Electrospray ionization mass spectrometry fingerprinting of propolis

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Crude ethanolic extracts of propolis, a natural resin, have been directly analysed using electrospray ionization mass (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) in the negative ion mode. European, North American and African samples have been analyzed, but emphasis has been given to Brazilian propolis which displays diverse and region-dependent chemical composition. ESI-MS provides characteristic fingerprint mass spectra, with propolis samples being divided into well-defined groups directly related to their geographical origins. Chemometric multivariate analysis statistically demonstrates the reliability of the ESI-MS fingerprinting method for propolis. On-line ESI-MS/MS tandem mass spectrometry of characteristic $[M - H]^-$ ion markers provides an additional dimension of fingerprinting selectivity, while structurally characterizing the ESI-MS marker components of propolis. By comparison with standards, eight such markers have been identified: *para*-coumaric acid, 3-methoxy-4-hydroxycinnamaldehyde, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3-prenyl-4-hydroxycinnamic acid, chrysin, pinocembrin, 3,5-diprenyl-4-hydroxycinnamic acid and dicaffeoylquinic acid. The negative mode ESI-MS fingerprinting method is capable of discerning distinct composition patterns to typify, to screen the sample origin and to reveal characteristic details of the more polar and acidic chemical components of propolis samples from different regions of the world.

Introduction

Propolis is an important natural resinous product with a variable chemical composition and a multitude of pharmacological, nutritional, and antimicrobial applications.[1,2] Bees use propolis to reinforce their combs and to keep the hive environment aseptic, extracting this resin from plants around their hives. For centuries propolis has been used mainly for its resinous and anti-microbial characteristics.[3] As propolis is not easily fractionated, at the beginning of the 20th century only its gross composition had been determined (resin, wax, volatile components and insoluble matter) and found to be qualitatively similar between samples of different origins.[3] The first compounds to be identified were cinnamyl alcohol and cinnamic acid,[4] vanillin[5] and then chrysin.[6] From the 1960's on, flavonoids[7–9] and other phenolic compounds[10,11] were identified. With the advent of modern chromatographic techniques frequently associated with mass spectrometry, many compounds have been isolated and identified in propolis, bringing the number of known components into the hundreds.[1] But the complex chemical composition of propolis is frequently updated due to many regional variations.

Propolis composition varies greatly according to the plants found around the hive, with buds from *Populus* species being the main source of resins for European and North American propolis.[12] In areas where these plants are not native, bees find other plant sources for these resins.[13,14] Because of the great diversity of Brazilian plant ecology, the composition of Brazilian propolis is both unique and greatly variable, qualitatively and quantitatively, and attempts have been made to typify Brazilian propolis according to its regional origin.[15,16] The rich composition of Brazilian propolis is being intensely studied, with new compounds being reported every year. It is important therefore, particularly for Brazilian propolis but also for propolis of other origins, to have a direct, fast and reliable

analytical procedure capable of providing fingerprint characterization of crude alcoholic extracts of propolis samples. Such a technique would allow one to rapidly typify the propolis sample, to screen for its geographical origin, and to identify the major class of natural product components, before initiating more refined separation, quantitation, and structural characterization procedures.

For many years mass spectrometry has been used in conjunction with gas chromatography (GC-MS) for the detailed analysis of the main volatile and semi-volatile components of propolis.[17–19] Propolis contains, however, many components that are not volatile enough for direct GC-MS analysis[20,21] even upon derivatization or high-temperature GC-MS.[19] But recently, electrospray ionization (ESI)[22] has revolutionized the way molecules are ionized and transferred to mass spectrometers for mass and structural analysis, and has greatly expanded the applicability of mass spectrometry for a variety of new classes of molecules with thermal instability, high polarity and high mass, even up to millions of mass units.[23] ESI mass (and tandem mass) spectrometry has gained widespread recognition mainly for its successful use in the structural analysis of bio-molecules,[24–26] but this general ionization technique has also opened new approaches for the detailed structural characterization of polar natural compounds.[27,28] ESI-MS has also been used as an efficient and fast fingerprinting method with direct insertion analysis of complex product mixtures such as those found in wine,[29] petroleum,[30] beer[31] and of natural product extracts.[32,33] ESI gently transfers ionized molecules directly from solution to the gas phase, being therefore applicable to thermally labile substances, from low to high molecular weight organic molecules with medium to high polarity, and practically all polar solvents may be used.[22] ESI is also fast and fully compatible with liquid chromatography, and has therefore been applied in high throughput screening procedures.[34] ESI is convenient for direct MS analysis of multi-component polar natural product extracts because most molecules

bearing acidic or basic sites will be detected as a single ion, either in their protonated [MH$^+$] or deprotonated [M − H]$^-$ forms. On-line tandem MS/MS with collision-induced dissociation (CID) of MH$^+$ or [M − H]$^-$ is used for more refined structural elucidation studies.[35]

In this study, we show that ESI-MS, particularly in the negative ion mode (ESI(−)-MS), is feasible for rapid fingerprinting of propolis and for MS characterization of the more polar acidic components. ESI in conjunction with tandem mass spectrometry (ESI-MS/MS) also provides fast and refined structural characterization of characteristic propolis marker components. Brazilian propolis was selected as a proof-of-principle case because of its great diversity representing therefore a challenging case for testing the ESI-MS propolis fingerprinting method. Samples of red, green and brown propolis from various regions in Brazil were selected for analysis, whereas samples from Africa, Europe and North America were used for comparison.

In addition, to statistically establish the correlation among the propolis samples, chemometric principal component analysis (PCA) has been applied. In chemometrics, PCA[36] is the fundamental basis of most methods of multivariate analysis;[37] it describes the variance in a set of multivariate data in terms of a set of underlying orthogonal variables. The original variables can be expressed as a particular linear combination of the principal components. In PCA, each PC accounts for a portion of the total variance of the data set. Often, a small set of principal components (2 or 3) can be used to resynthesize the data and thus reduce the dimensionality of the data set. Plotting the data in the space defined by the two or three largest PCs provides a rapid means of visualizing similarities or differences in the data set, improving sample discrimination. Chemometric procedures for classification, multivariate calibration and mixture resolution have been extensively applied to different types of mass spectra data, for instance for metabolite fingerprinting.[38] We have also used chemometric methods to help interpret MS data particularly for challenging cases of isomer distinction and quantitation.[39–42]

Experimental

Propolis samples

Samples of Brazilian propolis were collected from the following states: 10 from Minas Gerais (MG 1–10), 5 from São Paulo (SP 1–5), 14 from Paraná (PR 1–14), 1 from Mato Grosso do Sul (MS 1), 7 from Bahia (BA 1–7) and 1 from Alagoas (AL 1). Two Bulgarian samples were collected from the Balkan Mountains (BU1) and from the Black Sea Coast (BU2). One propolis sample from Maputo, Mozambique (MO1), one sample from England (UK1) and one sample from Finland (FI1) were also obtained, as well as two samples from North America, one from the state of New York (US1) and one from Indiana (US2).

Extraction procedure

All samples were ground prior to extraction. The samples were extracted by maceration for 7 days in a shaker, regulated at a speed of 100 opm and temperature of 30 °C, with 10 mL of absolute ethanol (Merck, Darmstadt, Germany) for every 3 g of crude propolis. The insoluble portion was then separated by filtration, the filtrates kept in a freezer at −16 °C overnight and filtered again at this temperature to reduce the wax content of the extracts. Solvent was then evaporated on a water bath at a temperature of 50 °C to obtain dry extracts of propolis.

General experimental procedures

Ethanolic extracts of propolis (EEP) were analyzed by direct infusion into the ESI source by means of a syringe pump (Harvard Apparatus) at a flow rate of 15 μL min^{-1}. ESI-MS and ESI-MS/MS (low energy CID) spectra were acquired using a hybrid high-resolution and high-accuracy (5 ppm) Micromass Q-TOF mass

spectrometer. For ESI in both the negative and positive ion modes, capillary and cone voltages were set to ±3000 V and ±40 V, respectively, with a de-solvation temperature of 100 °C. For CID in the negative ion mode ESI, the collision energy was optimized for each component, varying from 15 to 30 eV. The dry extracts of the propolis samples were dissolved in a solution of 70% (v/v) chromatographic grade methanol (Tedia, Fairfield, OH, USA) and 30% (v/v) deionized water. The solutions used for ESI(−)-MS analysis contained approximately 50 ng of dry propolis extract for every 1 mL of methanolic solution plus 5 μL of ammonium hydroxide (Merck, Darmstadt, Germany).

Statistical analysis of data

Principal Component Analysis (PCA) was performed using the 2.60 version of Pirouette software from Infometrix, Woodinville, WA, USA. The mass spectra were expressed as the intensities of individual [M − H]$^-$ ions (*i.e.* variables) of two of the most characteristic negative ion markers of each sample. The data was preprocessed using auto scale and the PCA method was run. The three principal components were able to explain 58% of the total variance in the data.

Results and discussion

As compared to the positive ion mode, the negative ion mode of ESI rendered by far the most informative results for fingerprinting of all samples of propolis analyzed. Therefore, although ESI in the positive ion mode has also been tested, only the results obtained by ESI-MS in the negative ion mode will be presented and discussed. Most of the main components of propolis are organic acids and phenols; hence they ionize easily in basic solutions by deprotonation, and are therefore transferred efficiently to the gas phase as [M − H]$^-$ negative ions. Samples of Brazilian propolis were also analysed more than once, over several months, showing quite similar ESI-MS spectra for those originated from the same region.

European and North American propolis

Two samples from North American (Indiana and New York) propolis, two samples of Bulgarian propolis, and single samples from England and Finland showed very similar ESI-MS spectra (Fig. 1), which confirmed the great similarity in their plant origins.[12] Major negative ion markers are those of *m/z* 253, 255, 269, 271, 285 and 313. Major ions of *m/z* 253, 255, 269, 271 were also detected in the single ESI-MS spectrum reported by Mauri and Pietta (2000)[32] for a single sample of (presumably) European propolis, and these ions were assumed to be the deprotonated forms of flavonoids common in European propolis, that is, chrysin, pinocembrin, apigenin/galangin and naringenin, respectively. Interestingly, the group of European and North American propolis samples we analysed showed a unique and characteristic ESI-MS negative ion marker of *m/z* 313. This ion is by far the most abundant in their ESI(−)-MS fingerprint spectra (Fig. 1), and this ion was also observed with lower intensity in the ESI(−)-MS spectrum of the European propolis sample analyzed by Mauri and Pietta (2000).[32]

African propolis

The sample of propolis from Mozambique displays also a unique, characteristic ESI-MS fingerprint spectrum (Fig. 1). Major negative ion markers are those of *m/z* 239, 255, 269, and 369. The uniqueness of the Mozambique sample is certainly a result of its rather characteristic African plant sources.

Propolis from the northeast of Brazil: The unique "Red Brazilian propolis"

The samples from the states of Bahia and Alagoas in the northeast of Brazil can be easily divided into two main groups (R1 and R2)

by visual inspection of their ESI-MS fingerprints (Fig. 1). The R1 group (3 samples: BA1, BA2 and AL1) displays a unique "ruby" red color, an uncommon feature for propolis, and most characteristic negative ion markers of m/z 255, 267, 271, 285, 519, and 601.

The samples belonging to the R2 group came from the state of Bahia (BA 3–7); they display a less intense reddish-brown color and characteristic ESI(−)-MS fingerprints. Many negative ion markers are of relatively high intensity (Fig. 1), mainly those of m/z 255, 281, 311, 325, 339 and 441. Propolis from this region of Brazil has only recently begun to be studied, therefore relatively little is known about its chemical composition and phytochemical origins and phytotherapic properties.

Propolis from the south and southeast of Brazil: The "Green and Brown Brazilian propolis"

The samples of propolis from the south and southeastern states of Brazil, which are distinguished by their green (G) or brown (B) colors, display ESI-MS fingerprints (Fig. 1) all characteristic and rather distinct from those of European and North American propolis, as well as from the samples of Red Brazilian propolis. They also vary significantly among themselves, and can be clearly divided into three groups, as discussed below.

The greatest number of samples belongs to the group of "green propolis" (G), with major ESI-MS negative ion markers of m/z 231, 255, 299, 315 and 363 (Fig. 1). Note that, owing to the high resolution of the orthogonal TOF mass analyzer, two ions of nominal m/z 299, that is 299.2 and 299.3, are detected, which enhances the selectivity of the fingerprinting characterization of G propolis (see insert in Fig. 1). G propolis clearly shows both m/z 299.2 and 299.3 ions, whereas the brown B1 and B2 samples show only one or none of these ions. The G group comprised all of the samples from the states of São Paulo (SP 1–5), Minas Gerais (MG 1–10), Mato Grosso do Sul (MS1) and 4 samples from the state of Paraná (PR1, PR3, PR4 and PR5). Two of the samples from the state of Minas Gerais were typical of G propolis, except for the absence of the ion of m/z 255 and the presence of the ion of m/z 321, which in one sample was nearly as intense as that of m/z 299. Several of the components identified in propolis samples of this group (Table 1) have also been identified in samples of *Baccharis*
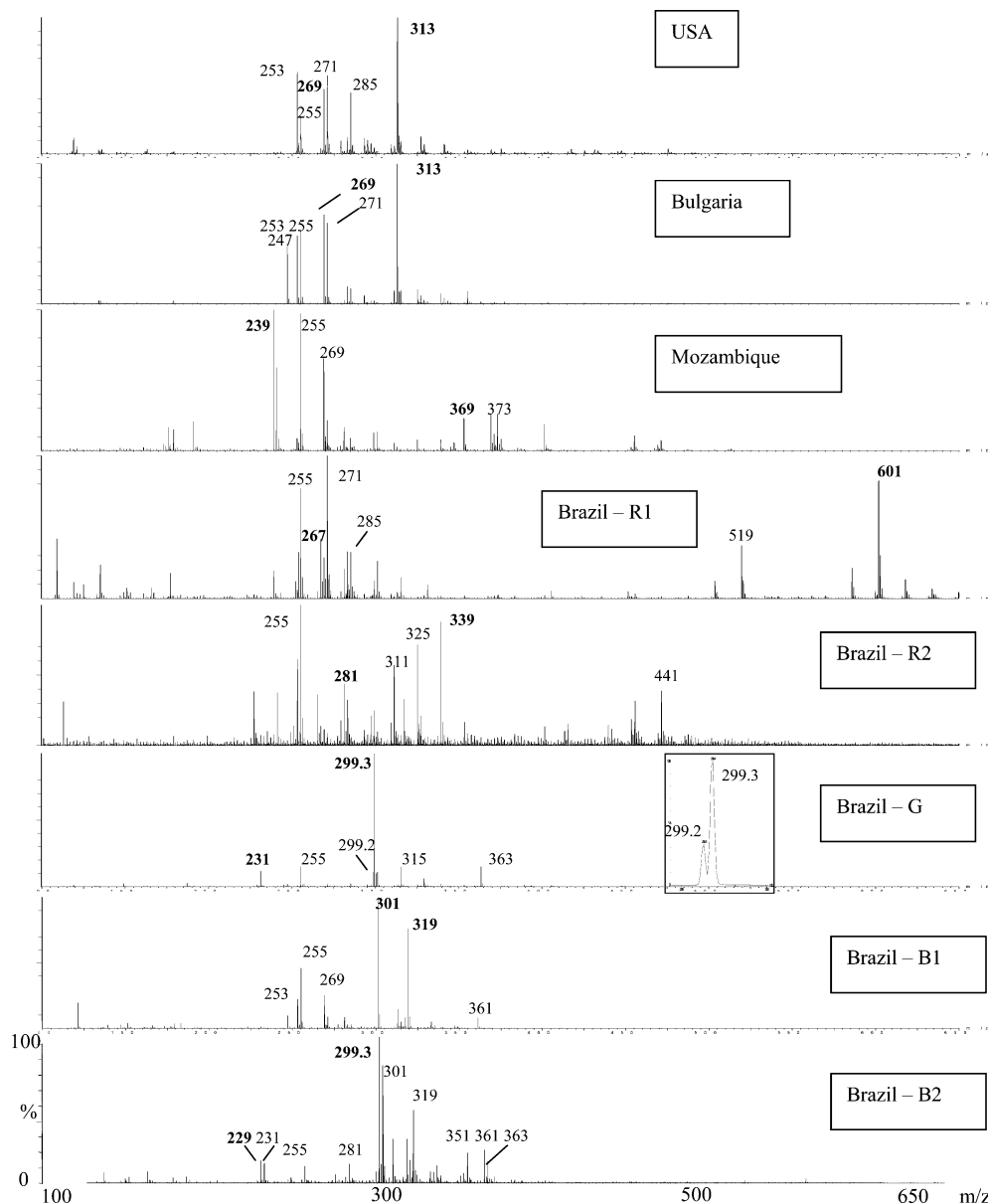
Fig. 1 ESI(−) mass spectra of ethanolic extracts of propolis from Europe, North America, Africa and different regions in Brazil. R1 and R2 are "red propolis" from the Northeast of Brazil; G is "green propolis" from the Southeast of Brazil while B1 and B2 are "brown propolis" from the South of Brazil. Main [M − H]⁻ ion markers are indicated, whereas the two most characteristic ion markers used for PCA are shown in bold.

dracunculifolia,[16,43,44] confirming this plant as an important source for propolis from the southeast of Brazil.

Two groups of "brown propolis" (B) were differentiated within the samples from the south of Brazil. The first group, B1, with all 4 samples coming from the state of Paraná (PR 8, PR9, PR10 and PR11), displays as the most characteristic ESI-MS negative ion marker that of *m/z* 301. In these B1 samples the ions of *m/z* 229 (characteristic of B2), 231 and 299 (characteristic of G) are not detected. Additional and also characteristic features of B1 are the ions of *m/z* 253, 255, 269, 319 and 361. The similarity between the negative ion markers of propolis from this sub-tropical region of Brazil and those from European and North American propolis indicate similar plant sources.

The second group of "brown propolis", B2, also came from the state of Paraná, in the South of Brazil (PR2, PR6, PR7, PR12, PR13 and PR14). The ion marker of *m/z* 299.3 is still the most abundant (as for G) and all the major negative ion markers of the G group are present. In addition, B2 display most of the characteristic negative ion markers of the B1 group, mainly those of *m/z* 301, 319, 351 and 361, but a unique negative ion marker for B2 is that of *m/z* 229. These results indicate that the resins for the propolis samples of the B2 group were obtained from more than one important plant source; probably owing to a superposition of the vegetation that originates both G and B1 propolis samples.

Structural characterization *via* tandem mass spectrometry

Although it is not the purpose of the present investigation to determine propolis composition by direct insertion ESI-MS, we have identified some of the major negative ion markers mainly to demonstrate the capability of such an approach for enhanced selectivity in bi-dimensional ESI-MS/MS propolis fingerprinting. Seven compounds present in propolis from the south and southeast of Brazil have been identified by comparison of their tandem mass spectra with those of standards acquired commercially or isolated in relation to previous research:[18,21] *para*-coumaric acid, 3-methoxy-4-hydroxycinnamaldehyde, 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopyran, 3-prenyl-4-hydroxycinnamic acid, chrysin, pinocembrin and 3,5-diprenyl-4-hydroxycinnamic acid (Table 1). These compounds have been reported previously in samples of Brazilian propolis and the activity of some of them against bacteria,[21] *Trypanossoma cruzi*[21] and *Candida*,[45] as well as their cytotoxic[46] and antioxidant[47] activities have been investigated. The ion of *m/z* 515 has been determined to be a deprotonated form of dicaffeoylquinic acid by comparison of its ESI-MS/MS mass

spectrum (Fig. 2) with spectra reported by Miketova *et al.*[48] It is not possible, though, to determine which isomer, or mixture of isomers, is present, as ESI-MS/MS cannot distinguish between them. Caffeoylquinic acids have been studied for their hepatoprotective, antioxidant, antiviral, antibacterial, antihistaminic[48] and macrophage enhancing[49] activities, and are at least partially responsible for these activities in Brazilian propolis.

The detection of characteristic negative ion markers of the same *m/z* ratio in propolis samples from different geographical regions led us to compare their structures by ESI-MS/MS. The [M − H]⁻ ions of *m/z* 247, 269, 271 and 313 found in the Europe, North America, and Brazilian (B1) samples (Fig. 1) display nearly identical tandem mass spectra (not shown), indicating the same structure. The [M − H]⁻ ions of *m/z* 253 and 255 observed in the B2 Brazilian propolis as well as in European and North American samples also display the same MS/MS spectra regardless the origin of the propolis sample. These ions were determined, by comparison with standards, to be the deprotonated forms of chrysin and pinocembrin, respectively.

A negative ion marker of *m/z* 255 is common for all propolis samples analyzed. The ESI-MS/MS spectra of those from the European, North American, African, B1 and R2 Brazilian samples clearly show, by comparison with a standard, that this ion corresponds to deprotonated pinocembrin. Distinct ESI-MS/MS spectra were obtained, however, for the ions of *m/z* 255 from the G, B2 and R1 Brazilian propolis samples, which certainly indicates a different structure, still under investigation.
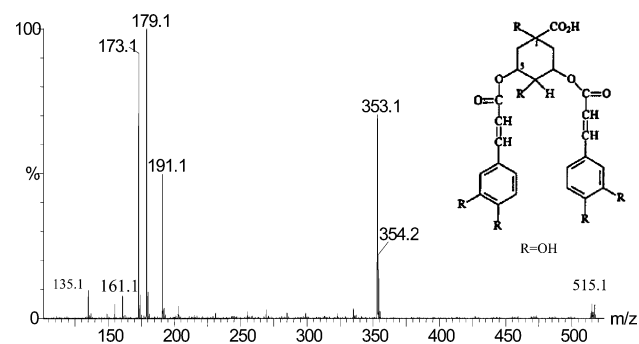
Fig. 2 ESI-MS/MS mass spectrum of the deprotonated molecule [M − H]⁻ of *m/z*, 515 from a propolis sample from the southeast of Brazil, type G. This ion has been identified as deprotonated dicaffeoylquinic acid.

Table 1 Compounds identified in ethanolic extracts of propolis from Europe, the USA, Africa and Brazil, using ESI(−)-MS/MS

[M − H]⁻	Name	Found in propolis[a] from:	Collision energy/eV	Main fragments *m/z* (relative abundance %)
163	*p*-Coumaric acid	Europe, USA, Brazil (G, B1, B2)	25	163 (10), 119 (100), 93 (5)
177	3-Methoxy-4-hydroxycinnamaldehyde	Brazil (B1, B2)	15	177(50), 162(100), 149(13), 134(8), 133(8), 121(5), 105(5)
229	2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyran	Brazil (B2)	20	229(20), 201(11), 185(100), 174(18), 170(40), 160(40), 146(20).
231	3-Prenyl-4-hydroxycinnamic acid	Brazil (G, B2)	20	231(18), 187(73), 176(5), 132 (100), 119(3).
253	Chrysin	Europe, USA, Brazil (B1)	30	107(45), 119 (42), 143 (100), 145(40), 208 (18), 253(70).
255	Pinocembrin	Europe, USA, Africa Brazil (B1, R2)	25	107 (71), 136 (20), 145 (55), 151 (100), 164 (20), 171 (63), 187 (20), 231 (85), 255(95).
299	3,5-Diprenyl-4-hydroxycinnamic acid	Brazil (G, B2)	25	299(15), 284(10), 255(95), 244(12), 200(100), 145(5)
515	Dicaffeoylquinic acid	Brazil (G, B2)	25	515(6), 353(72), 191(51), 179(100), 173(95), 161(8), 155(5), 135(10)

[a] G, B1, B2 and R2 refer to different types of propolis found within Brazil.

Chemometric analysis

Chemometric methods have been used to statistically establish the correlation among all the propolis samples investigated. The central idea of PCA is to reduce the dimensionality of a data set in which there are a large number of correlated variables, while retaining as much as possible the total information.[36,37] Owing to the great number of variables, we selected, for PCA analysis, the two most characteristic negative ion markers for each propolis group defined by the ESI-MS fingerprints. These ion markers are shown in bold in Fig. 1. The PC1 × PC2 × PC3 three-dimensional plot (Fig. 3), which covers 58% of the total data variance, clearly separates the propolis samples into the seven groups already detected by visual inspection of the ESI-MS fingerprint spectra (Fig. 1). This result statistically demonstrates, therefore, the reliability of the ESI-MS fingerprinting method for propolis.

Conclusion

As exemplified herein for samples of the highly diverse Brazilian propolis, as well as for several samples from Africa, Europe and North America, and as statistically demonstrated *via* chemometric analysis (PCA), ESI-MS in the negative ion mode provides an effective fingerprinting method for high-throughput screening of propolis. The ESI-MS negative ion markers are representative of the chemical composition of the samples, and therefore geographical origins are easily recognized. Such composition varies mainly as to the dominance by flavonoids, terpenoids or phenolic compounds, and are indicative of the main plant sources of the propolis samples. Using ESI($-$)-MS fingerprinting it was possible to distinguish regional patterns in the composition of the greatly diverse Brazilian propolis, including the characteristic red, brown and green Brazilian propolis, and to clearly differentiate them from European, North American and African propolis. As expected from similar plant sources, European and North American propolis samples display similar ESI-MS (as well as ESI-MS/MS) fingerprint mass spectra. Tandem mass spectrometry with collision-induced dissociation allows on-line structural identification of marker ions, adding an optional mass dimension for improved selectivity in more structurally refined, bidimensional ESI-MS/MS fingerprinting characterization of propolis. We are currently applying ESI-MS and ESI-MS/MS fingerprinting to screen com-

mercial preparations of propolis for their geographical regions, and to evaluate specific nutraceutical benefits.

References

1 M. C. Marcucci, *Apidologie*, 1995, **26**, 83.
2 A. H. Banskota, Y. Tezuka and S. Kadota, *Phytotherapy Res.*, 2001, **15**, 561.
3 E. L. Ghisalberti, *Bee World*, 1979, **60**, 59.
4 W. Kustenmacher, *Ber. Dtsch. Parm. Ges.*, 1911, **21**, 65.
5 K. Dietrich, *Pharm. Zentralhalle*, 1911, **52**, 1019.
6 G. F. Jaubert, *C. R. Hebd. Seances Acad. Sci.*, 1926, **184**, 1134.
7 V. R. Villanueva, D. Bogdanovsky, M. Barbier, M. Gonnet and P. Lavie, *Ann. Inst. Pasteur*, 1964, **106**, 292.
8 V. R. Villanueva, M. Barbier, M. Gonnet and P. Lavie, *Ann. Inst. Pasteur*, 1970, **118**, 84.
9 S. A. Popravko, A. I. Gurevich and M. N. Kolosov, *22nd International Beekeepers Congress Summaries*, 1970, 163–164.
10 L. Cizmarik and I. Matel, *Experientia*, 1970, **26**, 713.
11 L. Cizmarik and I. Matel, *J. Apic. Res.*, 1973, **12**, 52.
12 W. Greenaway, T. Scaysbrook and F. R. Whatley, *Bee World*, 1990, **71**, 107.
13 F. A. Tomás-Barberán, C. Garcia-Vigueira, P. Vit-Olivier, F. Ferreres and F. Tomás-Lorente, *Phytochemistry*, 1993, **34**, 191.
14 V. Bankova, R. Christov, A. Kujumgiev, M. C. Marcucci and S. Popov, *Z. Naturforsch.*, 1995, **50c**, 167.
15 M. C. Marcucci, Fapesp, INPI Patent no. 0006272–3, 2000.
16 Y. K. Park, S. M. Alencar and C. L. Aguiar, *J. Agric. Food Chem.*, 2002, **50**, 2502.
17 V. Bankova, A. Dyulgerov, S. Popov, L. Evsratieva, L. Kuleva, O. Pureb and Z. Zamjansan, *Apidologie*, 1992, **23**, 79.
18 M. C. Marcucci, J. Rodriguez, F. Ferreres, V. Bankova, R. Groto and S. Popov, *Z. Naturforsch.*, 1998, **53c**, 117.
19 A. S. Pereira, M. Norsell, J. N. Cardoso and F. R. Aquino Neto, *J. Agric. Food Chem.*, 2000, **48**, 5226.
20 V. Bankova, S. Popov and N. L. Marekov, *J. Chromatogr.*, 1982, **242**, 135.
21 M. C. Marcucci, F. Ferreres, V. C. Garcia-Vigueira, S. Bankova, S. L. de Castro, A. P. Dantas, P. H. M. Valente and N. Paulino, *J. Ethnopharmacol.*, 2001, **74**, 105.
22 R. B. Cole, *Electrospray Ionization Mass Spectrometry*, John Wiley and Sons Inc., New York, 1997.
23 S. D. Fuerstenau, W. H. Benner, J. J. Thomas, C. Brugidou, B. Bothner and G. Siuzdak, *Angew. Chem. Int. Ed. Engl.*, 2001, **40**, 542.
24 T. Kotiaho, M. N. Eberlin, P. Vainiotalo and R. Kostiainen, *J. Am. Soc. Mass Spectrom.*, 2000, **11**, 526.
25 V. Rioli, F. C. Gozzo, C. S. Shida, J. E. Krieger, A. S. Heimann, A. Linardi, P. C. Almeida, S. Hyslop, M. N. Eberlin and E. S. Ferro, *J. Biol. Chem.*, 2003, **278**, 8547.
26 K. J. Koch, F. C. Gozzo, S. Nanita, M. N. Eberlin and R. G. Cooks, *Angew. Chem. Int. Ed. Engl.*, 2002, **41**, 1721.
27 R. Stefani, M. N. Eberlin, D. M. Tomazela and F. B. Costa, *J. Nat. Prod.*, 2003, **66**, 401.
28 L. Hamerski, M. Furlan, D. H. S. Silva, A. J. Cavalheiro, M. N. Eberlin, D. M. Tomazela and V. S. Bolzani, *Phytochemistry*, 2003, **63**, 397.
29 H. J. Cooper and A. G. Marshall, *J. Agric. Food Chem.*, 2001, **49**, 5710.
30 C. A. Hughey, R. P. Rodgers and A. G. Marshall, *Anal. Chem.*, 2002, **74**, 4145.
31 (*a*) M. N. Eberlin, L. L. R. Rocha and A. A. Araujo, *Analyst* to be submitted; (*b*) P. Mauri, M. Minaggio, P. Simonetti, C. Gardana and P. Pietta, *Rapid Commun. Mass Spectrom.*, 2002, **16**, 743.
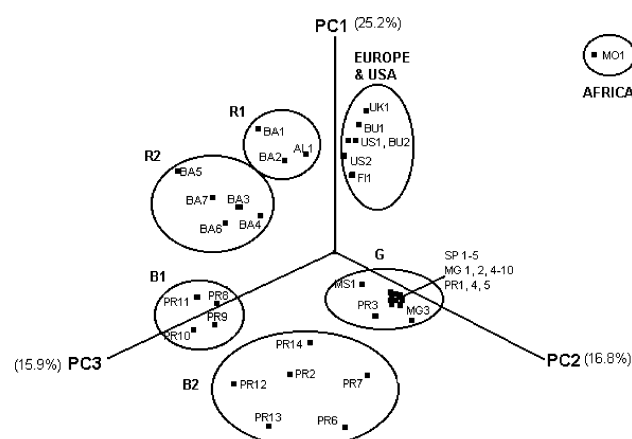
Fig. 3 Three-dimensional PC1 × PC2 × PC3 plot using abundances and *m/z*, ratios of a set of the most characteristic ESI-MS negative ion markers of propolis (see Fig. 1). Note that the samples are clearly separated into 6 groups (clockwise from PC1 axis): 1. Europe and the USA; 2. Green propolis from the southeast of Brazil (G); 3. Brown propolis from the south of Brazil (B2); 4. Brown propolis from the south of Brazil (B1); 5. Red propolis from the northeast of Brazil (R2) and 6. Red propolis from the northeast of Brazil (R1). The sample of African propolis stands isolated from all other samples. Percent of total variance represented by each PC are shown in parentheses.

32 P. Mauri and P. Pietta, *J. Pharm. Biomed. Anal.*, 2000, **23**, 61.

33 X. He, *J. Chromatogr., A*, 2000, **880**, 203.

34 M. A. Strege, *J. Chromatogr., B*, 1999, **725**, 67.

35 A. A. Sabino, A. H. L. Machado, C. R. D. Correira and M. N. Eberlin, *Ang. Chem. Int. Ed. Engl.*, 1998, **794**, 263.

36 I. T. Jolliffe *Principal Component Analysis*, Springer Verlag,New York 1996.

37 A. C. Rencher, *Methods of Multivariate Analysis*, Wiley, New York, 1995.

38 L. W. Sumner, P. Mendes and R. A. Dixon, *Phytochemistry*, 2003, **62**, 817.

39 C. G. Zampronio, L. A. B. Moraes, M. N. Eberlin and R. J. Poppi, *Anal. Chim. Acta*, 2001, **446**, 495.

40 C. G. Zampronio, L. A. B. Moraes, M. N. Eberlin and R. J. Poppi, *Talanta*, 2003, **60**, 37.

41 C. G. Zampronio, S. P. Gurden, L. A. B. Moraes, M. N. Eberlin, A. K. Smilde and R. J. Poppi, *Analyst*, 2002, **127**, 1054.

42 R. M. Alberici, C. G. Zampronio, R. J. Poppi and M. N. Eberlin, *Analyst*, 2002, **127**, 230.

43 M. C. Marcucci, F. Ferreres, A. R. Custodio, M. M. C. Ferreira, S. Bankova and C. Garcia-Vigueira, *Z. Naturforsch.*, 2000, **55c**, 76.

44 S. Kumazawa, M. Yoneda, I. Shihata, J. Kaneda, T. Hamasaka and T. Nakayama, *Chem. Pharm. Bull.*, 2003, **51**, 740.

45 A. C. H. F. Sawaya, A. M. Palma, F. M. Caetano, M. C. Marcucci, I. B. S. Cunha, C. E. P. Araujo and M. T. Shimizu, *Lett. Appl. Microbiol.*, 2002, **35**, 203.

46 M. Hirota, T. Matsuno, T. Fujiwara, H. Sugiyama and S. Mineshita, *J. Nat. Prod.*, 2000, **63**, 366.

47 K. Hayashi, S. Komura, N. Isaji, N. Ohishi and K. Yagi, *Chem. Pharm. Bull.*, 1999, **47**, 1521.

48 P. Miketova, K. H. Schram, J. Whitney, E. H. Kearns and B. N. Timmerman, *J. Mass Spectrom.*, 1999, **34**, 1240.

49 T. Tatefuji, N. Izumi, T. Ohta, S. Arai, M. Ikeda and M. Kurimoto, *Biol. Pharm. Bull.*, 1996, **19**, 966.