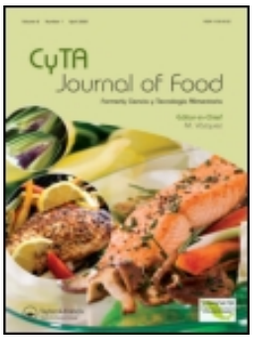

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Polyphenols and palynological origin of bee pollen of *Apis mellifera* L. from Brazil. Characterization of polyphenols of bee pollen

Polifenoles y origen palinológica de polen de abeja de *Apis mellifera* L. del Brasil. Caracterización de polifenoles de polen de abeja

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The aim of this work was to study the chemical composition, botanical origin and the antioxidant activity of ethanolic extracts of bee pollen. The antioxidant activity was measured by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH•) scavenging method and β-carotene-linoleic assay. The pollen extracts were purified using a XAD2 resin and the amount of phenolic compounds and flavonoids were identified by reverse phase-high performance liquid chromatography (RP-HPLC) and gas chromatography–mass spectrometry (GC–MS) analyses. The phenolic content of bee pollen extracts before and after the resin were 38.6 and 17.8 mg/g of gallic acid equivalent in bee pollen, respectively. Two different flavonoids (rutin and myricetin) which can be accounted by the high antioxidant activity of bee pollen extracts were identified and quantified. The total antioxidant capacity measured by the DPPH• radical method increased significantly in bee pollen extracts purified with hydrophobic resin: 24.84–94.75% (Palmeira). All samples were considered heterofloral, which were composed by pollen from *Myrtaceae eucalyptus*, Asteraceae and Brassicaceae families, and among others.

Keywords: antioxidant capacity; flavonoids; polyphenols; XAD2; HPLC; GC–MS; SEM

El objetivo de este trabajo fue estudiar la composición química, el origen botánico y la actividad antioxidante de los extractos etanólicos de polen de abeja de *Apis melífera* L. La actividad antioxidante fue medida por los métodos de secuestro del radical 2,2-diphenyl-1-picrylhydrazylhydrate (DPPH•) y por la oxidación acoplada del beta-caroteno/ácido linoleico. Los extractos etanólicos fueron purificados usándose la resina XAD2 y la cantidad de compuestos fenólicos y flavonoides fueron identificados por el análisis de RP-HPLC/UV-Vis y CG-MS. El contenido fenólico de los extractos de polen de abeja antes y después de la resina fueron 38,6 y 17,8 mg/g de GAE (equivalente en ácido gálico) en el polen de abeja, respectivamente. Fueron identificados y cuantificados dos distintos flavonoides (rutina y miricetina), los cuales pueden explicar la alta actividad antioxidante de los extractos de polen de abeja. La capacidad antioxidante total medida por el método del radical DPPH aumentó significativamente en los extractos purificados con la resina hidrófoba 24,84% para 94,75% (Palmeira/PR); 40,81% para 92,56% (São Joaquim/SC); y 14,91% para 94,05% (Encruzilhada/RS). Todas las muestras fueron consideradas heteroflorales, las cuales eran compuestas por el polen de *Myrtaceae eucalyptus* y de las familias Asteraceae y Brassicaceae entre otras.

Palabras clave: capacidad antioxidante; flavonoides; polifenoles; XAD2; HPLC; CG-MS

Introduction

Bee pollen contains important nutritional substances such as carbohydrates, proteins, amino acids, lipids, vitamins, minerals, and traces of micronutrients. Besides these substances, significant amounts of polyphenols have been detected (Carpes et al., 2008) with various therapeutic effects (Leja, Mareczek, Wyzgolik, Klepacz-Baniak, & Czekonska, 2007). Each pollen type has its own specific characteristics related to the genetics of the floral species and plantations visited by the bees. During the pollen harvest (collection), the bees might show preferences regarding the floral sources (Kreyer, Oed, Walther-Hellwig, & Frankl, 2004). Bee pollen can be *monofloral*, characterized by single botanic taxonomy in the pollen load of the "corbiculae", retaining the constant organoleptic and biochemical properties of the original plant. When the bees visit other flowers, or mix the "pollen loads" from various flowers, these are called *heterofloral* and present a variety of biochemical properties (Almeida-Muradian, Pamplona, Coimbra, & Barth, 2005).

The analysis through high performance liquid chromatography (HPLC) with photodiode array detector is the standard method for detection of polyphenols (Sivam, 2002). However, one of the main difficulties in determining phenolic compounds and flavonoids in bee products like pollen and honey through HPLC lies mainly in the extraction of flavonoids and the preparation of samples due to their high sugar content (Tomás-Barberán, Blázquez, García-Viguera,

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Ferreres, & Tomás-Lorente, 1992). Flavonoid glycosides often require hydrolysis, either acidic, basic or enzymatic, to remove the glucose molecule (Ferreres et al., 1994).

In the extraction of bee products, the liquid–liquid fractioning produces interface drawbacks, which do not allow full recovery of flavonoids. However, according to Ferreres et al. (1994), this problem can be solved with the use of a hydrophobic non-ionic polymeric resin Amberlite XAD-2. D'arcy (2005) used two methods to extract the phenolic compounds from honeys using hydrophobic resin: (1) mixing the honey solution with XAD-2 for 10 min before packing in glass column and (2) by adding the honey solution to the resin already packed (elution method). The recovery of all phenolic compounds was higher when the author used the method of mixing the honey solution with the resin XAD-2 before packaging.

Synthetic compounds butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) antioxidants are effective and widely used in food industry, but have also become controversial due to their potential adverse effects on health (Shahidi & Zhong, 2010). In this context, the demand for natural antioxidants has received special attention from researchers worldwide and from the food industry. Among natural products, pollen has been used empirically for its potential benefits to human health and for presenting valuable biological activities such as antioxidant (Almaraz-Abarca et al., 2007; Carpes, Begnini, Alencar, & Masson, 2007; Morais, Moreira, Féas, & Estevinho, 2011), antibacterial (Morais et al., 2011; Basim, Basim, & Özcan, 2006), antifungal (Özcan, Ünver, Ceylan, & Yestisir, 2004), and anti-inflammatory (Maruyama, Sakamoto, Araki, & Hara, 2010) properties. It has recently gained popularity as a healthy food supplement and has been extensively used in foods and beverages in different parts of the world. Several authors have reported the need for further studies to evaluate the relative composition of polyphenols in bee pollen and pollen extracts, as well as their chemical and biological specificities in order to evaluate their antioxidant role and contribution (Almaraz-Abarca et al., 2007; LeBlanc, Owen, Boue, DeLucca, & Deeby, 2009; Maruyama et al., 2010).

Therefore, the aim of this study was to evaluate the chemical composition, botanical origin, and the antioxidant activity of ethanolic extracts of bee pollen. The bee pollen samples were purified using a Amberlite XAD-2 resin. In addition, the phenolic compounds and flavonoids contents were quantified.

Material and methods

Chemicals and reagents

Amberlite® XAD-2, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), (±) α-tocopherol (VE), trans-β-carotene, Tween 40, and linoleic acid were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). The Folin–Ciocalteu reagent, 3,4,5-trihydroxybenzoic acid (gallic acid; GA), sodium carbonate, BHT, and BHA standards were obtained from Synth (Diadema, SP, Brazil). The authentic standards, ρ-coumaric acid, ferulic acid, cinnamic acid, GA, quercetin, kaempferol, caferide, apigenin, isosacuranetin, pinocembrin, chrysin, acacetin, galangin and myricetin, were obtained from Extrasynthese Co. All the solvents used for chromatography were of HPLC grade and all the other chemicals were of analytical-reagent grade.

Preparation of bee pollen ethanol extract (EPE) and purification

The extract was obtained from 100 g of bee pollen from samples PR 03, SC 03, and RS 09 in 100 mL of 70% ethanol (v/v), at 70°C in a water bath for 30 minutes (EPE). The extracts were acidified to pH 2, mixed with 100 g of Amberlite XAD2 and packed in glass column (25 cm × 2 cm). Each column was eluted with 100 mL of acidic water pH 2, 300 mL of distilled water pH 7 and then with 300 mL of methanol (Alencar et al., 2007). The methanolic fraction was evaporated in rotary evaporator at low temperature of 40°C. The recovered organic phase was used for analysis by HPLC and gas chromatography–mass spectrometry (GC–MS). The supernatants before and after purification with resin XAD2 were used for the determination of phenolic compounds, flavonoids, antioxidant activity, and chemical profile analysis through HPLC.

Total phenolic compounds

The content of total phenolic compounds was determined through Folin–Ciocateau spectrophotometric method using GA as standard. The pollen extracts were diluted 1:25 in water. To an aliquot of 0.5 mL of diluent sample, 2.5 mL of Folin–Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO, USA) was added and diluted in 1:10 distilled water. Two milliliters of sodium carbonate 4% were added to the reaction. After 2 h of rest, readings were performed in SHIMADZU spectrophotometer Model UV 1240 V at 740 nm. An analytical curve containing 10, 25, 40, 70, 85, and 100 mg/kg of GA was constructed and the results were expressed as mg/g of GAE (gallic acid equivalent) of bee pollen (Singleton, Orthof, & Lamuel-Raventos, 1999). All determinations were performed in triplicate.

Total flavonoids

The total flavonoids concentration was determined according to the method described by Park, Koo, Sato, and Contado (1995), with some modifications. An aliquot of 0.5 mL of pollen extract diluted in water (1:10) was transferred to a test tube and added 4.3 mL of 80% ethanol, 0.1 mL of aluminum nitrate at 10% and 0.1 mL of potassium acetate. After 40 min (of rest), the readings were made on SHIMADZU spectrophotometer Model UV 1240 V, 415 nm. An analytical curve containing 10, 25, 40, 70, 85, and 100 mg/kg of quercetin was constructed and the results were expressed in mg/g of quercetin in bee pollen. All determinations were performed in triplicate.

Determination of the antioxidant activity

Antioxidant activity by the coupled oxidation of β-carotene and linoleic acid

The measurement of the antioxidant activity was determined by coupled oxidation of β-carotene and linoleic acid, according to Ahn, Kumazawa, Hamasaka, Bang, and Nakayama (2004) and Haminiuk et al. (2011) methodologies.

About 10 mg of β-carotene was weighed, and dissolved in 100 mL of chloroform. Then, an aliquot of 3 mL of chloroform–β-carotene was added with 40 mg of linoleic acid and 400 mg Tween 40. The chloroform was removed using a nitrogen gas flow, and the residue obtained redissolved in 100 mL of aerated water for 30 min. Aliquots of 3 mL of the β-carotene/linoleic acid emulsion were mixed with 300 μL of EPE diluted to final concentration of 0.5 mg/mL in 70% ethanol. The reading of absorbance was performed in spectrophotometer at 470 nm, at baseline and intervals of 20 min during 2 h with incubation at 50°C for the oxidation reaction. The control sample contained 300 μL of 70% ethanol. The antioxidant activity was expressed as the percentage of relative inhibition in relation to control after 120 min of incubation, using the following equation:

$$\%\mathrm{AA} = 100 \times \left(\frac{DR_c - DR_S}{DR_c}\right) \quad (1)$$

where %AA is the antioxidant activity, DR_c is the degradation rate of control sample (=ln (a/b)/120); DR_S is the degradation rate of sample with the test substance (=ln (a/b)/120); a is the initial absorbance at time 0 and b is the absorbance after 120 min. All determinations were performed in triplicate.

Scavenging activity of the DPPH• radical

The measurement of the DPPH• radical scavenging activity was performed according to methodology described by Brand-Williams, Cuvelier, and Berset (1995). To evaluate the antioxidant activity, the ethanol pollen extracts (EPE) were reacted with the stable DPPH• radical in an ethanol solution. Ethanol pollen extracts stock solutions were diluted to final concentration of 0.5 mg/mL. The reaction mixture consisted of adding 0.5 mL of sample, 3 mL of absolute ethanol, and 0.3 mL of DPPH• radical solution 0.3 mM in ethanol. All determinations were performed in triplicate. The scavenging activity percentage (%AA) was determined according to Mensor et al. (2001).

$$\%AA = \left[\frac{(Abs_{sample} - Abs_{\mathrm{blank}}) \times 100}{Abs_{control}}\right] \quad (2)$$

where Abs represents absorbance read at 517 nm after 100 min of reaction.

Reverse phase-high performance liquid chromatography (RP-HPLC)

The ethanolic pollen extracts were analyzed through HPLC in a Shimadzu chromatograph equipped with RP-18 ODS-A column (4.6 mm × 250 mm × 5 μm) and a photodiode-array detector (PDA) (SPD-M10AVp, Shimadzu Co., Kyoto, Japan). The ethanolic pollen extracts were filtered through a 0.22 μm filter (Millipore) and injected 10 μL (EPE: 100 mg/mL) and 5 μL (EPE: 7 mg/mL) of the filtrate before and after the use of the hydrophobic resin respectively. The mobile phase consisted of a linear gradient of acetic acid: water (1:20) (solvent A) and methanol (solvent B) starting with 5% B and increasing to 60% B (40 min), keeping in 90% B (55–60 min) and decreasing to 5% B (60–70 min) with solvent flow rate of 1 mL/min. The polyphenols were identified based on the retention times and UV-Vis absorption pattern, and the peak purity confirmed by diode array detector (DAD). Quantitation was performed by external standardization (Alencar et al. 2007). The following authentic standards of phenolic acids and flavonoids were examined: ρ-coumaric acid, ferulic acid, cinnamic acid, GA, quercetin, kaempferol, caferide, apigenin, isosacuranetin, pinocembrin, chrysin, acacetin, galangin and myricetin.

Gas chromatography–mass spectrometry (GC–MS)

The analyses through GC–MS of pollen extracts were performed according to method described by Markham, Mitchell, Wilkins, Daldy, and Lu (1996). Aliquots of 400 μL of each extract were placed inside glass "vials" and 1 mL of ethereal diazomethane solution (CH_2N_2) was added to the methylation. Samples were kept in an ice bath for 4 h to complete methylation. The resulting solutions were dried with anhydrous magnesium sulfate and analyzed on a gas chromatograph coupled to a mass spectrometer. The chromatograph was equipped with an HP-5MS capillary column (30 mm × 0.25 mm × 0.25 μm) and a detector operating in the "scanning" mode (m/z 40–500). The temperature program was: 40°C (0 min) to 140°C (10°C min^{-1}), 140°C (0 min) to 300°C (5°C min^{-1}, 10 min). The samples (0.6 μL) were injected by an autoinjector using the "splitless" injection technique. The integration was done using specific software. The compounds detected in samples were identified by comparison with the data of mass spectra NIST107, NIST21, and WILEY139, workstation components CLASS-5000 version 2.2. The tool for extracting selective ions to verify the presence or absence of various classes of compounds expected in the process of identifying the components of samples was also used.

Botanical origin of the pollen loads

Approximately 2 g of each sample was considered representative for pollen analysis (Louveaux, 1990). The pollen loads were grouped up into subsamples according to their coloring and each subsample was weighed and metalized with gold and then analyzed by scanning electronic microscopy (SEM) in Digital Scanning Microscope DSM 940 A (Zeiss Co., Oberkochen, Germany). In this experiment, approximately 350 pollen grains of each sample were identified and counted and from this total the percentage of each pollen type was established as: "predominant pollen" or "dominant" (>45% do total) (PD); "Accessory or secondary pollen" (16–45%) (PA); "Important isolated pollen" (3–15%) (PII); and "Occasional isolated pollen" (<3%) (PIO) (Barth, 1989, 2004).

Statistical analysis

Assays were performed in triplicate for each sample. Results were expressed as mean values ± standard deviation (SD). In each parameter, the differences between the means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test with $p \leq 0.05$. The statistical analysis was carried out using the software SAS version 9 (2004) (SAS Institute Inc., Cary, NC, USA).

Results and discussion

In a preliminary study, 36 bee pollen samples from different states from southern Brazil (Parana, Santa Catarina and Rio Grande do Sul states) were collected. After a preliminary classification of 36 samples through the technique of scanning the UV-visible region, HPLC, high performance thin layer chromatography (HPTLC), content of phenolic compounds and antioxidant activity, it was possible to observe a great similarity in the absorption profile between samples from the same state, which enabled their classification into three major groups of bee pollen (Carpes et al., 2008). Thus, the bee pollen representative of Parana, Santa Catarina and Rio Grande do Sul states were the samples PR 03 (Palmeira/PR), SC 03 (São Joaquim/SC) and RS 09 (Encruzilhada do Sul/RS), respectively.

Content of phenolic compounds and total flavonoids

The use of hydrophobic polymer resin Amberlite XAD2 is very important in the recovery of phenolic compounds present in bee pollen. As shown in Table 1, the content of phenolic compounds and flavonoids in pollen extracts after the use of polymer resin ranged from 20.17 to 33.39 mg/g of GAE in bee pollen and 6.54 to 17.49 mg/g of quercetin in bee pollen, respectively. Sample SC 03 showed the largest variation in the content of total flavonoids after the use of resin Amberlitte XAD2 (from 28.33 to 6.54 mg/g of quercetin in bee pollen).

It was observed that, for the content of phenolic compounds, there was a significant change in the pollen extract after purification with hydrophobic resin. A decrease in the content of these compounds with significant loss of phenolics in all samples was found (Table 1). The reduction in the content of phenolic compounds in three samples after the use of resin (Table 1) was probably due to the removal of polar phenolic compounds that could be carried in the aqueous fraction. This reduction was also observed in the chromatograms of bee pollen extracts (Figures 1–3).

But, during the analysis of flavonoids, we found a significant increase in the content of flavonoids in the samples PR RS 03 and 09, demonstrating that the purification had concentrated such compounds in the extract. The pollen extract SC 03 had the highest content of phenolic compounds, regardless the purification with the resin and

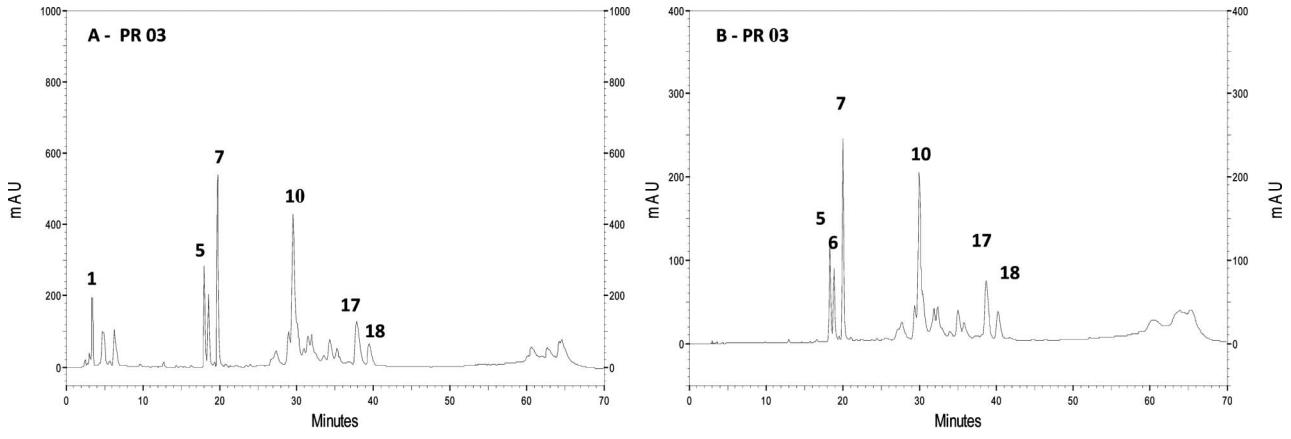
Table 1. Total phenolic content, total flavonoids, and antioxidant activity of bee pollen extracts before and after the use of hydrophobic resin.

Tabla 1. El contenido de fenoles totales, flavonoides totales, y la actividad antioxidante de extractos de polen de abeja antes y después del uso de la resina hidrófoba.

Bee pollen extracts (EPE)	Total phenolic content (mg/g of GAE in bee pollen)		Total flavonoids (mg/g of quercetin in bee pollen)		Antioxidant activity (%)*			
					β-carotene method		DPPH radical method	
	Before**	After**	Before**	After**	Before**	After**	Before**	After**
PR 03	46.43 ± 0.85^{Ab}	20.22 ± 0.25^{Bc}	8.55 ± 1.34^{Bc}	17.64 ± 0.41^{Aa}	76.42 ± 1.22^{Bc}	86.43 ± 0.38^{Ac}	24.84 ± 0.45^{Bb}	94.75 ± 0.42^{Aa}
SC 03	48.76 ± 0.78^{Aa}	33.53 ± 1.02^{Ba}	28.43 ± 0.73^{Aa}	6.58 ± 0.23^{Bc}	79.43 ± 0.38^{Bb}	89.22 ± 1.07^{Ab}	40.81 ± 0.38^{Ba}	92.56 ± 0.33^{Ac}
RS 09	30.55 ± 0.54^{Ac}	25.47 ± 1.43^{Bb}	11.75 ± 0.14^{Bb}	14.65 ± 0.88^{Ab}	92.86 ± 0.55^{Ba}	93.29 ± 0.37^{Aa}	14.91 ± 0.25^{Bc}	94.05 ± 0.52^{Ab}

Note: Data followed by same letter (capital letter) in a line are not statistically different ($p > 0.05$). Data followed by same letter (lowercase) in a column are not statistically different ($p > 0.05$). *Concentration of extracts 0.5 mg/mL. **Before and after the use of XAD2 resin.

Nota: Los datos seguidos por la misma letra (mayúscula) en una línea no son estadísticamente diferentes ($p > 0,05$). Datos seguidos por la misma letra (minúscula) en una columna no son estadísticamente diferente ($p > 0,05$). *La concentración de los extractos 0,5 mg/mL. **Antes y después del uso de resina XAD2.

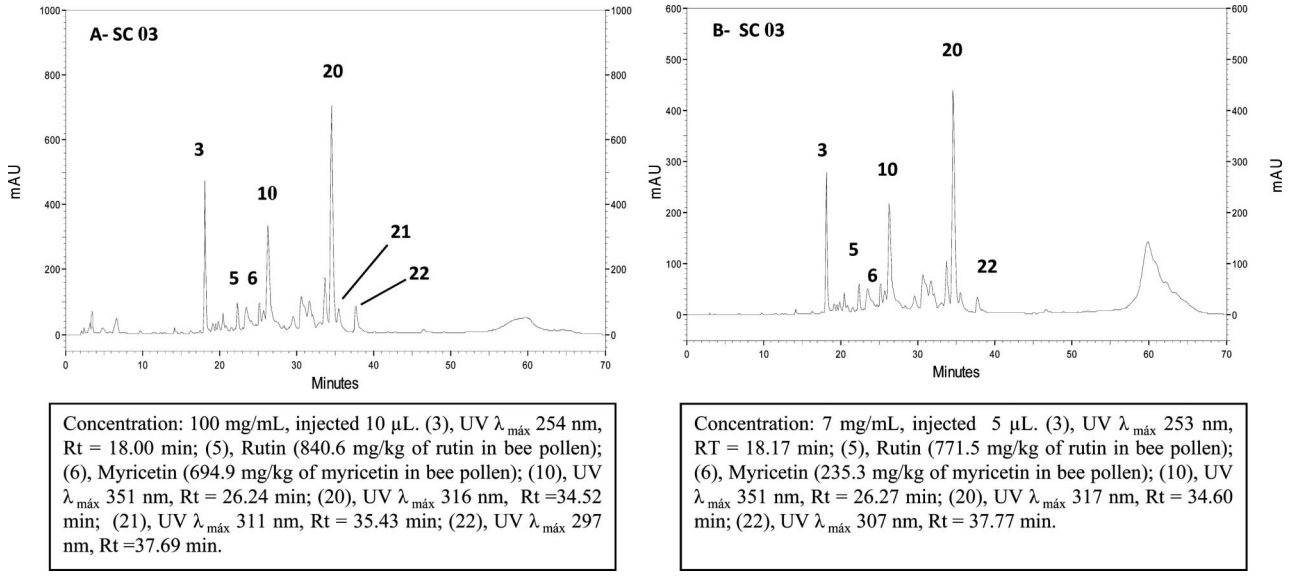


Concentration: 100 mg/mL, injected 10 µL. (1), UV $\lambda_{máx}$ 259 nm, Rt = 3.46 min; (5), UV $\lambda_{máx}$ 253 nm, Rt = 18.00 min; (7), UV $\lambda_{máx}$ 263 nm, Rt = 19.74 min; (10), UV $\lambda_{máx}$ 319 nm, Rt = 29.54 min; (17), UV $\lambda_{máx}$ 309 nm, Rt = 37.86 min; (18), UV $\lambda_{máx}$ 313 nm, RT = 39.48 min.

Concentration: 7 mg/mL, injected 5 µL. (5), UV $\lambda_{máx}$ 252 nm, Rt = 18.34 min; (6), UV $\lambda_{máx}$ 263 nm, Rt = 18.84 min; (7), UV $\lambda_{máx}$ 263 nm, Rt = 20.04 min; (10), UV $\lambda_{máx}$ 320 nm, Rt = 29.92 min; (17), UV $\lambda_{máx}$ 309 nm, Rt = 38.64 min; (18), UV $\lambda_{máx}$ 313 nm, Rt= 40.19 min.

Figure 1. Chromatographic profile (HPLC) of bee pollen extracts (PR 03 sample) before (A) and after (B) the use of XAD2 resin.

Figura 1. Perfil cromatográfico (HPLC) de los extractos de polen de abeja (muestra PR 03) antes (A) y después (B) del uso de resina XAD2.

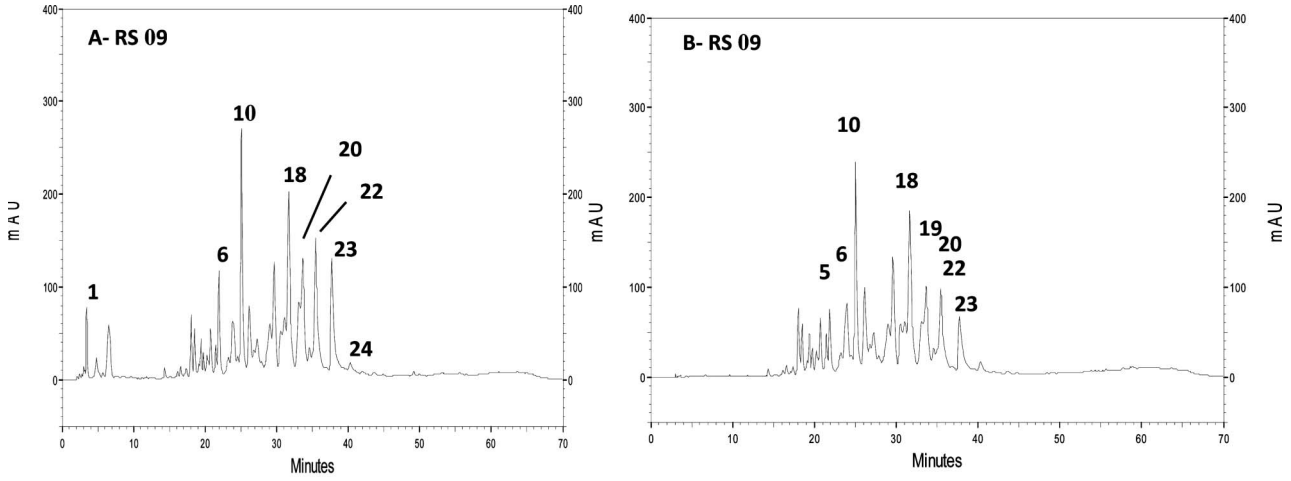


Concentration: 100 mg/mL, injected 10 µL. (3), UV $\lambda_{máx}$ 254 nm, Rt = 18.00 min; (5), Rutin (840.6 mg/kg of rutin in bee pollen); (6), Myricetin (694.9 mg/kg of myricetin in bee pollen); (10), UV $\lambda_{máx}$ 351 nm, Rt = 26.24 min; (20), UV $\lambda_{máx}$ 316 nm, Rt =34.52 min; (21), UV $\lambda_{máx}$ 311 nm, Rt = 35.43 min; (22), UV $\lambda_{máx}$ 297 nm, Rt =37.69 min.

Concentration: 7 mg/mL, injected 5 µL. (3), UV $\lambda_{máx}$ 253 nm, RT = 18.17 min; (5), Rutin (771.5 mg/kg of rutin in bee pollen); (6), Myricetin (235.3 mg/kg of myricetin in bee pollen); (10), UV $\lambda_{máx}$ 351 nm, Rt = 26.27 min; (20), UV $\lambda_{máx}$ 317 nm, Rt = 34.60 min; (22), UV $\lambda_{máx}$ 307 nm, Rt = 37.77 min.

Figure 2. Chromatographic profile (HPLC) of bee pollen extracts (SC 03 sample) before (A) and after (B) the use of XAD2 resin.

Figura 2. Perfil cromatográfico (HPLC) de los extractos de polen de abeja (muestra SC 03) antes (A) y después (B) del uso de la resina XAD2.



Concentration: 100 mg/mL, injected 10 µL. (1), UV $\lambda_{máx}$ 259 nm, Rt = 3.46 min; (6), Rutin (991.0 mg/kg of rutin in bee pollen); (10), UV $\lambda_{máx}$ 263 nm, Rt = 25.08 min; (18), UV $\lambda_{máx}$ 273 nm, Rt = 31.65 min; (20), UV $\lambda_{máx}$ 289 nm, Rt = 33.64 min; (22), UV $\lambda_{máx}$ 296 nm, Rt = 35.42 min; (23), UV $\lambda_{máx}$ 297 nm, Rt = 37.73 min; (24), UV $\lambda_{máx}$ 267 nm, Rt = 40.28 min.

Concentration: 7 mg/mL, injected 5 µL. (5), UV $\lambda_{máx}$ 253, Rt = 21.47 min; (6) Rutin (426.3 mg/kg of rutin bee pollen); (10), UV $\lambda_{máx}$ 264 nm, Rt = 25.05 min; (18), UV $\lambda_{máx}$ 273 nm, Rt = 31.63 min; (19), UV $\lambda_{máx}$ 290 nm, Rt = 33.64 min; 20(C), UV $\lambda_{máx}$ 296 nm, Rt= 35.43 min; (22), UV $\lambda_{máx}$ 297 nm, Rt = 37.75 min; (23), UV $\lambda_{máx}$ 267 nm, Rt = 38.55 min.

Figure 3. Chromatographic profile (HPLC) of bee pollen extracts (RS 09 sample) before (A) and after (B) the use of XAD2 resin.

Figura 3. Perfil cromatográfico (HPLC) de los extractos de polen de abeja (muestra RS 09) antes (A) y después (B) del uso de la resina XAD2.

the highest content of flavonoids, only before purification, because after purification the content of flavonoids was significantly reduced with the use of resin XAD2 ($p \leq 0.05$) (Table 1).

Antioxidant activity

With regard to antioxidant activity, analyzed by both methods (β-carotene and DPPH• radical scavenging activity), the use of hydrophobic resin changed the profile of the antioxidant activity ($p \leq 0.05$). An increase in the antioxidant activity was observed after the use of resin in all samples, probably due to the higher concentration of bioactive compounds in the extracts. The extract RS 09 showed a higher antioxidant activity by β-carotene method, before and after use of the resin, independent of purification.

The total antioxidant capacity measured by DPPH• test increased significantly in bee pollen extracts purified with hydrophobic resin ($p \leq 0.05$) (Table 1). Although the content

of total phenolic compounds decreased around two fold, the resin Amberlite XAD2 provided an adsorption of phenolic compounds with effective antioxidant activity, which could be a suitable treatment in the characterization of polyphenols in pollen (Table 1). There were statistical differences at 95% by Tukey test between samples in the analysis of DPPH• radical scavenging activity. Before purification, the sample of highest activity was SC 03 and after purification, the highest antioxidant capacity was PR 03 fit to the sample RS 09. Izuta et al. (2009) have studied the bee pollen from Spain, Brazilian green propolis, and Chinese red propolis, all were extracted with 95% ethanol at room temperature. In this study, the DPPH• radical scavenging activities of Brazilian green propolis and Chinese red propolis were stronger than those of other bee products (e.g. bee pollen). The IC50 value of the bee pollen extract (196.7 μg/mL) was four times higher than that of Brazilian propolis ethanol extract (52.0 μg/mL).

The antioxidant activity of bee pollen extracts by the β-carotene method had an increase, though less significant (Table 1). In this method, the complex interfacial phenomenon called *polar paradox* influences the behavior of compounds with antioxidant properties. Thus, a possible explanation for the slight increase in the antioxidant activity may be the removal of polar compounds present in the extract. The polar antioxidants remaining in the aqueous phase are more diluted and are thus less effective in protecting the lipid. Koleva, Van Beek, Linssen, Groot, and Evstatieva (2002) studied the antioxidant activity measured by the β-carotene method in extracts of *Sideritis Labiatae* from Bulgaria and found that the most active inhibitors of β-carotene bleaching were mainly non-polar extracts.

High performance liquid chromatography – HPLC/PDA

According to chemical evidences outlined in the chromatograms obtained, (Figures 1–3) the bee pollen from Southern Brazil has a different chemical composition. Several peaks at different retention times showed a complex composition (Figures 1A, 2A, and 3A). The chemical profile obtained through the HPLC/PDA technique revealed compounds with high polarity, probably phenolic acids, and a large variety of more polar compounds, probably flavonoids. The chromatographic profile of bee pollen extracts indicated the presence, on average, of approximately 24 different compounds.

When comparing the chromatograms of ethanolic pollen extracts before and after purification with the resin, the presence of new peaks and the disappearance of others could be observed. Compounds with retention time less than 18 min disappeared probably because they were more polar and were dragged after washing with water (Figure 1B). According to D'arcy (2005), the new compounds detected may have originated from chemical reactions of compounds such as chlorogenic acid and ferulic acid with Amberlite XAD2. However, the author suggests further studies to confirm these statements.

Sample PR 03 showed a chromatographic profile different from other samples, with more pronounced peaks at the retention time around 20–30 min (Figure 1A). This sample, after the use of resin, had only the removal of compounds with retention time less than 18 min, and after this time, the chromatographic profile remained the same as the extract without resin, i.e. without any probable interaction with the hydrophobic resin (Figure 1B).

In sample SC 03, it was possible to identify and quantify a possible structure related to rutin flavonoids and myricetin, with levels of 840.6 mg/kg and 694.9 mg/kg of pollen, respectively (Figure 2A).

The presence of these two compounds may explain the high antioxidant activity of this sample. The resin XAD2 allowed the removal of the few polar compounds present in this extract, which were solubilized and dragged after washing with acidic water. It was possible to identify and quantify flavonoids rutin and myricetin, with levels of 771.5 mg/kg and 235.3 mg/kg of pollen, respectively (Figure 2B).

Chromatograms corresponding to bee pollen RS 09 had the largest number of peaks (24 peaks) when compared with the other two samples (Figure 3). However, only rutin could be identified at a concentration of 991.0 mg/kg of pollen from sample RS 09 (Figure 3A). After purification, rutin was also found, but in lower concentration (426.3 mg/kg of pollen) (Figure 3B) and it could be observed a shift in the retention time of some peaks (peak 10 and 20) and the suppression of others (peak (24), UV λ_{max} 267 nm, RT = 40.28 min) (Figure 3B).

The presence of rutin in bee pollen from southern Brazil indicates the biological and nutritional quality of pollen due to its high antioxidant activity. It is interesting to note that a minimum of 200 mg/kg of rutin in bee pollen is necessary to standardize the pollen marketed in Spain (Serra Bonvehí, Soliva Torrentó, & Centelles Lorente, 2001). Yao (2002) reported that Amberlite XAD2 did not allow the recovery of phenolic acids such as GA and ellagic acid in acidic solutions of honeys (pH 2). D'arcy (2005) optimized the extraction of phenolic compounds with resin XAD2 and found caffeic, ferulic, and ρ-coumaric acids, besides flavonoids quercetin, hesperetin, and chrysin in honeys from Australia. According to D'arcy (2005), the recovery of flavonoids such as hesperetin and chrysin were higher than 80% with the use of Amberlite XAD2 in honey solution. These results agree with those presented by Martos, Cossentini, Ferreres, and Tomas-Barberan (1997). This confirms that the method used in this study was suitable for the extraction of flavonoids in bee pollen.

Serra Bonvehí et al. (2001) identified 13 phenolic compounds in bee pollen from Spain. Among them, seven were phenolic acids such as 3,4-dihydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, ethyl ester of the 4-hydroxybenzoic acid, trans-cinnamic acid and flavonoids rutin, quercetin, myricetin, kanferol, and isorhamnetin. Leja et al. (2007) studied the phenolic constituents (total phenolics, phenylpropanoids, flavonols, and anthocyanins) of bee pollen from 12 different species from the Krakow region in Poland. This study found a wide variation of phenolic compounds in the species investigated and in most samples tested, the high antioxidant activity was related to the content of phenylpropanoids.

Study carried out by Tomás-Barberán, Tomás-Lorente, Ferreres, and Garcia-Viegueira (1989) showed that bee pollen from *jara* contained mainly quercetin and isorhamnetin-3-glucoside, besides trace concentrations of myricetin traits and kaempferol-3-glycosides. This flavonoid is similar to that found in natural pollen from *jara*, which suggests that this flavonoid pattern can be used as chemical marker. Similarly, the compound 8-methoxy-kaempferol-3-glucoside can be

considered as a biochemical marker of bee pollen from *almond* because it is not present in pollen from other plants. Thus, according to these studies, flavonoid patterns may also be used as biochemical markers as for the botanical origin of pollen.

Maruyama et al. (2010) detected by HPLC/PDA in Spanish pollen, flavonoids such as kaempferol 3-glucoside, quercetin-7-rhamnoside, isorhamnetin, kaempferol, and quercetin. These compounds are directly related to anti-inflammatory effect demonstrated by this pollen.

Ferreres, Pereira, Valentão, and Andrade (2010) evaluated non-colored flavonoids in the bee pollen from Lousã (central Portugal) by HPLC–PDA–MS with an electrospray ionization (ESI) interface. Twelve non-colored flavonoids were characterized, being kaempferol-3-O-neohesperidoside the major compound, besides others in trace amounts. These include quercetin, kaempferol, and isorhamnetin glycosides, with several of them being isomers. Acetylated derivatives are also described.

Chemical composition analysis through GC/MS

Ethanolic pollen extracts were purified through the hydrophobic resin XAD-2 and were submitted to analysis of the chemical profile through GC–MS. Sample PR 03 presented chromatographic profile containing 10 peaks and four compounds were identified (Table 2, Figure 4A). Representative compounds of the sample were fatty acids (carboxylic), identified as methyl esters of C16, C18:2, and C18:3, with predominance of the latter (42.4%). These compounds accounted for 59.5% of the total composition. Benzoic acid methyl ester was identified, representing 3.9% of the sample.

The sample SC 03 showed relatively simple chromatographic profile, with nine peaks detected and five compounds identified (Table 3, Figure 4B). The same three fatty acids were identified, as those observed in sample PR 03. Thus, the hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester and then 9,12,15-octadecatrienoic acid methyl ester accounted for 83.6% of compounds found in sample SC 03 (Table 3). Aromatic carboxylic acids were also identified (phenolic acids) relative to peaks 1 (benzoic acid methyl ester) and 2 (4-methoxy benzoic methyl ester), contributing with 6.7% in the total composition of sample SC 03 (Figure 4B).

Sample RS 09 showed chromatographic profile represented by 11 compounds of which 8 were identified through mass spectrometry. Two phenolic acids were identified (benzoic acid methyl ester and phenylacetic acid methyl ester), accounting for 15.3% of the sample composition, and compound relative to benzoic acid methyl ester was also detected in samples PR 03 and SC 03. The same three fatty acids were identified (peak 6, 8 and 9) as those found in samples PR 03 and SC 03, which contribute with 70.4% of the total composition. Other acids were also found, one short-chain diacid C3 (peak 1) C19 (peak10) (Figure 4C). The compound corresponding to peak 5, with retention time of 20.45 min is also common in the three samples; however it could not be identified (Table 4, Figure 4C).

Studies by Nahar, Russel, Middleton, Shoe, and Sarker (2005) showed that methanolic extracts of the seeds of *Ilex aquifolium* analyzed by reversed-phase preparative HPLC contained 2,4-dihydroxyphenylacetic acid and 2,4-dihydroxyphenylacetic acid methyl ester. In the DPPH• assay, the phenylacetic acid derivatives showed significant levels of free radical scavenging (antioxidant) activity compared to that of the positive control, quercetin. Various parts of *Ilex aquifolium* are still today included in traditional medicinal preparations to treat liver, stomach and intestinal cancers, dropsy, fever, gout, jaundice, malaria, warts, swelling and tumors.

In this study, EPE were previously analyzed by Infrared (IR) and the frequencies of stretching of OH group, COOH groups were observed between 3000 and 3500 cm^{-1}, confirmed by the presence of the carbonyl C=O of the carboxylic group between 1600 and 1750 cm^{-1}. Fatty acids in the form of methyl esters could also be observed, which are predominant in these samples. The phenolic acids found in the three samples may be responsible for the high antioxidant activity of bee pollen; however, many compounds have not been identified through GC–MS with the derivatization technique used in this study (methylation with diazomethane). It is also known that this derivatization technique can degrade phenolic compounds, thus justifying the non-detection of flavonoids in the samples.

It is known that a large number of analytical methods are suggested in literature for the separation and identification of phenolic compounds. Most of these protocols are based on

Table 2. Gas chromatography/mass spectrometry analysis of bee pollen extract (PR 03 sample).

Tabla 2. Análisis de cromatografía de gases/espectrometría de masas del extracto de polen de abejas (muestra PR 03).

Peaks #	Chemical compounds	Rt (min)	m/z (%)
1	Benzoic acid, methyl ester	7.30	136 [35, M^+], 105 (100), 77 (83), 51 (60)
2	nd	19.71	224 [28, M^+], 118 (100), 209 (40), 91 (20), 179(12), 77 (12), 41(12)
3	nd	19.86	224 [32, M^+], 118 (100), 209 (40), 41 (16), 91 (16), 77 (12), 179 (12), 193 (12), 96 (8), 65 (4)
4	nd	21.65	161 [M^+], 43 (100), 55 (52), 79 (52), 93 (32), 105 (12), 121 (12), 135 (4), 149 (4)
5	Hexadecanoic acid, methyl ester	22.17	270 [M^+], 74 (100), 43 (68), 87(60), 55 (16), 143 (8)
6	9,12-octadecadienoic acid, methyl ester	25.38	294 [M^+], 41 (100), 67 (92), 81 (72), 88 (55), 95 (40), 109 (12), 123 (8), 135 (8), 150 (4)
7	9,12,15-octadecatrienoic acid, methyl ester	25.53	292 [M^+], 41 (100), 55 (68), 79 (76), 93 (36), 108 (20), 121 (8), 135 (6), 149 (6)
8	nd	28,66	176[4, M^+], 59 (100), 70 (60), 84 (45), 95 (20)
9	nd	29.37	234 [4, M^+], 98 (100), 41 (96), 85 (80), 55 (48), 79 (36), 112 (10), 126 (4), 154 (4), 194 (4)
10	nd	31.54	194[4, M^+], 91(100), 117(16), 65(8)

Note: Rt, Retention time (min); m/z, mass-to-charge ratio; nd, not detected.

Nota: Rt, Tiempo de retención (min); m/z, la masa-a-carga; nd, no detectado.

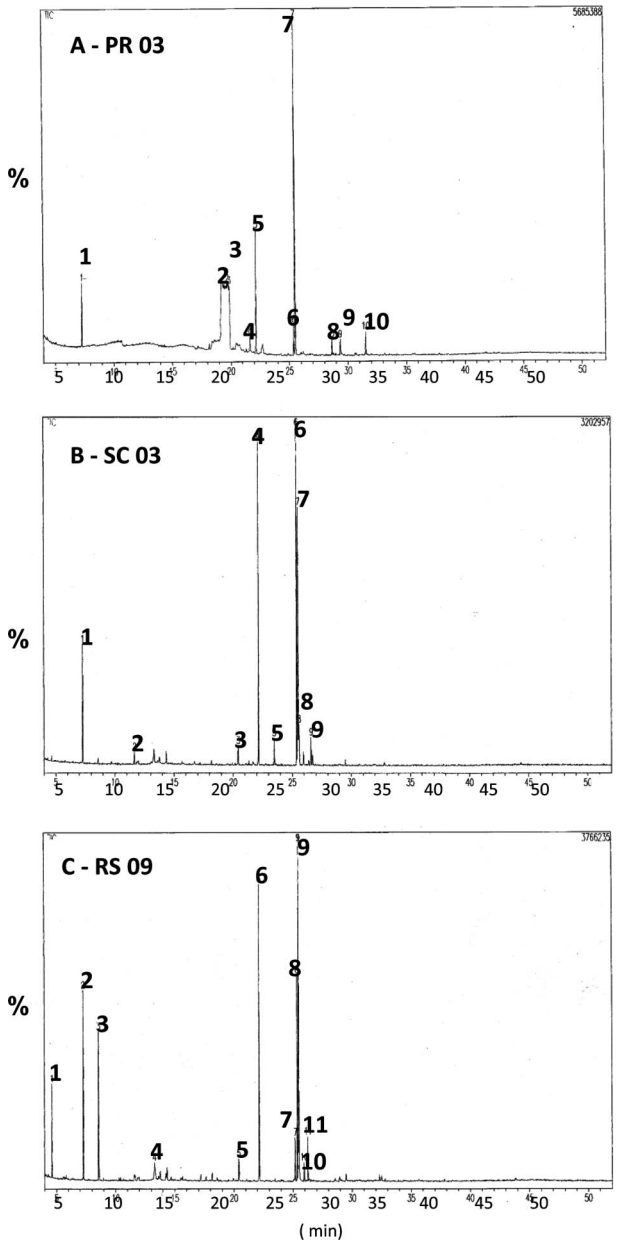


Figure 4. Chromatographic profile (CG-MS) of bee pollen extracts. (A) PR 03; (B) SC 03; (C) RS 09.

Figura 4. Perfil cromatográfico (CG-MS) de los extractos de polen de abeja. (A) PR 03; (B) SC 03; (C) RS 09.

high-efficiency liquid chromatography with spectrophotometry in the UV region because no derivatization before analysis is required (Justesen & Knusthesen, 2001; Matilla & Kumpulainen, 2002). However, compared to mass spectrometry, the UV-Vis spectrum does not provide sufficient data for the identification of these compounds (Chen, Zuo, & Deng, 2001). Thus, GC–MS can provide more detailed and specific results, as long as the derivatization methodology is appropriate. The study of not volatile and thermolabile phenolic compounds through GC–MS involves their conversion into volatile and thermotolerant compounds by a suitable chemical derivatization (Zuo, Wang, & Zhan, 2002).

According to Proestos, Serreli, and Komaitis (2006), silylation is an alternative procedure for the GC–MS analysis of volatile and thermolabile compounds, like most phenolic compounds. Silylation is a nucleophilic substitution reaction in which one active hydrogen of —OH, —COOH, =NH, —NH2 or —SH is replaced by a trimethylsilyl group. Silyl compounds must be of low basicity and capable of stabilizing a negative charge in the transition state (Chu, Chang, Liao, & Chen, 2001). However, the silylation process needs further studies to identify the silyl derivatives, since it depends on skills and laboratory structures, use of toxic and specific reagents such as n o-bis trimethylsilyl trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), hexamethyldisiloxane (HMDS), dimethyldichlorosilane (DMDCS) and toluene (Proestos et al., 2006).

Gas chromatography of flavonoids seems to be a topic of continuing interest, since it has been studied by several authors (Chen & Zuo, 2007; Liu et al., 2007). The high performance liquid chromatography–mass spectrometry (HPLC–MS) has been widely used for the identification of phenolic compounds in plant extracts; however, few Brazilian laboratories have this equipment and experience in the analysis of phenolic compounds and flavonoids, besides being a very expensive technique. According to Proestos et al. (2006), the use of HPLC–MS provides an important advantage in combining the ability to separate the liquid chromatography with the power of mass spectrometry, as a method of identification and confirmation of chemical compounds.

Botanical origin

The "pollen loads" studied here, consisted of a mixture of pollen types from many different floral species, whose coloring varied from light yellow to darker colors, such as

Table 3. Gas chromatography/mass spectrometry analysis of bee pollen extract (SC 03 sample).

Tabla 3. Análisis de cromatografía de gases/espectrometría de masas del extracto de polen de abejas (muestra SC 03).

Peaks #	Chemical compounds	Rt (min)	m/z (%)
1	Benzoic acid, methyl ester	7.29	136 [32, M^+], 105 (100), 77 (92), 51 (68)
2	4-methoxy benzoic, methyl ester	11.66	166[24, M^+], 135(100), 40(65), 77(32), 63(16), 92(16), 107(16)
3	nd	20.45	224 [24, M^+], 118 (100), 40 (32), 209 (32), 91(20), 77(16), 193(12), 65(8), 103(8), 179 (8)
4	Hexadecanoic acid, methyl ester	22.18	143[8, M^+], 74(100), 43 (72), 87(60), 55(36)
5	nd	23.48	157[4, M^+], 88(100), 43(80), 55(44), 101(36), 70(20)
6	9,12-octadecadienoic acid, methyl ester	25.40	294 [M^+], 41 (100), 67 (82), 81 (64), 82 (36), 96 (32), 110 (12), 135 (4), 150 (4)
7	9,12,15-octadecatrienoic acid, methyl ester	25.51	292 [M^+], 41 (100), 55 (72), 79 (56), 95 (28), 108 (12)
8	nd	25.60	137[M^+], 41(100), 55(96), 69(44), 83(28), 97(16), 110(8)
9	nd	26.61	150[M^+], 41(100), 67(84), 55 (80), 81(60), 95(36), 110(12)

Note: Rt, Retention time (min); m/z, mass-to-charge ratio; nd, not detected.

Nota: Rt, Tiempo de retención (min); m/z, la masa-a-carga; nd, no detectado.

Table 4. Gas chromatography/mass spectrometry analysis of bee pollen extract (RS 09 sample).

Tabla 4. Análisis de Cromatografía de gases/espectrometría de masas del extracto de polen de abejas (muestra RS 09).

Peaks #	Chemical compounds	Rt (min)	m/z (%)
1	Propanedioic acid, dimethyl ester	4.63	132 [M^+], 59 (100), 101(64), 42(52), 74(40)
2	Benzoic acid, methyl ester	7.29	136 [32, M^+], 105 (100), 77 (92), 51(68)
3	Phenylacetic acid, methyl ester	8.59	150 [16, M^+], 91(100), 65 (16), 40(8)
4	1-Dodecene	13.29	111 [7, M^+], 43(100), 55(76), 69(40), 83(28), 97(16)
5	nd	20.45	224 [24, M +], 118 (100), 209(32), 91(20), 40 (16), 77 (12), 193(12), 96(8), 179(8), 165 (4)
6	Hexadecanoic acid, methyl ester	22.18	143 [8, M^+], 74(100), 43 (48), 87(60), 55(32)
7	nd	25.21	161 [4, M^+], 41(100), 79(96), 67(52), 91(48), 55(44), 105 (20), 119 (8), 133 (4), 147 (4)
8	9,12-octadecadienoic acid, methyl ester	25.39	294 [M^+], 41 (100), 67 (88), 55(84), 81 (64), 82 (37), 96 (36), 110 (12), 135 (4), 150 (4)
9	9,12,15-octadecatrienoic acid, methyl ester	25.53	292 [M^+], 41 (100), 79 (72), 55 (68), 67(56), 93 (32), 108 (16), 121(8), 135(4), 149(4)
10	Nonadecanoic acid, methyl ester	25.97	312 [M^+], 74(100), 43(80), 87(60), 55(36), 143(8)
11	nd	26.29	161 [M^+], 41(100), 67(76), 81(56), 55(52), 95(28), 100(8), 121(8)

Note: Rt, Retention time (min); m/z, mass-to-charge ratio; nd, not detected.

Nota: Rt, Tiempo de retención (min); m/z, la masa-a-carga; nd, no detectado.

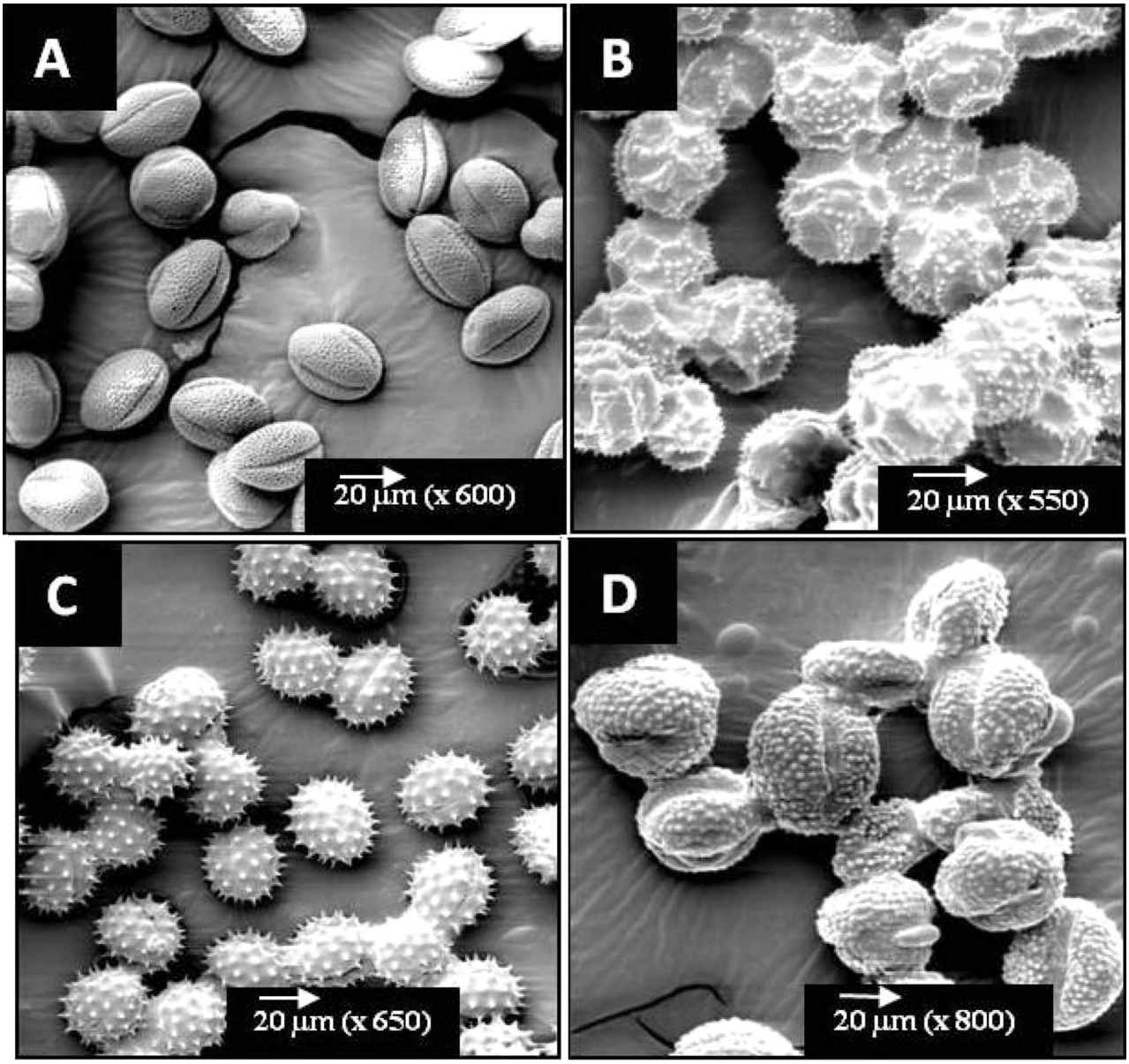


Figure 5. Pollen types observed in pollen loads in the PR 03, SC 03 and RS 09 samples. (A) Brassicaceae; (B) *A. elephantopus*; (C) *A. eupatorium*; (D) *A. gochnatia.*

Figura 5. Tipos de polen observados en las cargas de polen en las muestras PR 03, SC 03 y RS 09 samples. (A) Brassicaceae; (B) *Asteraceae elephantopus*; (C) *Asteraceae eupatorium*; (D) *Asteraceae gochnatia.*

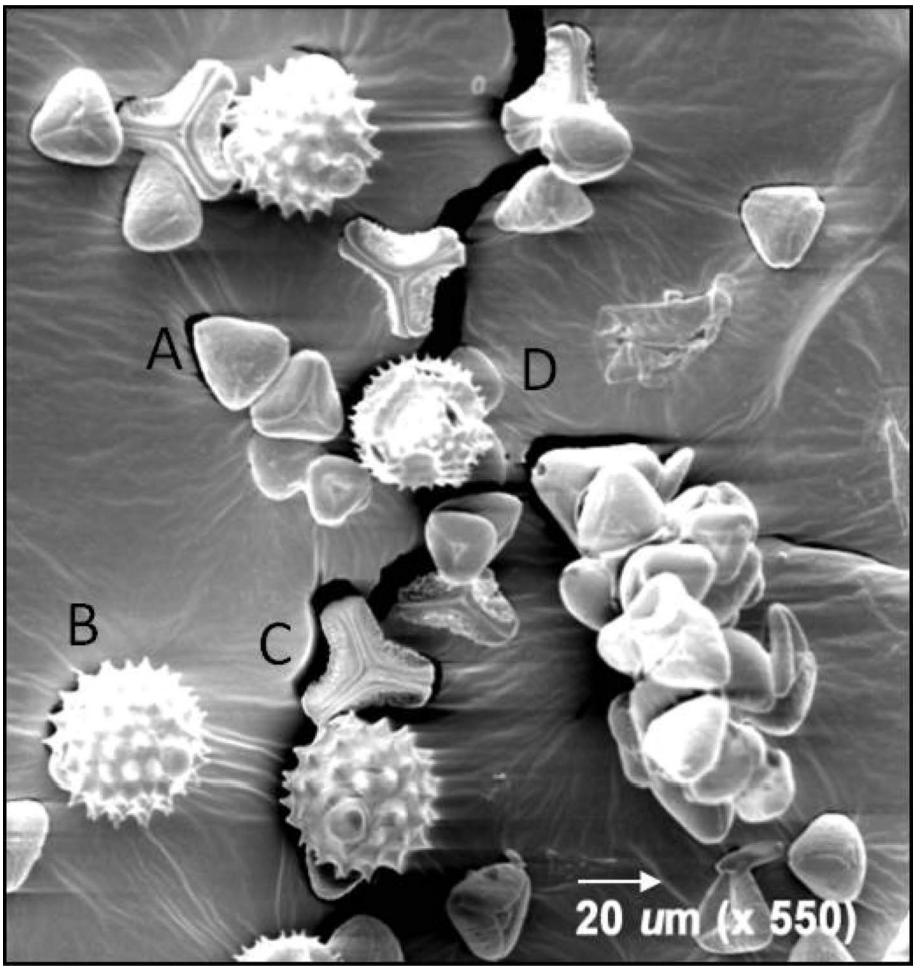


Figure 6. Pollen types observed in pollen loads in the RS 09 samples. (A) *M. eucalyptus;* (B) *A. baccharis;* (C) *L. struthanthus*; (D) *A. elephantopus.*

Figura 6. Tipos de polen observados en las cargas de polen en las muestra RS 09. (A) *Myrtaceae eucalyptus;* (B) *Asteraceae baccharis;* (C) *Loranthaceae struthanthus*; (D) *Asteraceae elephantopus*.

purple and brown. All the samples (PR 03, SC 03, RS 09) were classified as heterofloral, the samples contained at least two pollen types (Figure 5). In the sample PR 03, Brassicaceae family (Figure 5A) was the dominant pollen (>45%) however, the pollen type from the *Asteraceae elephantopus* (Figure 5B) was observed as occasional isolated pollen (<3%). The pollen-type *Asteraceae eupatorium* (Figure 5C) and *Asteraceae gochnatia* (Figure 5D) were found in the SC 03 sample as important isolated pollen (3–15%). Four pollen types were identified in the sample RS 09, *Myrtaceae eucalyptus* as dominant pollen, *A. elephantopus* and *Asteraceae baccharis* as accessory pollen (15–45%) (Figure 6). In this sample, the pollen type *Loranthaceae struthanthus* (Figure 6) was present as important isolated pollen (3–15%). The pollen-type *M. eucalyptus* (Figure 6) was present as dominant pollen too in SC 03 sample. Nevertheless, it is known that honey from the Southern region of Brazil is predominantly of Asteraceae (Compositae), especially *Senecio brasiliensis*, ''maria-mole'' and *Lithrea* sp. ''aroeira'' (Barth, 1989). In other studies, Barth and Dutra (2000) confirmed that the pollen types of various species of Asteraceae, *M. eucalyptus* and *Mimosa scabrella*, frequently occur in the honey from the Southern region of Brazil. Morais et al. (2011) also found in pollen collected in Natural Parks in Portugal, Asteraceae and Myrtaceae families. These families were also detected in pollen from Viana do Castelo, Portugal, and the family Myrtaceae was classified as dominant pollen (Estevinho, Rodrigues, Pereira, & Feás, 2012).

Conclusions

This study has fostered important data for the determination of the total antioxidant capacity and identification of some flavonoids and phenolic acids in bee pollen samples from southern Brazil. The resin Amberlite XAD2 provided the adsorption of phenolic compounds of effective antioxidant activity and may be a suitable treatment in the characterization of polyphenols from bee pollen.

Some polyphenols could be identified with the aid of instrumental techniques such as HPLC–PDA and GC–MS; however, many of the phenolic compounds were not identified and thus, there is need to supplement this study with more appropriate techniques such as HPLC–MS and nuclear magnetic resonance (NMR) to identify more accurately the structure the compounds. These scientific data on the concentration of total polyphenols and flavonoids in bee pollen will enable the complete chemical

characterization of bee pollen from southern Brazil and its consolidation as a safe and nutritious food to consumers and as functional raw-material for the food industry.

The pollen types found in greater number in the samples were from the Asteraceae family. The pollen-type Brassicaceae was found as dominant in PR 03 while in SC 03 and RS 09 the *M. eucalyptus* was dominant pollen type.

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