Talanta 81 (2010) 100–108

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Fingerprinting of propolis by easy ambient sonic-spray ionization mass spectrometry

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ARTICLE INFO

Article history:
Received 30 June 2009
Received in revised form
16 November 2009
Accepted 17 November 2009
Available online 24 November 2009

Keywords:
Easy ambient sonic-spray ionization mass spectrometry
Fingerprinting
Principal component analysis
Propolis
Natural product extracts

ABSTRACT

Chemical profiles of a representative set of 49 propolis ethanolic extracts collected worldwide (North and South America, Europe, Asia and Oceania) were obtained via easy ambient sonic-spray ionization mass spectrometry (EASI-MS). This simple and easily implemented fingerprinting technique analyses directly (without any pre-separation or sample manipulation) a tiny droplet of the ethanolic extract placed on a inert surface under ambient conditions. Data acquisition took about a minute per sample with no substantial sample carry-over. Extraction of propolis with ethanol by using an ultrasonic bath method gave EASI-MS data similar to the traditional maceration method, reducing total analysis time for the crude propolis resin from a week to just ca 1 h. Principal component analysis of the EASI-MS data is shown to group samples according to the plant sources of their resins, which characterizes their geographical origin.

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1. Introduction

Propolis is an important apicultural product with variable chemical composition and worldwide use due to several pharmacological and nutritional applications [1]. Bees use propolis to reinforce their combs and to keep the hive environment aseptic, collecting this resin from plants around their hives. In regions of temperate climate such as Europe [2] and North America [3] *Apis mellifera* bees obtain resins mainly from the buds of species of *Populus*. The composition of propolis from these regions is therefore very similar and the main constituents are flavonoids, aromatic acids and their esters [1].

In tropical and sub-tropical areas of South America, the chemical composition of propolis shows a much greater regional variation. For example, *Clusia major* and *Clusia minor* are indicated as the origin of the resins in Venezuelan propolis [4], whereas *Baccharis dracunculifolia* is the main source for green Brazilian propolis [5]. In temperate areas of South America, such as Chile, the composition is again similar to that of colder climates, with flavonoids being the most important components [6].

Some studies have reported on the composition of *A. mellifera* propolis from other regions of the world such as New Zealand [7] and Korea [8], including flavonoids as important constituents of propolis from these regions. For the majority of Asian or African countries, however, the composition and plant origins of propolis samples are only recently being studied [9–11]. Both gas chromatography coupled to mass spectrometry (GC/MS) [7] and high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC/ESI-MS) have been used to compare *A. mellifera* propolis from different regions [9,10] as well as its plant origins [12].

Very little is known about the composition and plant origins of stingless bee propolis. GC/MS was used to analyze content of propolis of stingless bees from Brazil [13–15] and Mexico [16]

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doi:10.1016/j.talanta.2009.11.043

Table 1
Sample number, species of bee, region of origin, group placed by principal component analysis and PC1, PC2, PC3 coordinates relating to Fig. 3.

	Type of bee	Origin	PCA group	PC1	PC2	PC3
A1	*A. mellifera*	MENDONZA/ARGENTINA	1, 2	109.7544	27.90731	15.93457
A2	*A. mellifera*	INDIANA/USA	2	52.74798	63.68331	17.63283
A3	*A. mellifera*	RIO GRANDE DO NORTE/BRAZIL	4C	−52.3585	23.55575	−23.7305
E4	*A. mellifera*	C/SPAIN	1	30.28264	−52.5617	−18.2593
A5	*A. mellifera*	C/CANADA	2	69.05376	67.02587	20.41561
A6	*A. mellifera*	SERGIPE/BRAZIL	4B	−68.819	16.792	−40.8666
A8	*A. mellifera*	AMAZONAS/BRAZIL	4A	−74.8835	12.3794	−55.5771
A9	*A. mellifera*	ALAGOAS/BRAZIL	4B	−66.9181	5.461559	−35.5465
A11	*A. mellifera*	ALAGOAS/BRAZIL	4A	−66.1101	16.47319	−56.8684
A12	*A. mellifera*	ALAGOAS/BRAZIL	4A	−54.7884	24.22638	−50.3004
A14	T. angustula	SÃO PAULO/BRAZIL	3	−79.9943	−21.2002	93.37627
A15	*A. mellifera*	PICHIRROPULLI/CHILE	4C	−57.4579	42.59583	−38.8034
A16	*A. mellifera*	COLLIPOLI/CHILE	2	54.29689	14.01309	10.39098
A17	*A. mellifera*	RADAL/CHILE	2	61.29895	43.17845	32.8277
A19	*A. mellifera*	REMANSO/VENEZUELA	4B	−68.3021	7.749347	−30.9521
A20	*A. mellifera*	LLIANO/VENEZUELA	4B	−66.5348	6.017807	−28.7062
O21	*A. mellifera*	C/NEW ZEALAND	1, 2	80.8157	−27.7756	−8.19677
O22	*A. mellifera*	C/AUSTRALIA	2	41.50467	35.04181	13.00326
A23	*A. mellifera*	RIO GRANDE DO SUL/BRAZIL	2	56.88296	72.48175	27.7651
E25	*A. mellifera*	TIMISOARA/ROMANIA	1, 2	78.05702	−40.6424	−12.6777
A26	*A. mellifera*	CURNAVACA/MEXICO	1, 2	111.4547	−1.74428	−0.34212
E27	*A. mellifera*	C/HUNGARY	1, 2	86.7058	−5.71544	7.352558
E30	*A. mellifera*	ALENTEJO/PORTUGAL	1	14.61484	−58.2377	−22.4967
L31	*A. mellifera*	TALAGAN/IRAN	1	13.23686	−79.823	−32.3319
L32	*A. mellifera*	ZANJAN/IRAN	1	−3.08482	−63.5916	−31.6722
L33	*A. mellifera*	KARAJ/IRAN	1	−1.25621	−80.1186	−2.21457
L34	*A. mellifera*	CHOJIR/IRAN	1	31.61997	−55.2673	−27.6179
L35	*A. mellifera*	BEHBAHAN/IRAN	1	1.321666	−87.3484	24.83115
L36	*A. mellifera*	C/IRAN	1	24.86318	−59.8615	−32.5948
K37	*A. mellifera*	CHILGOK/KOREA	1, 2	72.63493	−24.1862	−1.97269
K38	*A. mellifera*	SANGJU/KOREA	2	35.82546	32.1337	16.11394
K39	*A. mellifera*	GEOCHANG/KOREA	2	49.8798	46.33692	6.845568
K40	*A. mellifera*	CHEONGJU/KOREA	1, 2	80.80489	−45.9642	−6.47927
K41	*A. mellifera*	MUJU/KOREA	2	33.22769	11.4308	7.399349
K42	*A. mellifera*	CHEJU/KOREA	5	−53.0561	3.068734	12.56824
K43	*A. mellifera*	POCHEON/KOREA	5	−73.6454	5.615676	13.59627
I44	Mellipona	BANGALORE/INDIA	3	−81.1342	−23.9405	75.47089
I45	*A. cerana*	BANGALORE/INDIA	4C	−23.2798	39.87898	−8.3786
I47	*A. dorsata*	BANGALORE/INDIA	4C	−18.2782	66.27965	−19.19
I48	*A. florea*	BANGALORE/INDIA	4C	−64.3161	28.55444	−35.5526
I49	*A. mellifera*	BANGALORE/INDIA	2	23.30111	41.68868	9.641777
I50	Trigona	BANGALORE/INDIA	3	−83.8034	−22.066	74.8331
F1	*A. mellifera*	INDIANA/USA (A2)	2	21.13958	43.15217	8.997267
F2	*A. mellifera*	ALAGOAS/BRAZIL (A11)	4A	−68.0198	10.15408	−42.9789
F3	Mellipona	BANGALORE/INDIA (I44)	3	−85.8498	−21.9058	85.83662
F4	Trigona	BANGALORE/INDIA (I50)	3	−82.4408	−21.8111	73.28023
F5	*A. mellifera*	MUJU/KOREA (K41)	2	48.73756	10.56655	9.808963
F6	*A. mellifera*	TIMISOARA/ROMANIA (E25)	1, 2	79.94204	−28.355	0.032237
F7	*A. mellifera*	POCHEON/KOREA (K43)	5	−69.6736	4.67353	6.352797

C, comercial sample and F1–F7, fast extraction in ultrassound bath.

showing the composition of native bee propolis to be different from that of *A. mellifera* bees from the same regions. In more recent studies [17,18], negative ion mode eletrospray ionization-mass spectrometry [ESI(−)-MS] fingerprinting was used to compare the composition of ethanolic extracts of samples of native Brazilian stingless bee propolis with extracts of possible plant sources. Although the composition of the resins depended on the species of bee and the geographic region from where the sample was taken, *Schinus terebenthifolius* was found to be an important plant source for propolis of stingless bees in all regions of Brazil.

To determine the plant sources of propolis samples, the identification of key characteristic marker compounds in both the extract and the sample permit secure correlation [17,18]. This fingerprinting approach constitutes therefore a fast and reliable method, especially applicable for the analysis of numerous samples and indicated for the qualitative distinction between samples with complex chemical composition. Therefore, fast MS fingerprinting methods can be successfully applied to the high-throughput direct analysis of crude propolis samples, especially to determine sample origin. We performed the first systematic study applying ESI-MS fingerprinting to propolis [19], analyzing over 40 propolis samples, mainly from Brazil. Since then, ESI-MS fingerprinting has been applied to a wide variety of food and beverage samples [20–22] as well as natural products [23].

Recently, a series of new ambient mass spectrometric techniques such as desorption electrospray ionization—DESI [24], direct analysis in real time—DART [25], electrospray laser desorption ionization—ELDI [26], matrix assisted laser desorption electrospray ionization—MALDESI [27], atmospheric solids analytical probe—ASAP [28], extractive electrospray ionization—EESI [29], desorption atmospheric pressure photoionization—DAPPI [30], and easy ambient sonic-spray ionization—EASI [31] have been introduced, which allow MS analysis with great speed directly for samples at ambient conditions.

EASI, which was originally termed DeSSI [32], is one of the simplest, gentlest and most easily implemented ionization methods. EASI uses super-sonic-spray ionization to create minute droplets which become charged due to statistical imbalance distribution of

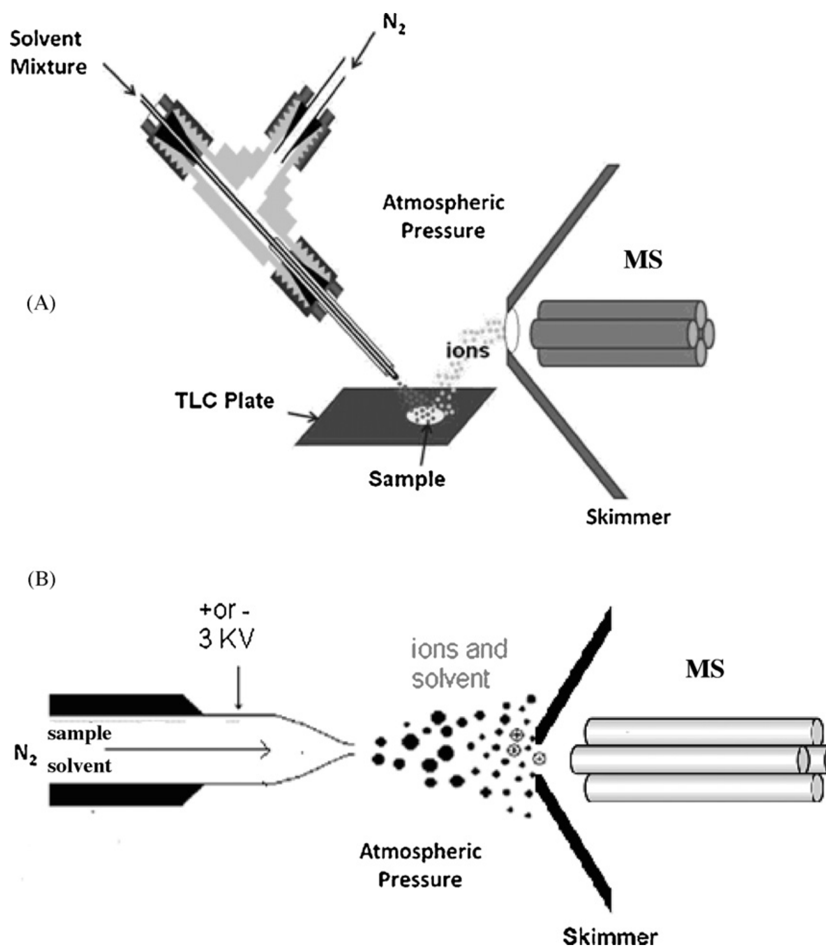

Fig. 1. (A) Schematic of the EASI source, which uses super-sonic-spray ionization to create very minute charged droplets of the solvent. The stream of the super-sonic charged droplets strikes the sample on the surface, ionizes the neutral molecules in the sample and transfers them to the gas phase and (B) schematic of a traditional ESI source in which the sample is introduced in a solution with the ESI solvent.

charge (cations and anions). The dense stream of the super-sonic charged droplets causes analyte pick up from the surface, ionization of neutral molecules and their transfer to the gas phase. EASI displays, therefore, several unique features: requires no voltage to eliminate counter-ions, no voltage switches from positive to negative ion modes and vice-versa, no heating for desolvation and is less prone to analyte oxidation. Comparisons of EASI and DESI for the same sample [32] showed that there were less background cluster ions from solvent ionization (noise) and in some cases higher abundance of the analyte ions in the spectrum, when EASI was used. Otherwise the EASI-MS data are quite similar to ESI-MS or SSI-MS data.

EASI has already been applied with success to different analytes and matrixes such as biodiesel [33] and drug tablets [32]. It has also been coupled to membrane interface mass spectrometry—MIMS [31] and thin layer chromatography—TLC [33] among others.

Initial studies of propolis activity gave not much importance to the composition and plant sources of this resin. In recent years, however, phytochemists and pharmacologists have come to perceive that different propolis samples vary greatly in composition and biological activity [34]. It is important therefore to evaluate the chemical composition of a propolis sample before undertaking a study of its biological activity. For example, poplar type propolis has demonstrated anti-inflammatory activity that has not yet been determined for red Cuban propolis, whereas there is a known allergen in poplar propolis (3,3-dimethylallyl caffeate) that is not present in propolis from other plant sources [34]. It is hoped that this type of comparative study will permit the indication of a specific type of propolis for a determined biological activity.

The present study has evaluated the applicability of EASI-MS fingerprinting with principal component analysis (PCA) for the direct, fast and reliable characterization of propolis samples from different origins, and to determine the plant origins of their resins.

2. Experimental

2.1. Reagents and samples

All reagents used were of analytical grade. HPLC grade methanol was purchased from Merck SA (Rio de Janeiro, Brazil). Deionized water was obtained from a Milli-Q (Millipore) purification unit. A total of 42 samples of propolis ethanol extracts were analyzed and are listed in Table 1. Some of the samples were crude propolis and were extracted in ethanol, using the method described in Sawaya et al. [19]. In brief, 600 mg of ground crude propolis were macerated in 2 mL of pure ethanol for 1 week at a temperature of 30 °C, and then filtered to remove the insoluble portion and the wax. This procedure mimics the procedure most frequently used by beekeepers. Commercial samples of ethanolic propolis extracts were analyzed in the same solvent and concentration as bought. A second, faster, extraction procedure was used to repeat the extraction of some samples (F1–F7 in Table 1), with the same proportion of ground propolis to ethanol and were extracted in an ultrasound bath for 30 min, centrifuged, kept in a freezer until precipitation of the wax, and then the supernatant solution was extracted and used.

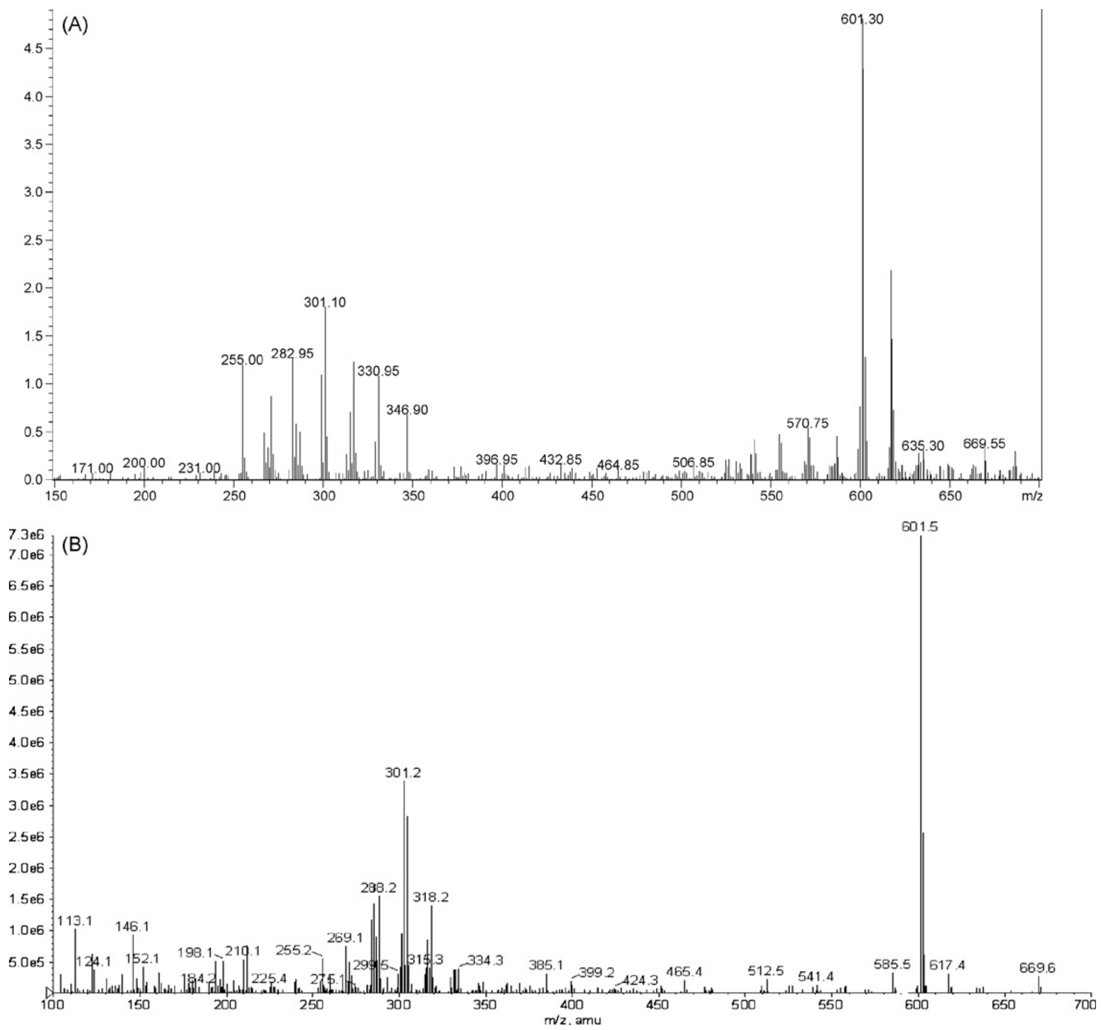

Fig. 2. Mass spectra of sample A12—red propolis from Alagoas, Brazil using (A) EASI-MS and (B) ESI-MS.

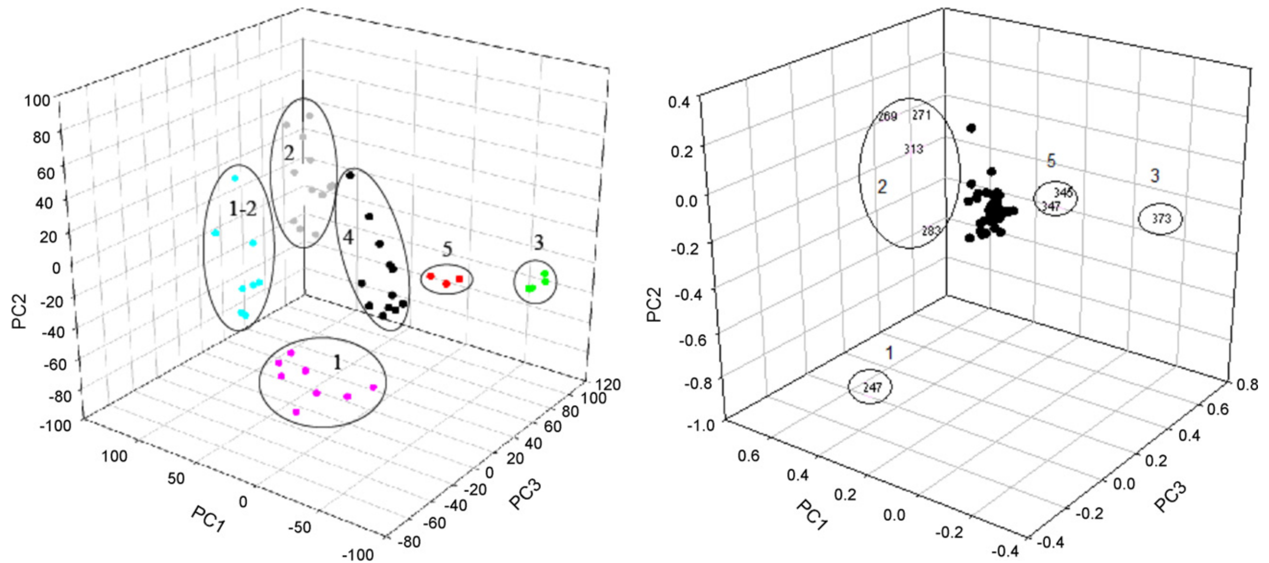

Fig. 3. 3D PCA of the EASI-MS fingerprints of the samples shown in Table 1. The loadings at the bottom (ions responsible for grouping the samples) are numbered in the same way as the scores (groups of samples) on top.

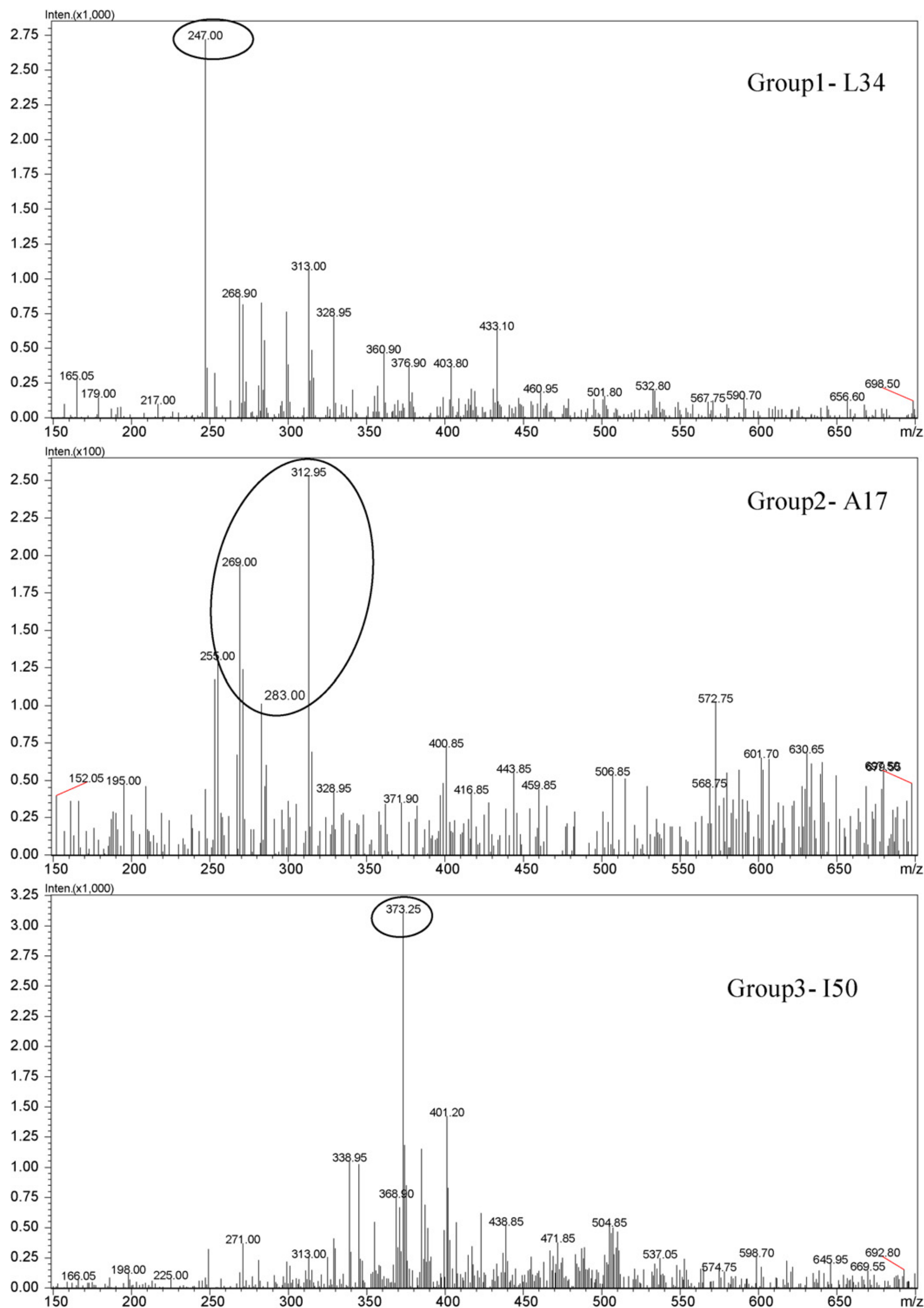

Fig. 4. Representative EASI-MS fingerprints from samples from groups 1 to 3. Characteristic ions are circled.

2.2. General experimental procedure

Experiments were performed on a Shimadzu 2010 EV quadrupole mass spectrometer using a home-made EASI source. Although the structure of the EASI source is similar to that of DESI [24], the main difference is that no heating or voltage is applied to the EASI source; hence they differ greatly in regard to the mechanism of ionization. In EASI, the solvent droplets end up being charged (both positively and negatively) due to a natural statistical imbalance distribution of cations and anions in the very minute droplets formed during sonic spraying. In DESI, the droplets are either positively or negatively charged with the assistance of high voltage applied to the spray capillary.

The mass spectrometer was operated in the negative ion mode with a detector voltage of -1.5 kV. Approximately 20 μL of the propolis extracts were deposited on the surface of a TLC plate (Merck, 60 G). Ammonium hydroxide solution (0.5%) was added to the methanol:water solvent (70:30) to help induce ionization in the negative mode, and this solvent mixture was pumped through the EASI source at a rate of 20 μL min^{-1} using a Harvard Apparatus syringe pump. Nitrogen was used as a nebulizing gas. Although mass spectra were acquired over the 100–1000 m/z range, ions if

interest were found only between 150 and 650 *m*/*z* and the spectra are presented in this range.

The ESI-MS was acquired on a Q-trap (Applied Biosystems) Mass Spectrometer with an ESI source operated in the negative ion mode under the following main conditions: capillary voltage: 4kV, DP: 40 V, source temperature 200 °C, and nitrogen as nebulizing gas, using the Q1 full scan mode. Fig. 1 shows schematic drawings of both types of sources.

2.3. Chemometric 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 the all ions with intensities over 20% in the fingerprints of each sample, resulting in a total of 88 variables for 49 samples, with mean-centering preprocessing and step validation.

3. Results and discussion

Fig. 1 shows schematic drawings of both EASI and ESI sources and Fig. 2 shows, as illustrative examples and for comparative purposes, an ESI-MS and an EASI-MS of sample A12 (red Brazilian propolis from Alagoas). Both EASI and ESI mass spectra indicate similar chemical profiles with only variations in ion abundances. On the whole, the spectra obtained by EASI-MS were similar to those obtained by ESI-MS in previous studies [19].

Fig. 3 shows the three-dimensional PCA plots of the EASI-MS of all the samples (Table 1) and their grouping (scores) according to most characteristic marker ions (loadings). The 3D plot was chosen due to the large number of samples and variables, and over 90% of the variance was explained by the 3 selected factors. Table 1 also presents the PC1, PC2 and PC3 coordinates for each sample. A clear clustering pattern directly related to specific marker ions was observed for samples in groups 1, 2, 3 and 5; but not for samples in group 4, which were then subjected to a second PCA. Fig. 4 shows representative EASI-MS of samples from groups 1 to 3.

Samples placed in group 1 are from Portugal, Spain and Iran and display a common and most abundant anion of *m*/*z* 247. This anion was also observed in ESI-MS of samples of Italian propolis [30] where it was identified as a dihydroxyflavone. Other less abundant ions corresponding to well known flavonoids: *m*/*z* 253 (chrysin), *m*/*z* 255 (pinocembrin), *m*/*z* 269 (apigenin/galangin), *m*/*z* 271(pinobanksin), *m*/*z* 283 (caffeic acid phenethyl ester, CAPE) and *m*/*z* 313 (pinobanksin acetate), found in propolis derived from *Populus* resins can also be observed [35]. Iranian propolis has been shown to have antioxidant properties and to contain between 1 and 8% of flavonoids and 3–8% of phenolic compounds [36]. This composition is in agreement with the characteristic ions observed in the EASI-MS of the Iranian samples classified among group 1 samples.

Group 2 is composed of samples from several countries of temperate climate, and different continents (Korea, Australia, India, USA, Canada, Chile and South Brazil). The characteristic ions of this group again correspond to *Populus* flavonoids (*m*/*z* 269: apigenin/galangin; *m*/*z* 271: pinobanksin; *m*/*z* 313: pinobanksin acetate and *m*/*z* 283: caffeic acid phenethyl ester). The major components of the Korean samples placed in this group (K39—Geochang, K41—Muju and K38-Sangju) were identified by Ahn et al. [8] using HPLC. These flavonoids are also quite common in propolis from the South of Brazil and Central Chile [6] and their ions have been observed in ESI-MS of samples from the USA and Europe [19]. Group 1,2 comprises samples containing marker ions from both group 1 and group 2 with similar intensities. Samples

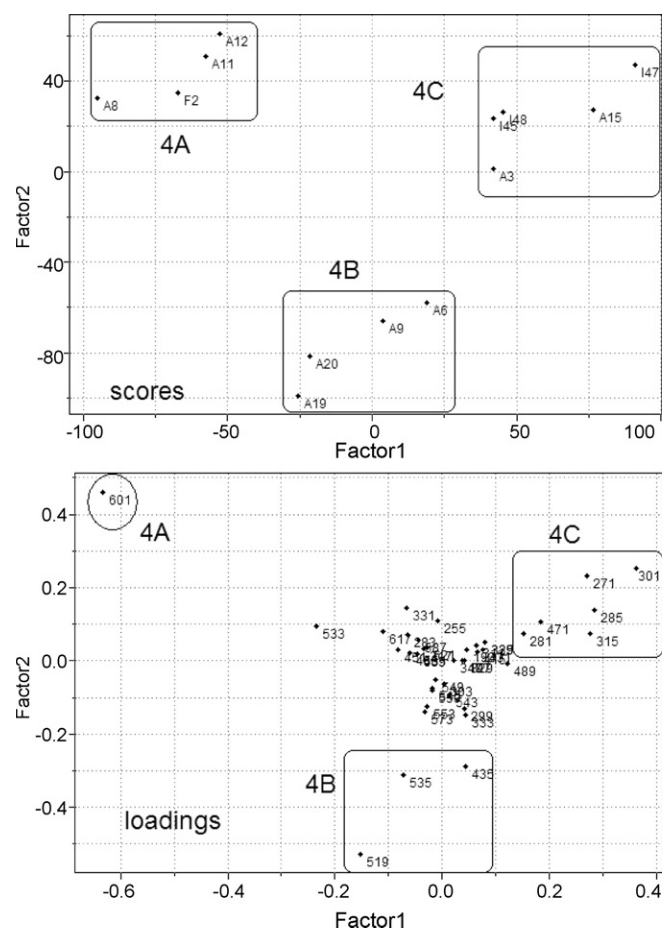

Fig. 5. 2D PCA of EASI-MS fingerprints of samples placed in group 4 (in Fig. 3). Three sub-groups were identified (4A–C). The loadings at the bottom (ions responsible for grouping the samples) are numbered in the same way as the scores (groups of samples) on top.

from this group came from Argentina, Korea, New Zealand, Mexico, Romania and Hungary.

Over half the samples analyzed had compounds derived from *Populus* resins as characteristic ions, indicating a clear preference for this plant genus by *A. mellifera* bees. The main difference of the EASI-MS between groups 1, 1–2 and 2 are the relative intensities of the marker ion of *m*/*z* 247. This difference may be related to the varying species of *Populus* encountered by the bees; to differences in climate and soil which lead to variations in the biochemical pathways of plant secondary metabolites or to differences in the seasons when the resins were collected. This result is similar to variations observed in a previous study [19] in the composition of green propolis in Brazil.

Group 3 is characterized by the marker ion of *m*/*z* 373 and clearly has a different plant source from the previous samples. This group is made up of stingless bee propolis from Brazil (A-14) and India (I44 and I50). The EASI-MS of these Indian propolis samples are quite similar to that of Brazilian *Tetragonisca angustula* propolis whose resins are derived from *Schinus terebenthifdolius*, a common plant throughout the Americas [17]. Although this species is not typical of India, other species of this genus can be found there. Further studies may be able to determine the plant source of these samples.

In Mexico and in Brazil [16,18], stingless bees frequently use resins from different plant sources than *A. mellifera* bees. This also seems to be the case in India, as the Indian *A. mellifera* propolis sample was placed in group 2 and has *Populus* resins as the charac-

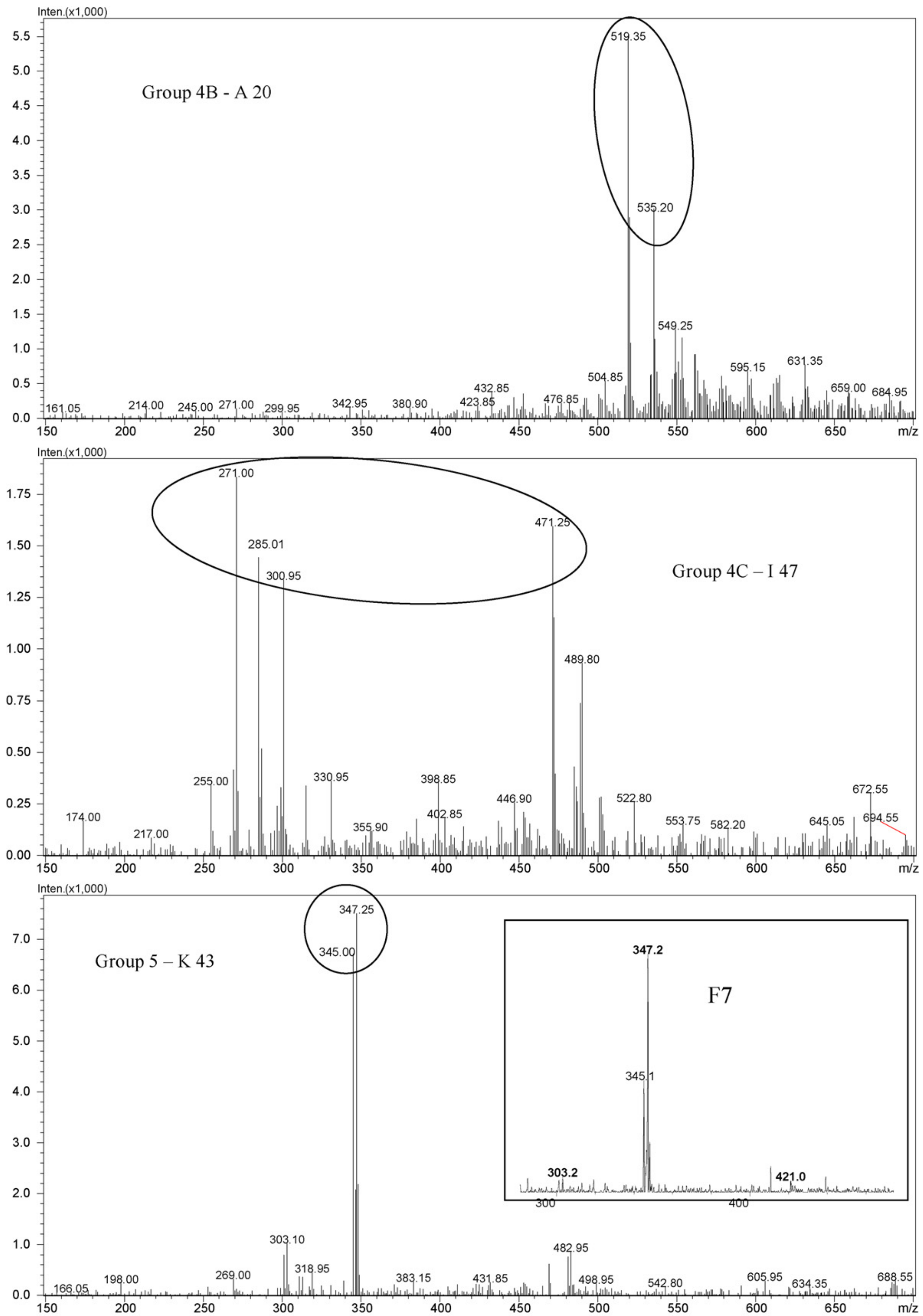

Fig. 6. EASI-MS fingerprints of representative samples of groups 4B, 4D and 5. Most characteristic ions are circled. Inset in group 5 shows the similarity between extracts obtained by maceration (K43) and by fast ultrasound bath extraction (F7) of the same sample.

teristic ions of its EASI-MS whereas the stingless bee propolis has a different composition.

Several samples were placed in group 4 (Fig. 3), but no clear marker ions could be identified. Therefore, the data from these EASI-MS were analyzed separately by a second PCA (Fig. 5), which divided them into three sub-groups (4A–4C). Group 4A is composed of 3 propolis samples characterized by ion of *m*/*z* 601. This ion corresponds to two prenylated benzophenones isolated from red Brazilian propolis and identified by Trusheva et al. [37] and which stood out as the major marker ion of red R1 Brazilian propolis in previous ESI-MS studies [19]. The samples in this group were red propolis samples from the states of Alagoas and Amazonas in Brazil, and Fig. 2B shows a typical spectrum.

Group 4B is composed of red propolis samples from Venezuela, and from the states of Alagoas and Sergipe in Brazil. The marker ions for this group are those of *m*/*z* 435, 519 and 535. Propolis from Venezuela has not been extensively studied yet, but two studies indicate that prenylated benzophenones are its most characteristic compounds [4,38] derived from flowers of the *Clusia* genus. Fig. 6 shows a characteristic EASI-MS for group 4B.

Group 4C is composed of samples from Brazil, Chile and India whose EASI-MS were quite different from the other samples from the same countries. Their main marker ions are those of *m*/*z* 271, 281, 285, 301, 315 and 471 (Fig. 6). Not enough is known about propolis from these regions to base an attribution of their plant source.

Group 5 is characterized by ions of *m*/*z* 345 and 347, which are observed in two samples of Korean propolis. The composition of one of these samples (K43, from Pocheon) was reported to be different from the other samples of Korean propolis [8]. This sample had a much lower concentration of flavonoids and polyphenols and intense but unidentified ions corresponding to less polar substances observed at the end of the HPLC analysis. The (K42) sample from Cheju presents a spectrum that is also different from the other Korean samples and similar to K43, from Pocheon (Fig. 6).

To test a extraction procedure for propolis faster than the common maceration, seven samples of different origins and compositions (A2, A1, E25, K41, K43, I44 and I50) were extracted in an ultrasound bath (see F1–F7 in Table 1) and then analyzed by EASI-MS. In all these cases, the different procedures produced extracts with similar EASI-MS and therefore they were placed in the same group by PCA. The inset in Fig. 6 (group 5) shows the similarity between the spectrum of the sample extracted by maceration (K43) and by fast ultrasound extraction (F7). This procedure reduces the extraction time of crude propolis from 1 week to less than 1 h, which is important for high throughput analysis of samples.

Identification of compounds based on a single mass is doubtful, therefore only those compounds which have been identified previously on these same samples (such as the Korean, Venezuelan and certain Brazilian samples) are proposed. The use of a triple-quadrupole or ion-trap mass spectrometer, to obtain MS/MS dissociation data of ions, and comparison to standards, should provide secure characterization. Ultra-high resolution and accuracy MS data from FT-MS measurements are being collected in our laboratory to identify a larger number of components in these novel types of propolis. However, fingerprint analysis, combined with similarity evaluation, is a rapid method or propolis profiling and it is often unnecessary to identify the individual compounds that make up the spectrum [11] when the main purpose of a study is the qualitative grouping of samples.

Initial GC–MS studies of propolis focused on the more volatile constituents of propolis, or necessarily undertook derivatization of the extracts [13–15] as a sample preparation procedure. Furthermore, a single sample could take an hour to be analyzed. As most of the active components in propolis are polar, HPLC–ESI-MS is substituting GC–MS to compare *A. mellifera* propolis from different regions [9,10] as well as its plant origins [12]. Although this method requires less sample preparation than GC, a typical chromatographic analysis takes between 30 min and 1 h, using around 1 mL min^{-1} of solvent throughout. Although the HPLC–MS chromatograms of propolis are useful for the quantification of individual components in propolis, the qualitative characterization of samples is just as effectively carried out using MS fingerprinting techniques. For instance, the grouping of Korean propolis samples by EASI-MS in our results is the same as by HPLC–MS [8]. The EASI-MS fingerprints of propolis samples are qualitatively similar to those obtained by direct insertion ESI-MS in previous studies [17–19] but can be obtained more quickly, for dozens of samples on a roll, without any pre-separation or sample preparation procedure. As only the solvent passes through the syringe and PEEK, and the contact with the sample is done by the charged droplets produced by solvent sonic spraying, it needs no equipment cleaning between samples. No carry-over was detected in our experiments with more than 40 propolis samples run subsequently.

4. Conclusion

EASI provides a simple voltage-free ionization method that could be coupled to miniature mass spectrometers for a robust instrument for fingerprinting analysis or portable devices for field work. Fingerprinting characterization of a large number of propolis samples and their grouping according to characteristic ion profiles could then be performed via EASI-MS in such instruments. Propolis extracts can be analyzed quickly, with no sample manipulation and no pre-separation steps. On average an acquisition speed of 1 min per sample was easily achieved with no instrumental cleaning or observable carryovers between samples up to 50 samples in a row. EASI-MS data was quite reproducible, permit the grouping of samples from similar plant and geographical origin, and may permit the identification of the plant sources of propolis resins particularly when the negative ion mode is used. EASI-MS data can be used therefore to screen for plant origins of propolis resins by comparing propolis extracts with extracts of possible plant sources.

Acknowledgements

We thank FAPESP and CNPq for financial assistance. ACHFS would like to thank CAPES for a postdoctoral fellowship and Maria Cristina Marcucci Ribeiro, Lidia Chitmia, Leonardo S. Santos and Cesar Vicente Ferreira Batista for samples of propolis.

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