85.46
100		90.46	89.98	86.32	93.30	94.44	94.15	91.02	94.49	91.23
500		92.22	87.29	84.78	94.10	93.81	95.13	94.23	94.01	93.64
1000		94.52	90.21	87.63	93.71	94.50	93.21	94.00	95.10	96.15

(0.2 μm) for HPLC analysis. Extracts were conserved at –20 °C and protected from light.

2.5. Analysis of flavonoids

A KONIK KNK-500-A-Series Analytical HPLC system (Spain) equiped with a UVIS 200 UV-V detector was used. The column was a LiChroCART® 250-4 LiChrospher® 100 RP-18 endcapped, 5 μm (Merck, Germany) kept at 20 °C. Elution was made with a flow rate of 1.0 ml/min using a gradient of methanol (solvent A) and water: formic acid, 19:1 v/v (solvent B) as follow: isocratic elution 40% A, 0–15 min; linear gradient from 40% to 50% A, 15–20 min; linear gradient from 50% to 55% A, 20–30 min; linear gradient from 55% to 70% A, 30–50 min; linear gradient from 70% to

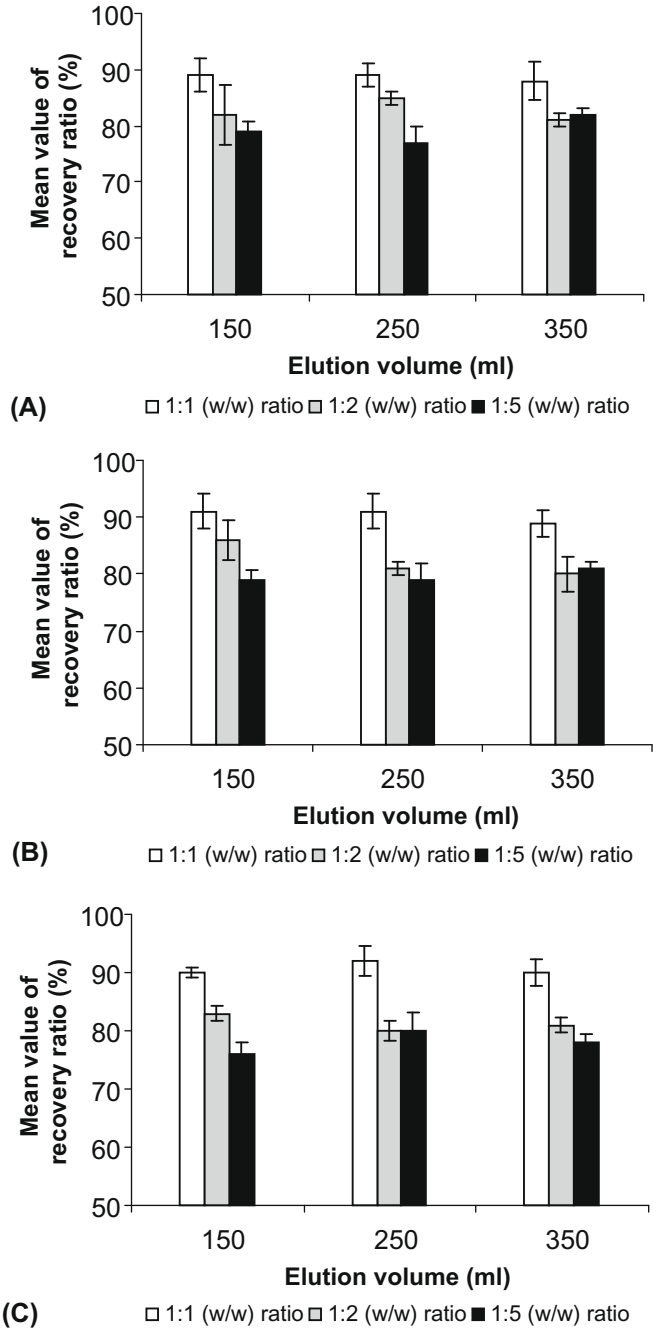

Fig. 1. Mean value of recovery ratio (%) for myricetin (A), quercetina (B) and luteolin (C) for light coloured honeys.

90% A, 50–52 min and finally isocratic elution 90% A during an additional 8 min. Injection volume was 5 μl and the chromatogram was registered at 340 nm. Identification of chromatographic peaks was based on retention times by comparison with known standards.

2.6. Quantification of flavonoids

Myricetin, quercetin and luteolin were quantified using the external standard method based on peak area. Analyses were made by plotting a calibration curve. To make the calibration curve of each flavonoid, from each stock solution appropriate volumes were diluted with methanol to obtain working solutions in the concentration range of 0.01 to 0.20 μg/μl that were correlated with the measured area. The area of these peaks was plotted and the corresponding concentration of flavonoids was calculated based on the calibration curve. For each sample, the quantitative analyses were performed in triplicate. Results were statistically compared based on an ANOVA test ($p = 0.05$) using the Microcal Origin 5.0 software program.

3. Results and discussion

3.1. Pollen analysis

Table 2 shows the identity of the honeys studied according to the botanical source and predominant pollen found, being identified as monofloral and mixed honeys. This classification considers a honey as mixed when it contains either two or three species as secondary pollen (Sabatino et al., 2006). In particular, when *Eucalyptus* sp. pollen was higher than 45% (dominant pollen) but lower than 70%, and other pollen type was present as secondary pollen class (45–16%) honeys were also classified as mixed.

Light coloured mixed honeys (M1, M2 and M3) obtained from the Buenos Aires province, had an important contribution of *Eucalyptus* sp. pollen with a mean incidence of 33–62%. In addition, samples also showed in general a pollen contribution from *Lotus* sp., *Melilotus* sp. and *Trifolium* sp. as secondary pollen. On the other hand, for M1 honey the accompanying pollen types (pollen traces, 3–1%) found was from Asteraceae (*Heliantheae* sp., *Carduus* sp., *Centaurea* sp.) and Lamiaceae (*Mentha* sp.). In M2 honey sample, the minor pollen was represented by Fabaceae (*Lotus* sp.), whilst pollen traces came from Asteraceae (*Helianthus* sp., *Carduus* sp., *Asterae* and *Anthemis* sp.), Brassicaceae (*Brassica* sp.). The M3 honey showed minor pollen contribution from Fabaceae (*Medicago sativa*, *Trifolium* sp.). Asteraceae (*Helianthus* sp., and *Taraxacum* sp.), Lamiaceae (*Lavandula* sp., *Thymus* sp.) and Brassicaceae (*Raphanus*) were identified in the pollen traces class.

Monofloral honeys corresponded to *Lotus* sp. (M4 and M5) and *Eucalyptus* sp. (M6 and M7). In *Lotus* sp. honeys, the dominant species were *Lotus* sp (41–44%) and *Eucalyptus* sp. (52–54%). Monofloral honeys of *Eucalyptus* sp. contained 78–82% of *Eucalyptus* sp. pollen. Among *Lotus* sp. honeys the minor pollen class belonged to Asteraceae (*Helianthus* sp.). In addition, pollen traces corresponding to Asteraceae (*Carduus* sp., *Centaura* sp.), Lamiaceae (*Mentha* sp., *Thymus* sp.) and Boraginaceae (*Echium* sp.) were identified. In *Eucalyptus* sp. honeys, minor pollen included Fabaceae (*Lotus* sp., *Melilotus* sp., *Trifolium* sp., *Robinia pseudoacacia),* Asteraceae (*Heliantheae*).

For mixed honeys from the Mendoza province (M8, M9 and M10), the minor pollen was represented by Zygophyllaceae (*Larrea* sp.) and Compositae (*Baccharis* sp.) with Rosaceae (*Prunus* sp.) present as pollen traces. The mixed honeys that came from Santiago del Estero (M11, M12 and M13) included pollen types belonging to Anacardiaceae (*Schinus* sp.) and Zygophyllaceae (*Larrea* sp.) as minor pollen, whilst Leguminosae (*Geoffroea* sp.) was present as pollen traces. In general, both M14 and M15 samples were characterised by Leguminosae (*Prosopis* sp.) as minor pollen, whilst, the pollen traces types found were Compositae (*Baccharis* sp.), Anacardiaceae (*Schinus* sp.).

3.2. Optimisation of the flavonoid extraction method

3.2.1. Light coloured honey

The evaluation of the resin/honey (w/w) ratio was made with 30 g of resin that was washed successively with distilled water and methanol. Thus, the assays were performed using 30, 60 and 150 g of the honey identified as a *control sample*. Table 3 shows the flavonoid recovery (expressed as a percentage) for each condition evaluated. For each resin/honey (w/w) ratio we checked that

Table 4
Influence of the resin/honey (w/w) ratio and elution volume on flavonoids recovery (%) from control dark coloured honey.

Spiking level of flavonoid (ppm)	Retention index (min)	Elution volume (ml)								
		150 Resin/honey ratio (w/w)			250 Resin/honey ratio (w/w)			350 Resin/honey ratio (w/w)		
		1:1	1:2	1:3	1:1	1:2	1:3	1:1	1:2	1:3
Myricetin	13									
10		84.31	86.00	76.81	88.21	86.74	73.45	84.12	79.63	82.49
100		92.15	76.36	81.23	86.40	83.70	75.68	86.44	81.47	82.00
500		90.55	89.12	80.23	91.45	85.12	78.00	91.00	82.45	83.71
1000		89.36	78.39	78.36	90.56	84.11	81.24	92.33	79.44	80.14
Quercetin	23									
10		82.14	83.45	78.25	86.60	81.23	75.68	85.12	75.67	79.36
100		87.46	90.14	76.35	89.69	82.44	83.45	91.23	78.42	81.25
500		92.04	87.77	79.56	91.55	79.36	80.24	90.11	81.77	82.44
1000		95.00	81.56	81.36	95.00	82.00	78.35	89.47	83.70	80.41
Luteolin	31									
10		90.47	82.56	76.33	90.14	78.60	74.67	93.33	83.07	76.23
100		88.46	84.98	79.27	89.66	78.22	82.11	87.45	81.40	78.14
500		89.22	83.00	75.36	91.12	82.47	82.70	89.03	79.46	80.16
1000		90.66	81.23	73.66	93.36	81.33	79.00	90.44	80.00	79.00

both the washed solution (distilled water) and the elution volume (methanol) were not limiting on the extraction procedure of flavonoids.

The volume of the washed solution that permitted the total removal of sugars for 30, 60 and 150 g of the *control honey* was: 80, 120 and 300 ml of water acidified (pH 2), and 200, 300 and 640 ml of distilled water (pH 7), respectively. On the other hand, the volume necessary for the complete desorption of flavonoids was shown to be dependent on the quantity of sample assessed (Table 3).

Fig. 1 shows that for the control light coloured honey, the recovery values of myricetin, quercetin and luteolin did not show significant differences when 250 and 350 ml of methanol was used as a desorption solvent. The range of recovery for 250 ml of eluent solvent was found to be between 90% and 94%, whilst for 350 ml of eluent varied between 91% and 93%. On the other hand, a significant decrease (78–94%) on the recovery range was measured with 150 ml of eluent. Thus, the recovery of flavonoids with 250 ml of methanol using XAD-4 resin was similar to the recovery percentages obtained from XAD-2 resin for honeys of different floral sources (Yao et al., 2003).

The results obtained for the evaluation of the resin-honey (w/w) ratio did not show significant differences in the recovery values with 1:1, 1:2 and 1:5 ratios for 250 and 350 ml of the eluent used. For 150 ml of methanol, a decrease on the flavonoids recovery was observed as the amount of honey applied increased. Thus, the results show that this volume of methanol was not enough to remove the flavonoids present when the matrix was more saturated.

3.2.2. Dark coloured honey

Table 4 depicts the optimisation of the resin/honey (w/w) ratio for *control dark coloured honey* using the same experimental conditions than that considered for light honeys. For the total removal of sugars, the acidified water used was 100, 180 and 370 ml for 30, 60 and 150 g of the honey sample, respectively. Whilst the distilled water used was 200, 380 and 700 ml for 30, 60 and 150 g of the honey, respectively.

In most cases, maximal recoveries were obtained with the 1:1 resin/honey (w/w) ratio (Fig. 2). In addition, for this ratio we checked that the minimum elution volume was not limiting on the flavonoid recovery.

For this *control honey*, the recovery percentages of each one of the flavonoids studied were not influenced by increasing volumes of methanol used as eluent solvent.

For 250 ml of methanol, extraction yields of 91% and 77% for 1:1 and 1:5 resin-honey (w/w) ratios were obtained, respectively.

In relation to the efficiency of the Amberlite XAD-4 resin, an increase in the resin-honey (w/w) ratio assessed produces a saturation of the active reticules of the resin (macroreticular structure) that avoided the adsorption of the flavonoid compounds that would be eluted with acidified water in the washing step of the column. In addition, an increase in the honey quantity meant an increase in the content of sugar, pigments and other substances diminishing the adsorption capacity of the resin. Thus, for dark honeys a 1:1 resin-honey (w/w) ratio would be a more appropriate ratio for an efficient extraction of phenolic compounds and flavonoids.

The HPLC chromatograms of both the light coloured honey and the dark coloured honey (used as *control honeys*) are showed in Fig. 3. These chromatograms show the presence of myricetin, quercetin and luteolin in honey samples that were previously spiked with the standards for the recovery assays.

3.3. Flavonoid contents of honeys

The contents of the main flavonoids in honeys that came from different regions of Argentina have been studied, being the samples gathered in relation to their geographic origin and floral source. Table 5 shows myricetin, quercetin and luteolin contents of mixed and monofloral Argentinean honeys. These compounds were present in all samples studied. In general, the predominant flavonoid was quercetin representing around 45% of the total flavonoid content in both the light and dark honeys. For honeys analysed in this study (mainly monofloral honeys), there is no available data for comparing the results obtained. However, considering values reported elsewhere for flavonoid contents of different floral species honeys, levels similar or higher than 0.20 mg/100 g honey seemed to be significant for defining a flavonoid profile characteristic of a geographical region (Yao et al., 2003).

3.3.1. Honey samples obtained from the middle-east region of Argentina

The honeys from the middle-east region of Argentina arising from the southeast zone of the Buenos Aires province corresponded, in all cases, to light coloured honeys regardless of their mixed or monofloral origin. The province is situated in the zone called 'the wet Pampa' that is an extended plain tempered region with rain levels between 500 and 1000 mm/year. Clover, lotus,

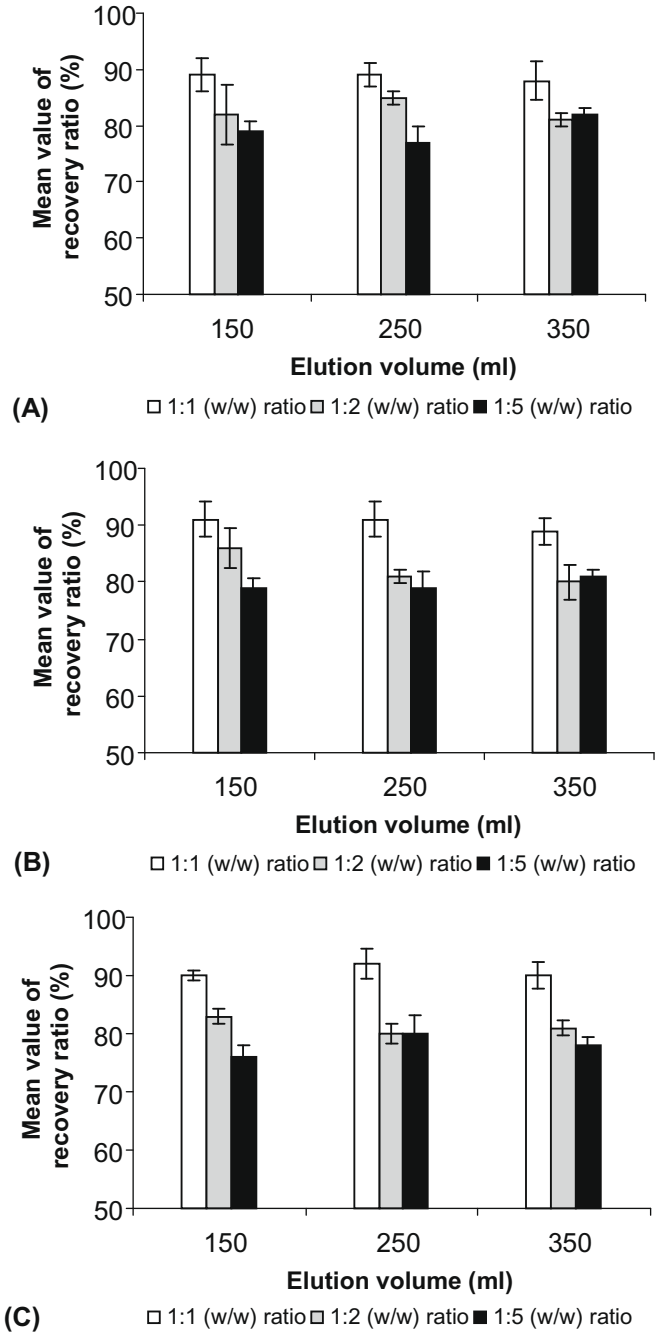

Fig. 2. Mean value of recovery ratio (%) for myricetin (A), quercetina (B) and luteolin (C) for dark coloured honeys.

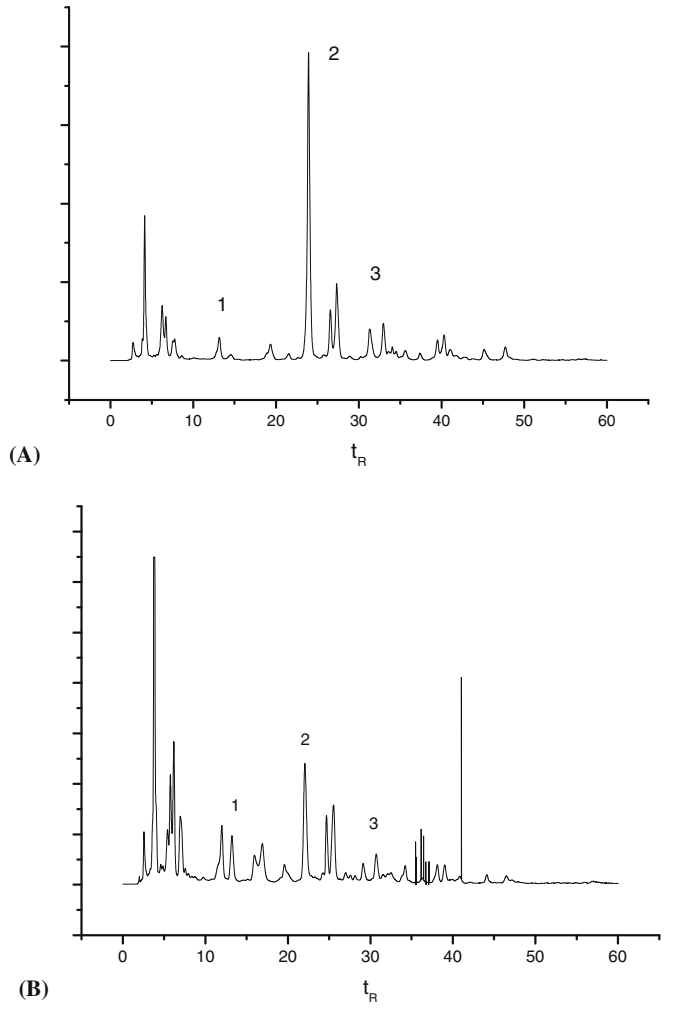

Fig. 3. HPLC chromatograms of flavonoids aglycones in light coloured honey (A) and dark coloured honey (B) recorded at 340 nm. Peak: myricetin (1), quercetin (2) and luteolin (3).

eucalypts, sunflower and thistle are part of the vegetation of apicultural interest (Cabrera & Argentina, 1976), with their pollen commonly represented in honeys that shows the importance of these plants in this phytogeographical region (Malacalza, Caccavari, Fagúndez, & Lupano, 2000; Sabatino et al., 2006).

Eucalyptus sp. and *Lotus* sp. monofloral honeys showed differences in total flavonoid contents with average levels of 1.64 and 0.81 mg/100 g of honey, respectively. In mixed honeys, the content of total flavonoids was an average 0.89 mg/100 g of honey, which was slightly higher than *Lotus* sp. honeys (Table 5).

Moreover, differences in levels of analysed flavonoids between mixed and *Eucalyptus* sp. honeys were greater than these observed between mixed and *Lotus* sp. ones.

For our monofloral *Eucalyptus* sp. honeys quercetin mean content (1.02 mg/100 g honey, Table 5) was significantly higher compared with Australian *Eucalyptus* sp. honey which recorded values between 0.19 and 1.30 mg/100 g honey with a mean value of 0.66 mg/100 g honey. Moreover, the quercetin contents in our *Eucalyptus* sp. honeys were higher than some values reported for European *Eucalyptus* sp. honeys that varied between 0.07 and 0.48 mg/100 g honey (Tomás-Barberán et al., 2001).

3.3.2. Honey samples obtained from the centre-west region of Argentina

The samples from the centre-west region of Argentina arising from the Mendoza province showed the highest contents of total flavonoids with an average value of 1.50 mg/100 g of honey (Table 5). In addition, it is worth mentioning that the values of myricetin for this group of honeys were the highest compared to other honey samples, ranging between 32% and 53%.

It is well known that climatic and geographical characteristics such as temperature, humidity and geochemical characteristics have influences on the predominant type of vegetation in a region. The Mendoza province is located at the centre-west region of Argentina, having a arid/semi-arid climate, with rain levels between 500 and 1000 mm/year, and temperatures that oscillate from 23 °C in summer to 5 °C in winter in the valley and monte

Table 5
Flavonoid contents of mixed and monofloral honeys gathered according to their geographic origin and floral sources.

Honey sample	Content of flavonoids (mg/100 g of honey)			
	Myricetin	Quercetin	Luteolin	Total
M1[a]	0.16	0.47	0.23	0.86
M2[a]	0.22	0.55	0.16	0.93
M3[a]	0.24	0.39	0.24	0.87
Mean ± Ds	0.21 ± 0.03	0.47 ± 0.07	0.21 ± 0.04	
%	23	52	23	
M4[a]	0.19	0.34	0.14	0.67
M5[a]	0.32	0.42	0.23	0.97
Mean ± Ds	0.25 ± 0.02	0.38 ± 0.04	0.18 ± 0.04	
%	30	47	22	
M6[a]	0.27	0.83	0.61	1.71
M7[a]	0.12	1.19	0.25	1.56
Mean ± Ds	0.19 ± 0.03	1.02 ± 0.10	0.43 ± 0.04	
%	23	45	31	
M8[b]	0.55	0.67	0.24	1.46
M9[b]	0.64	0.58	0.27	1.49
M10[b]	0.54	0.68	0.33	1.55
Mean ± Ds	0.58 ± 0.04	0.64 ± 0.04	0.28 ± 0.04	
%	39	43	19	
M11[c]	0.19	0.56	0.30	1.05
M12[c]	0.23	0.64	0.34	1.21
M13[c]	0.25	0.59	0.28	1.12
Mean ± Ds	0.22 ± 0.02	0.60 ± 0.03	0.31 ± 0.02	
%	19	53	27	
M14[d]	0.31	0.29	0.25	0.85
M15[d]	0.27	0.26	0.22	0.75
Mean ± Ds	0.29 ± 0.10	0.27 ± 0.08	0.23 ± 0.09	
%	37	34	29	

[a] Honey samples arise from the middle-east of Argentina (Buenos Aires province).
[b] Honey samples arise from the centre-west of Argentina (Mendoza province).
[c] Honey samples arise from the north of Argentina (Santiago del Estero province).
[d] Honey samples arise from the north of Argentina (Chaco province).

zones. Some of the predominant vegetation includes Zygophyllaceae (*Larrea* sp.), Compositae (*Baccharis* sp., *Cicorium intybus*, *Taraxacum officinale*, *Tessaria dodonaefolia*), Leguminosae (*Geoffroea decorticans*, *Prosopis* sp., *Melilotus* sp.), Anacardiaceae (*Schinus* sp.), Salicaceae (*Populus* sp., *Salix* sp.) and Rosaceae (*Pyrus communis*, *Cydonia oblonga*, *Prunus domestica*, *Prunus dulcis*).

Regions characterised by sunny and dry climates have showed to provide good conditions for synthesis and accumulation of flavonols in other products of vegetal origin such as red wines.

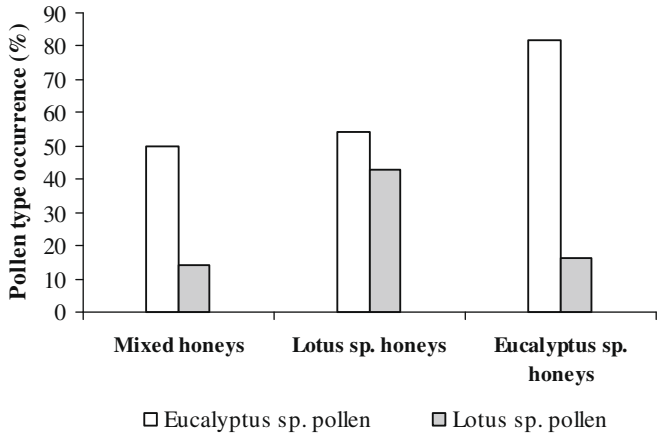

Fig. 4. Pollen type occurrence (%) for mixed and monofloral honeys.

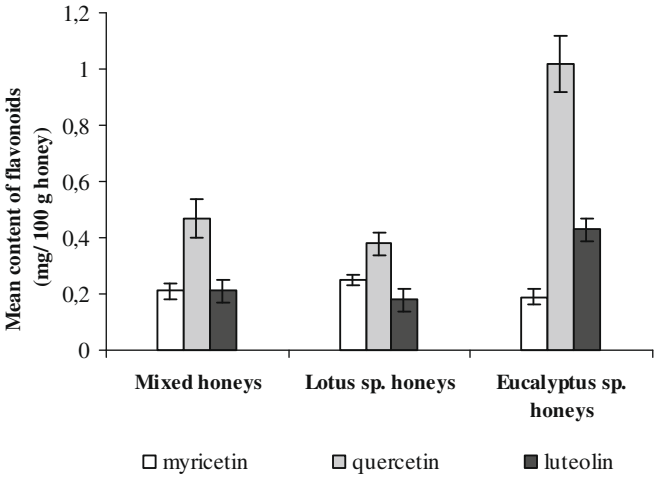

Fig. 5. Mean value for flavonoid contents in mixed and monofloral honeys.

Thus, environmental conditions affect the wine maturation and consequently the concentration of phenolic compounds came from different regions (Tsanova-Savova & Ribarova, 2002). Therefore, the contents of quercetin and mainly of myricetin could be considered as markers for honeys of the centre-west region of Argentina.

3.3.3. Honey samples obtained from the north region of Argentina

Among dark coloured honeys, samples from Santiago del Estero showed an average of flavonoids lower (1.13 mg/100 g of honey) than honeys belonging to centre-west region of the country (Table 5). On the other hand, the former samples had significant content of quercetin which accounted for around 53%. The Santiago del Estero province belongs to the phytogeographical region named "Parque Chaqueño seco", being located at the north of the country. The dry and warm climate provides conditions for the presence of plants and trees of apiculture interest such as the following families: Anacardiaceae (*Schinopsis*); Apocinaceae (Aspidosperma quebracho blanco Schltdl.); Bignoniáceas; Cactaceae (*Ficus* sp., *Optunia* sp.); Leguminosae (*Cercidium* sp., *Prosopis* sp., *Caesalpinia* sp., *Medicago* sp., *Melilotus* sp.); Myrtaceae (*Eucalyptus* sp.); Rhamnaceae (*Condalia* sp., *Ziziphus* sp.); Ulmaceae (*Celtis* sp.).

Flavonoid contents of honey samples from the Chaco province (laid in the north of the country) were analysed (Table 5). In general, these samples showed lower contents of myricetin, quercetin and luteolin compared with honeys from Mendoza and Santiago del Estero provinces. The Chaco province has moderate winters and hot summers with low to moderate rain levels. Some of the most common families of native tree species are: Anacardiaceae (*Schinopsis balansae*); Apocinaceae (Aspidosperma quebracho blanco Schltdl.); Bignoniaceae (*Tabebuia ipe*); Labiatae (*Acacia caven*); Leguminosae (*Geoffroea decorticans*, *Prosopis* sp.); Myrtaceae (*Eucalyptus* sp.); Rhamnaceae (*Ziziphus* sp.); Zygophyllaceae (*Bulnesia sarmienti*).

Santiago del Estero and Chaco domains are characterised by periods of dry and sunny climate. However, the honeys coming from these zones showed differences in the total flavonoid contents. It is interesting to note that for honeys from the Santiago del Estero, average levels of quercetin were approximately twice of that obtained for samples from the Chaco province. In spite of the fact that both provinces belong to the same phytogeographical region ("Gran Chaco Americano"), the differences observed in flavonoid contents could be attributed to the presence of specific vegetation in each region combined with some variability of the geochemical conditions.

3.4. Relationships between flavonoid contents of monofloral honeys and floral source

Fig. 4 shows the content of *Eucalyptus* sp. and *Lotus* sp. pollen among the mixed and monofloral honeys arising from the Buenos Aires province. The average occurrence of *Eucalyptus* sp. pollen was around 50% for mixed honeys, 52% for *Lotus* sp. monofloral honeys and 80% for *Eucalyptus* sp. monofloral honeys, showing a correlation with the different contents of quercetin and luteolin for each group of honeys (Fig. 5).

In *Eucalyptus* sp. honeys, quercetin and luteolin were the predominant compounds, corresponding to 62% and 26% of total flavonoid content, respectively. Moreover, these flavonoid amounts were around 50% higher than those found for quercetin and luteolin in both mixed (southeast zone of Buenos Aires province) and *Lotus* sp. honeys (Fig. 5).

On the other hand, *Lotus* sp. honeys showed lower contents of quercetin and luteolin than mixed honeys. In contrast, the content of myricetin was significantly higher than that found in mixed honeys (Fig. 5).

The pollen composition of *Lotus* sp. honeys showed an occurrence of *Eucalyptus* sp. and *Lotus* sp. types around 52% and 42%, respectively. *Eucalyptus* sp. pollen occurrence was quite similar to that found in mixed honeys. Whilst, in *Eucalyptus* sp. honeys, pollen of *Lotus* sp. was around 14%.

Lotus is a common flowering plant (perennial herbaceous plant) and *Eucalyptus* is represented for a diverse genus of trees (and a few shrubs). They are important sources of both nectar and pollen, whilst *Eucalyptus* plants also contribute to propolis. It is well known that nectar, pollen and propolis are good sources of phenolic compounds that are incorporated into honeys at varying levels giving them a characteristic profile for these compounds (Weston, 2000). Flavonoids almost exclusively occur in nectar and pollen as flavonoid glycosides that are hydrolysed in the bees stomach into aglycones, which are the constituents of honeys. Thus, the results obtained showed that *Eucalyptus* contributes to the contents of quercetin and luteolin. Meanwhile, *Lotus* could contribute to the myricetin levels, being the most evident in *Lotus* sp. honeys. Therefore, these results suggest that the presence of the flavonoids analysed could be associated to the floral origin of these honeys.

4. Conclusion

Regarding the flavonoid extraction method used in this study, it is worth noting that there are no previous reports utilising Amberlite XAD-4 resin for flavonoid isolation from honeys. Amberlite XAD-2 resin has been suggested as the most suitable non-ionic polymeric resin in flavonoid analysis from water extracts containing sucrose natural matrices (Tomás-Barberán, Blázquez, García-Viguera, Ferreres, & Tomás-Lorente, 1992). Thus, this resin has been widely used for both the quantitative analysis and the profile investigation of flavonoids from honeys.

Our results about the recovery efficiency of flavonoids shows similar values to those obtained with XAD-2. Nevertheless, we could remark that our results with Amberlite XAD-4 resin are potentially significative considering that the recovery assays were performed from complex natural matrices which natural components influencing the flavonoid recovery.

It is interesting to mention that for light coloured honeys there were no differences observed in the recovery values for the different resin/honey (w/w) ratio assessed (mean: 90%) when a sufficient volume of eluent solvent was used. This observation supports the idea that molecules such as pigments, plentifully present in dark honeys, could impair adsorption of flavonoids onto the resin.

The results of the present study contribute to the knowledge of major flavonoids composition of Argentinean honeys, and allow the comparison in this aspect with other honeys coming from different countries.

Considering the results obtained from the analysis of flavonoids for the samples used in this research, it is possible to remark that the differences on the contents of myricetin, quercetin and luteolin depend on the specific climatic and geographical conditions of each phytogeographical region of Argentina. Particularly, the analysis of flavonoid contents of monofloral honeys showed that for *Eucalyptus* sp. and *Lotus* sp. honeys there is a relationship between the dominant pollen and the contribution of a main flavonoid in particular.

Finally, we conclude that the contents of the flavonoid aglycones studied could be associated with the botanical species of the region establishing a pattern on the identification of local honeys.

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