Evalution of Phenolic Compounds in Brazilian Propolis from Different Geographic Regions

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Chemometrics has been shown quite efficient to uncover relationships between chemical composition of a sample and its geographical origin. Forty propolis samples originated from the the South and South East of Brazil were analyzed by HPLC and 18 compounds of interest were studied which included: caffeic, *p*-coumaric and ferulic acids, and some of their derivatives, pinobanksin, a derivative of kaempferol and five phenolic compounds (assigned as 3-prenyl-4-hydroxycinnamic acid (PHCA); 2,2-dimethyl-6-carboxyethnyl-2H-1-benzopyran (DCBE); 3,5-diprenyl-4-hydroxycinnamic acid (DHCA); compound E (still unknown) and 6-propenoic-2,2-dimethyl-8-prenyl-2H-1-benzopyran acid (DPB). Principal Component Analysis (PCA) indicated three different groups of propolis samples, having the same typical chromatogram, evaluated by HPLC. Samples from the South East group were rich in derivatives of kaempferol. Samples from the South group I had a high content of DPB compound, but a low concentration of kaempferol derivatives and of DCBEN compound. Samples from the South group II were characterized by a high concentration of DCBEN, DHCA, *p*-coumaric and DPB compounds. Therefore, the identification of new compounds in Brazilian propolis can give useful information about the plant sources of a given geographic region.

Introduction

Propolis, a resinous substance collected by honeybees from different plant exudates, is used to fill gaps and to seal parts of the hive. Propolis has many biological activities such as, antibacterial, antiviral, fungicidal, antitumoral, among others (Ghisalberti, 1979; Marcucci, 1995). At least 200 compounds have been identified in different samples, where a simple sample might have more than 100 compounds with include fatty and phenolic acids and esters, substituted phenolic esters, flavanoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, β-steroids, aromatic aldehydes, alcohols, sesquiterpenes, naphthalene and stilbene derivatives (Greenaway *et al.,* 1987; Greenaway *et al.,* 1991; Bankova *et al.,* 1992a; Marcucci, 1995).

Propolis has different botanical origins providing a distinct chemical composition. Previous studies of the chemical composition revealed that the resinous secretions of the black poplar (*Populus nigra*) could be considered as the main source of European propolis (Bankova *et al.,* 1992b; Garcia-Viguera *et al.,* 1993). Since there are no poplars in tropical regions, the chemical composition of South American propolis is potentially of interest. Investigations about the bee glue from Venezuela

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(Tomas-Barberan, *et al.,* 1993) and Brazil (Aga *et al.,* 1994) showed an unusual chemical composition, and, as expected, polyphenols from poplars were totally absent. With the purpose of uncovering the phenolic composition of Brazilian propolis, the identification and quantification of these compounds were carried out by High Performance Chromatography (HPLC).

Chemometrics is a powerful statistical tool used to uncover "relationships" between certain compounds present in a sample based on its geographic and botanical origin. Chemometrics is becoming necessary nowadays in chemistry, specially when a large amount of data is generated for each sample. In the present investigation, Principal Component Analysis (PCA) (Sharaf *et al.,* 1986; Malinowski, 1991) were used with the purpose to study the relationship between the chemical composition of propolis, as given by HPLC, and its geographical origin and botanical source.

Experimental

The propolis samples were collected in different places in Brazil. The samples and their geographic origin are listed in Table I.

Propolis extraction

One-hundred and fifty milligrams of each sample was cut in small pieces, dissolved in 5 ml of methanol, filtered, passed by a mini-sart membrane (0.45 μm Sartorius, Germany) and stored in a dark recipient until further analysis by HPLC.

HPLC analysis

Forty propolis samples from different geographical regions of Southern and South East Brazil were analyzed by HPLC (Merck-Hitachi, Germany), equipped with a pump (model L-6200, Merck-Hitachi, Germany) and a diode array detector (L-3000, Merck-Hitachi, Germany). Separation was achieved on al Lichrochart 125–4 column (Merck, Darmstadt, Germany) (RP-18, 12.5 × 0.4 cm, 5 μm particle size) using water, formic acid (95:5, v/v) (solvent A) and methanol (solvent B). The elution was carried out with a linear gradient and a flow rate of 1 ml/min $^{-1}$. The detection was monitored at 280 and 340 nm and the compounds identified using standards as references (Garcia-Viguera *et al.,* 1992; Garcia Viguera *et al.,* 1993). For data analysis the Merck-Hitachi D-6000 (Chromatography Data Station – DAD Manager) was used.

Spatial distribution of samples

In order to study the influence of geographical distribution, analysis was carried out on the sampling points by the georeferencing GIS* procedure. This procedure was based on the original city of the sample and on an spatial distribution of all Brazilian cities. This spatial analysis pinpoints the predominant propolis samples specifically situated at a common region. For example, the generic samples **a** to **c** from a specific geographic region (e.g. region **A**) have the predominance of compound **zz.** On the other hand, samples **d, e,** and **f** in region **B** have **yy** element as the most predominant one.

Results and Discussion

Eighteen different compounds have been identified and quantified in different propolis samples in Southern and South East of Brazil (Table I). These included caffeic acid (Caf) (retention Time (R_t) of: 1.55 minutes) and derivatives (Caf_1, R_t: 6.44 min; Caf_2, R_t: 12.10 min), *p*-coumaric acid (*p*-C, R_t: 2.96 min) and derivatives (p-C_1, R_t: 16.87 min; p-C_2 R_t: 17.25 min; p-C_3, R_t: 20.60 min; p-C_4, R_t: 21.63 min; p-C_5, R_t: 24.85 min; p-C_6, R_t: 25.75 min), ferulic acid (Fer, R_t: 3.99 min), pinobanksin (Pink, R_t: 11.30 min), a kaempferol derivative (Kaemp., R_t: 15.06 min) and five phenolic compounds labeled as PHCA (R_t: 17.38 min), DCBEN (R_t: 19.70 min), DHCA (R_t: 27.55 min), E (R_t: 31.03 min) (still unknown) and DPB (R_t: 31.42 min) (Fig. 1). DCBEN and DHCA were firstly isolated by Bohlman *et al.* (1981) and Labbe *et al.* (1986). Aga *et al.* (1994) also reported the isolation of the same compounds. In a recent review, all of these compounds were isolated and identified by Banskota *et al.* (1998).

The propolis samples originated from different locations from the South East Brazilian States (Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP)) and from Southern States (Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS)) (Fig. 2).

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* Geographic Information System software for Desktop Computer: Maptitude by Caliper Inc.

Table I. Location and plant sources of propolis samples.

nº	Location	Plant source	G.R.*	nº	Location	Plant source	G.R.*
11	Arroio dos Ratos-RS	2	SI	43	Bambuí-MG	3	SE
12	Taquara-RS	2	SI	49	Cambará do sul-RS	4	SI
14	Ponta Grossa-PR	2 + 5	SI	50	Pelotas-RS	4	SI
16	Alegrete-RS	4 + 2	SI	54	Bagé-RS	4	SI
20	Jarinu-SP	1 + 3	SE	62	Mafra-SC	?	SII
21	Mogi das Cruzes-SP	1	SE	63	Mafra-SC	5	SII
22	Estiva-MG	3	SE	64	S.J.do Triunfo-PR	6	SII
23	Mairiporã-SP	1 + 3	SI	65	Irati-PR	6	SII
24	Araraquara-SP	1	SI	66	Pinhão-PR	6	SI
25	Mairiporã-SP	1	SE	67	Balsa Nova-PR	6	SII
26	Ibiuna-SP	3	SE	68	Itaiópolis-SC	5	SII
27	Indaiatuba-SP	1	SE	74	Dois Córregos-SP	1	SE
28	Paty do Alferes-RJ	1 + 3	SE	82	Prudentópolis-PR	6	SII
33	Barra do Piraí-RJ	3	SE	87	Aramif.Pinhal S.Bento	6	SI
34	Caratinga-MG	1	SI	89	S.Lour.do Oeste-SC	6	SII
35	Sengés-PR	2 + 6	SI	91	Caxias do Sul-RS	4	SII
37	S.J.dos Pinhais-PR	3	SI	92	Curitiba-PR	6	SII
39	Sta.Helena-PR	6	SI	93	Curitiba-PR	6	SI
40	Paraná (?)-PR	6	SI	95	Tijucas do Sul-PR	6	SI
42	Tabatinga-SP	?	SE	97	Mandirituba-PR	6	SII

* G. R.: Geographic Region (SE, South Eastern; SI, South I; South II) identified by principal component analysis. Plant Source: 1, Eucalyptus from São Paulo State; 2, Eucalyptus from Paraná, Santa Catarina and Rio Grande do Sul States; 3, Native Forest from São Paulo, Rio de Janeiro and Minas Gerais States; 4, Native Forest from Rio Grande do Sul State; 5, Native Forest from Santa Catarina State, 6, Native Forest from Paraná State.

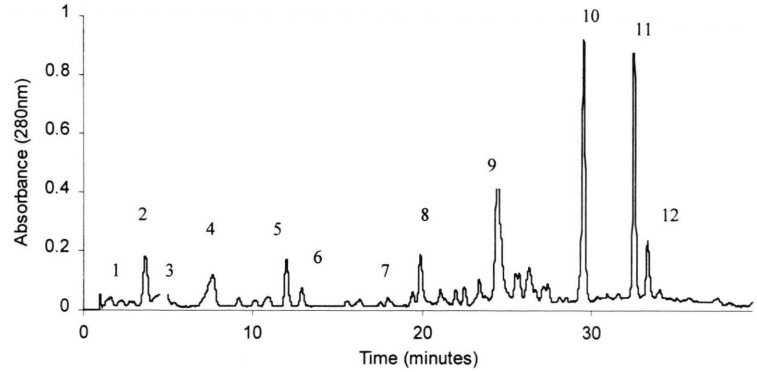

Fig. 1. Typical chromatogram of some compounds of interest present in propolis. Separation at λ=280 nm (see experimental conditions). Legend: 1, caffeic acid (Caf); 2, *p*-coumaric acid (p-C); 3, ferulic acid (Fer); 4, caffeic acid derivative (Caf_1); 5, pinobanksin; 6, caffeic acid derivative (Caf_2); 7, kaempferol derivative (Kaemp); 8, PHCA; 9, DCBEN; 10, DHCA; 11, E (unknown) and 12, PCB.

Table II shows the concentration range of phenolic compounds identified in different propolis samples, according to their retention time. A preliminary screening analysis has shown that the compound *p*-C6 was detected in only one sample, and *p*-C_3, *p*-C_4, *p*-C_5 and Caf_2 in a few samples. Principal component analysis performed with all the eighteen variables from the data set indicated that only seven variables had scores significantly different from zero. In this manner, from the eighteen variables experimentally measured, only seven would be suitable for this data analysis.

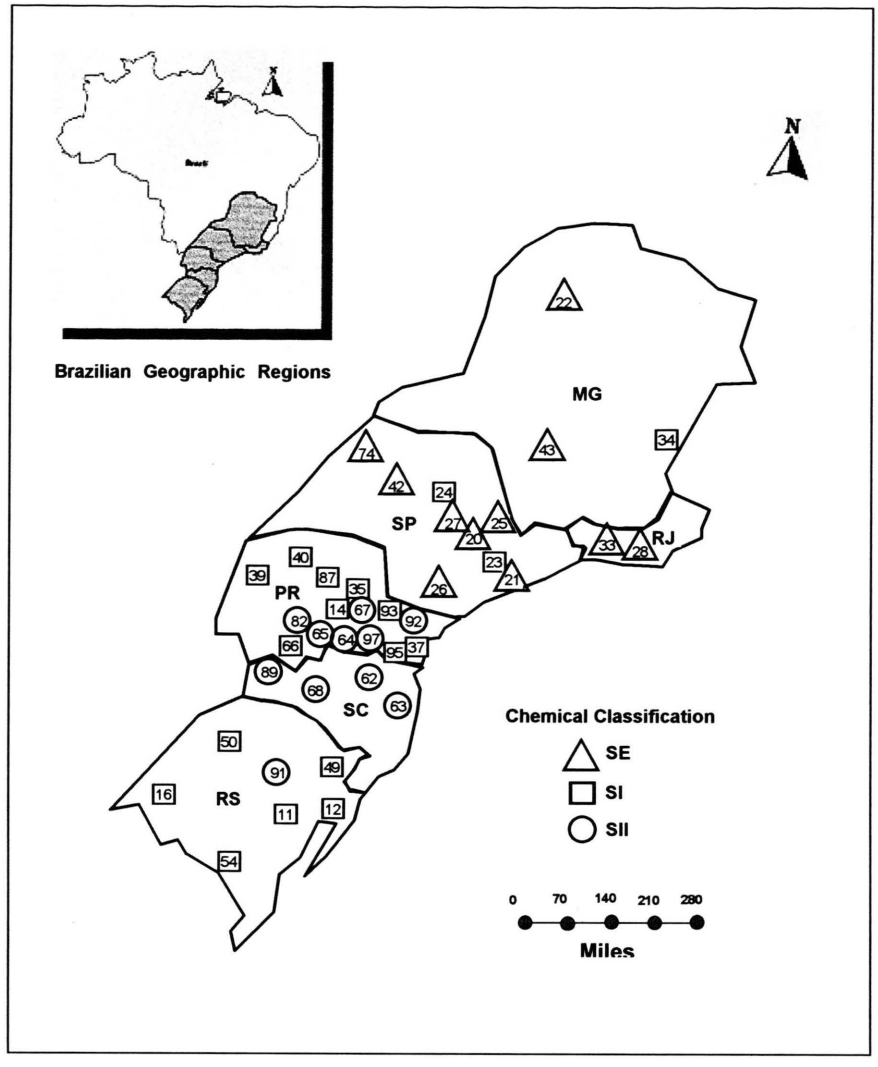

Fig. 2. Geographic distribution of samples belonging to groups SE, SI and SII.

Looking at the modeling power* of all variables analysis, the DCBEN, DHCA, DPB, *p*-C and kaempferol derivative presented values ≥ 0.6, while the variables E and pinobanksin (pink), approximately 0.3. The selected variables for the exploratory data analysis were PHCA, DCBEN, DHCA, DPB, *p*-coumaric (p-C), and a kaempferol derivative. The variable E was excluded based on the fact that it did not give any contribution in the cluster's separation. It was present in all samples in large amounts. The chosen variables were the largest peaks as seen in the chromatogram (Fig. 1). The reduction of the variable number brings more advantages to process the chemometric analysis.

Principal Component Analysis (PCA) indicated three different groups of propolis samples, having the same typical chromatogram, evaluated by HPLC. Samples originated from South East are characterized by having a high concentration of kaempferol derivative, a fair amount of DBP and absence or low content of DCBEN. Samples from

* Modeling power, MP, is a very useful for variable selection and it is defined as: MP = 1 – Sj/Sj(x) where S^2_j is the variable residual variance and $S^2_j(x)$ is the total variance of the variable.

Table II. Concentration range (mg/g) of phenolic compounds identified in different propolis samples.

Compound*	Concentration range (mg/g of sample)
Caffeic acid	0.17–1.72
p-Coumaric acid	0.15–9.09
Ferulic acid	0.11–7.34
Caffeic acid derivative 1	0.12–42.01
Pinobanksin	1.82–13.25
Caffeic acid derivative 2	0.57–42.81
p-Coumaric acid derivative 1	0.17–71.96
p-Coumaric acid derivative 2	0.38–50.02
3-Prenyl-4-hydroxycinnamic acid (PHCA)	0.88–5.02
2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran (DCBEN)	1.18–22.06
p-Coumaric acid derivative 3	0.67–24.33
p-Coumaric acid derivative 4	0.53–39.25
Kaempferol derivative	3.64–191.19
p-Coumaric acid derivative 5	0.80–43.33
p-Coumaric acid derivative 6 (detected in only one sample)	1.94
3,5-Diprenyl-4-hydroxycinnamic acid (DHCA)	1.54–17.86
E compound	2.07–63.04
6-Propenoic- 2,2-dimethyl-8-prenyl-2H-1-benzopyran (DPB)	1.51–23.61

* The compounds are listed according to their retention time.

SI group have a high content of DPB, but absence or low concentrations of kaempferol derivative and DCBEN. Samples of the South II are characterized by a high content of DCBEN, DHCA, *p*-coumaric acid and DPB compounds. Except for three samples, kaempferol derivative is absent in this group.

Bonhevíand Ventura Coll (1994) reported the results of cluster analysis of propolis using the chemical composition that they found as follows: vanillin, ferulic acid, rutin, 4-hydroxybenzoic ethyl ester, quercetin, kaempferol, apigenin, isorhamnetin, galangin, acacetin, pinocembrin, tectocrysin and total phenol compounds. These authors argued that it was possible to separate different groups based on flavonoid patterns and botanical and geographical origins, confirming the results of this study. They concluded that the flavonoids pattern of propolis that they studied were sufficiently distinct to permit the discrimination of propolis from China, Uruguay and Brazil. Flavonoids are the main constituents of propolis from the temperate zone. In most of the Brazilian propolis investigated until now, flavonoids are absent (Bankova *et al.,* 1995) except the kaempferol derivative and pinobanksin that we reported here and trace amounts of two dihydroxydimethoxy flavanones (Bakova *et al.,* 1995).

With respect to the vegetation source for propolis, it can be said that the propolis collected from *Eucalyptus* plantation are rich in kaempferol derivative and poor in DCBEN, while the propolis originated from native vegetation (specially from South II) are rich in DCBEN, *p*-C, DHCA and DPB compounds.

In order to find more definitive answers with respect to the vegetation, new studies are being developed, by using plant resins to compare with the compounds present in propolis samples of the same geographic region. There are many investigations about the origin of propolis from temperate zones, and it was concluded that poplar species appeared to be the main source of propolis (Bankova *et al.,* 1995), but there are no poplar buds in tropical areas. *Baccharis* spp. and *Araucaria heterofila* are probably the main plant sources of Brazilian propolis (Banskota *et al.,* 1998). Prenylated coumaric acids (PHCA, DHCA) and benzopyranes (DCBEN) originating from prenylated coumaric acids have been found in different *Baccharis* species (Boudourova-Krasteva *et al.,* 1997) and diterpenes in *Araucaria heterophylla.* Therefore, the identification of new compounds in Brazilian propolis can give useful information about the plant sources of a given geographic region of collection.

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