Research Article

Phytochemical Analysis

Received: 2 April 2012, Revised: 27 August 2012, Accepted: 2 September 2012 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/pca.2404

Pterodon pubescens Oil: Characterisation, Certification of Origin and Quality Control via Mass Spectrometry Fingerprinting Analysis

E. C. Cabral,[a] L. Sevart,[b] H. M. Spindola,[b] M. B. Coelho,[a] I. M. O. Sousa,[b] N. C. A. Queiroz,[b] M. A. Foglio,[b] M. N. Eberlin[a] and J. M. Riveros[c]*

ABSTRACT:

Introduction – The oil obtained from *Pterodon pubescens* (Leguminosae) seeds are known to display anti-cancer, anti-dermatogenic and anti-nociceptive activitiy. Phytochemical studies have demonstrated that its main constituents are diterpenoids with voucapan skeletons. Considering the potential biological activities of the oil, rapid and efficient methods for assessing its quality would facilitate certification and quality control.

Objective – To develop a direct mass spectrometric fingerprinting method for the *P. pubescens* seed oil that would focus on the major diterpenoids constituents, enabling quality control, origin certification and recognition of marker species in commercially available products.

Method – Two techniques were used: (i) direct infusion electrospray ionisation (ESI) mass spectrometry after solvent extraction and dilution and (ii) ambient desorption/ionisation via easy ambient sonic-spray ionisation, EASI(+)–MS, performed directly on the seed surface or at a paper surface imprinted with the oil.

Results – From a combination of ESI–MS, HRESI–MS and ESI–MS/MS data, 12 diterpenes were characterised, and typical profiles were obtained for the oil extract or the crude oil via both ESI–MS and EASI–MS. These techniques require no or very simple sample preparation protocols and the whole analytical processes with spectra acquisition take just a few minutes.

Conclusion – Both techniques, but particularly EASI–MS, provide simple, fast and efficient MS fingerprinting methodologies to characterise the *P. pubescens* oil with typical (di)terpene profiles being applicable to quality control and certification of authenticity and origin.

Keywords: EASI; ESI; mass spectrometry; *Pterodon pubescens* oil

Introduction

The genus *Pterodon* (Leguminosae) comprises four species native to Brazil: *P. pubescens* Benth (synonymy – *P. emarginatus* Vog.), *P. polygalaeflorus* Benth., *P. appariciori* Pedersoli and *P. abruptus* Benth (Harborne, 1967; Rai *et al*., 2010). The species *P. pubescens*, commonly known as sucupira branca or faveira, is a tree found in central Brazil and its fruits are commercially available in popular markets due to their anti-rheumatic, analgaesic and anti-inflammatory properties (Menna-Barreto *et al*., 2008).

The oil obtained from *P. pubescens* fruits, also known commonly as 'sucupira', has shown chemoprophylactic actions in schistosomiasis (Katz *et al*., 1993) due to its toxic and mutagenic effects (Sabino *et al*., 1999). Other biological activities have also been reported such as for arthritis (Katz *et al*., 1993) and as anti-dermatogenic, anti-nociceptive (Silva *et al*., 2004; Coelho *et al*., 2005) and immunomodulation agents (Coelho *et al*., 2004). Protection against infection with cercariae of *Schistosoma mansoni* (Menna-Barreto *et al*., 2008) was attributed to the 14,15-epoxy-geranylgeraniol and later to two other linear diterpenes, namely 14,15-dihydroxy-14 and 15-dihidroxygeranylgeraniol geranylgeraniol, that occur in *P. pubescens* with a characteristic floral odour. Menna-Barreto *et al.* (2008) also reported geranylgeraniol to have anti-*Trypanosoma cruzi* properties (Rai *et al*., 2010).

Phytochemical studies of *Pterodon* demonstrated the presence of alkaloids in the bark, isoflavones and some triterpenes in the wood, and diterpenes and isoflavones in the fruit oil (Braz Filho *et al*., 1971). The essential constituents of this genus are linear and cyclic diterpenoids (Scheme 1) with voucapan skeletons (Spindola *et al*., 2009).

The furan diterpenes of this genus have shown anti-oedema, analgaesic, anti-inflammatory, and anti-proliferative activities in strains of human cancer cells (Braz Filho *et al*., 1971). Among those shown in Fig. 1, diterpenes **4**, **5**, **7**, **12** and **13** have been isolated from *P. pubescens*. Recently, diterpenes **1**, **2**, **9** and **14**, which have been reported only for the genus, were also isolated. The cyclic diterpene **15**, which had not been previously reported in the species and genus, was recently isolated (Spindola *et al*., 2009).

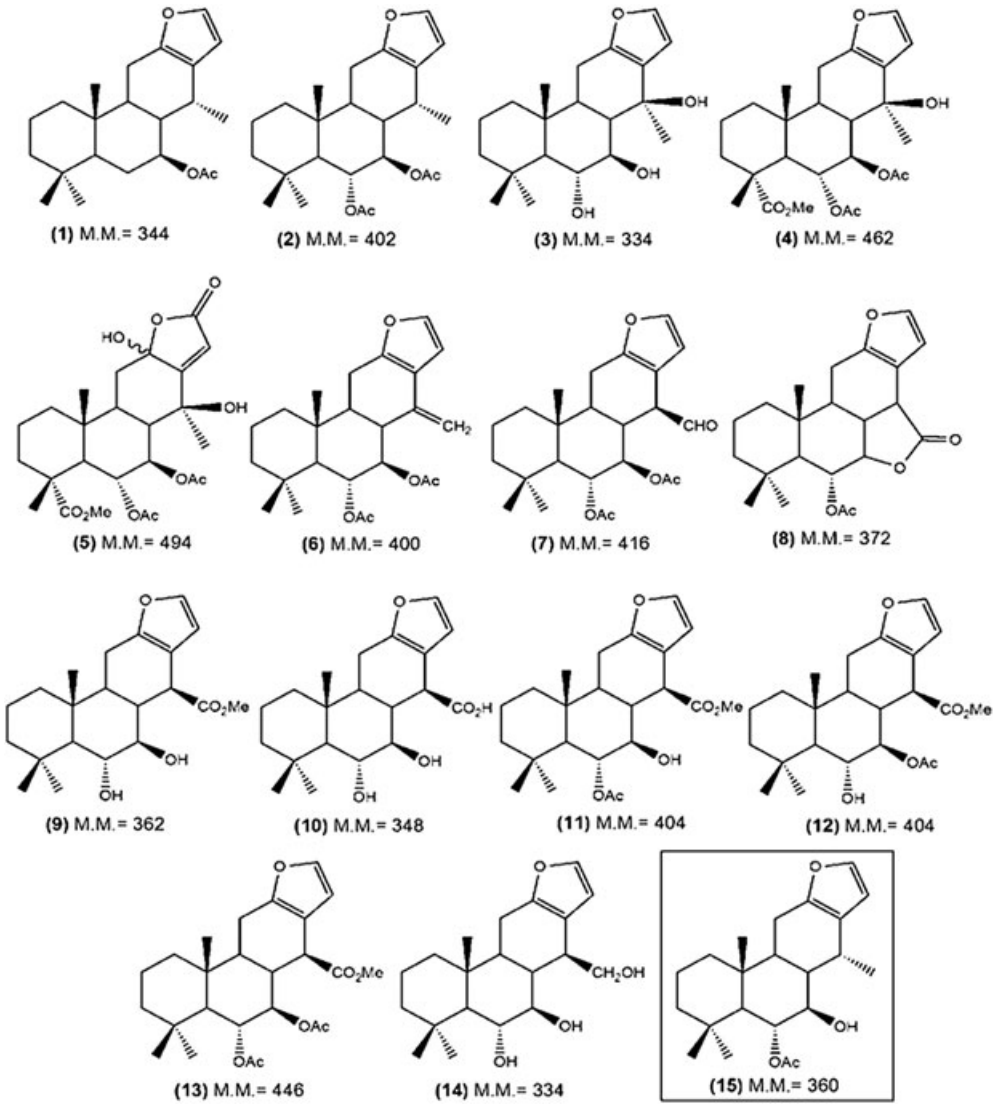
Considering the biological activities of the 'sucupira' species and the commercialisation of its fruit and alcoholic extracts in popular markets, methods for assessing the quality of these

* Correspondence to: J. M. Riveros, Institute of Chemistry, University of São Paulo – USP, Av. Lineu Prestes 748, 05508-900, São Paulo, Brazil. Email: jmrnigra@usp.br

[a] ThoMSon Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas – UNICAMP, 13084-970, Campinas, SP, Brazil

[b] CPQBA – University of Campinas – UNICAMP, 13083-970, Campinas, SP, Brazil

[c] Institute of Chemistry, University of São Paulo – USP, Av. Lineu Prestes 748, 05508-900, São Paulo, Brazil



Scheme 1. Characteristic structures of diterpenes of the genus *Pterodon*.

products are needed. Mass spectrometry (MS) is currently the gold standard technique for the analysis of complex chemical mixtures due mainly to its unmatched ability to detect, count and characterise atoms and molecules of many types, compositions and sizes (Marshall and Rodgers, 2004). Although previous separation is needed, particularly for precise quantitation, direct MS analysis using soft ionisation techniques performed at atmospheric conditions, such as electrospray ionisation (ESI–MS) of complex mixtures, has been shown to provide fast and reliable characterisation of complex mixtures via distinctive chemical profiles (pca2404-bib-0003Araújo *et al.*, 2005; Catharino *et al.*, 2007; Roesler *et al.*, 2007). We have used the MS fingerprinting approach to characterise numerous samples and have shown it to provide reliable qualitative distinction (see for instance: Sawaya *et al.*, 2004; pca2404-bib-0004Araújo *et al.*, 2005; Catharino *et al.*, 2005; Marques *et al.*, 2006; de Souza *et al.*, 2007).

Recently, MS analysis was simplified via the introduction of a series of ambient desorption/ionisation techniques (Chen *et al.*, 2009; pca2404-bib-0002Alberici *et al.*, 2010; Ifa *et al.*, 2010; Weston, 2010). These techniques require no, or very simple, sample preparation protocols and we have introduced such a technique, termed easy ambient sonic spray ionisation (EASI), and have demonstrated the efficacy of EASI–MS in a wide range of applications (Ferreira *et al.*, 2009; Lalli *et al.*, 2010; Sawaya *et al.*, 2010). The EASI technique has been shown to function as a soft, low-noise, reproducible, and sensitive (high signal to noise ratio) method inherently free of electrical, or discharge or redox interferences. It uses no heating, no voltages, no lasers and no corona discharges and is based solely on sonic spray ionisation, which produces, when assisted by a cylinder of compressed N_2 or even a can of compressed air (Schwab *et al.*, 2012), a supersonic stream of very minute bipolar charged droplets of the solvent, which are used to bombard the sample surface. The EASI–MS technique also has been demonstrated to work efficiently for the direct characterisation and quality control of oils (Simas *et al.*, 2010, 2010; Riccio *et al.*, 2011; Cardoso *et al.*, 2012) and fuels (pca2404-bib-0001Alberici *et al.*, 2010).

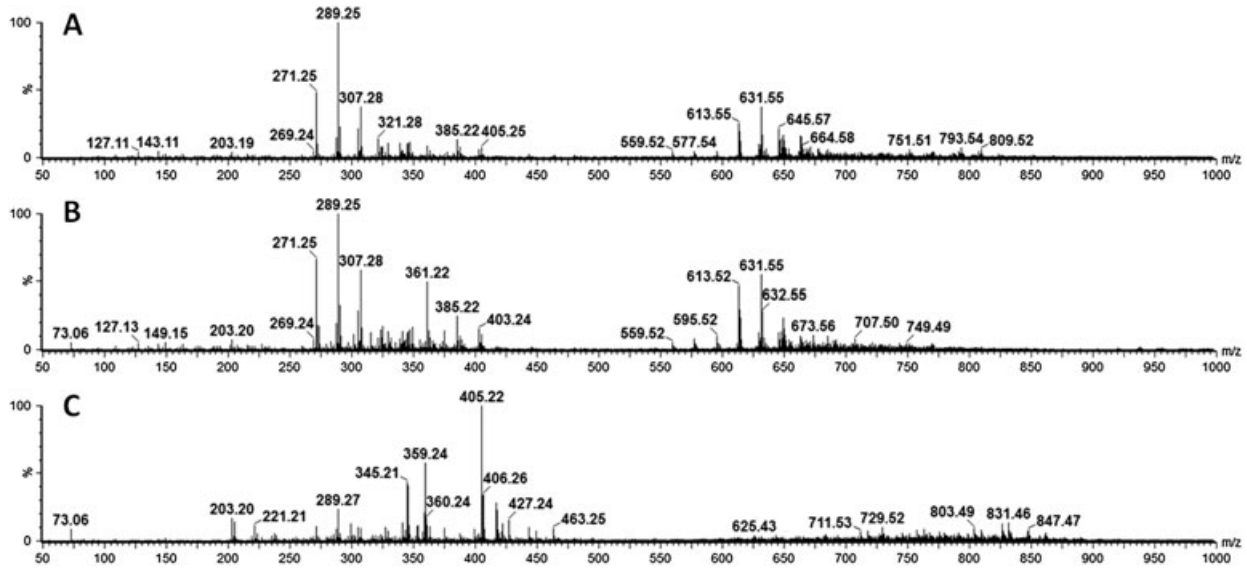


Figure 1. ESI(+)–MS fingerprintings of methanol solutions of (A) *Pterodon pubescens* oil sampled in the wild (natural), (B) the oil from a dichloromethane extract and (C) a sample of a commercial oil.

In this study we have tested the ability of both direct infusion ESI–MS and the more direct and less demanding desorption/ionisation EASI–MS to function as proper MS fingerprinting methods in providing fast, nearly direct and efficient characterisation, quality control, origin certification and recognition of chemical species present in the fruit oil of *P. pubescens*, focusing on the major diterpenoid constituents with known biological activities.

Experimental

Plant material

The fruits of *P. pubescens* were collected in São Carlos (São Paulo), under the supervision of Professor Jorge Yoshio Tamashiro from the Department of Botany, Institute of Biology (IB), University of Campinas (UNICAMP). The voucher specimen (UEC 1402) was deposited in the Herbarium of the IB-UNICAMP. Fruits were collected in Ponto Chique (Minas Gerias), Sorriso (Mato Grosso), Bom Jesus da Lapa (Bahia) and Aracaju (Sergipe). We also used a sample of commercial oil purchased at a popular market and a fresh oil sample taken directly from the schizogenous glands of fruit in the wild (natural oil).

Oil extraction

Clean fruits of *P. pubescens* were ground with dry ice in an industrial blender (Poli LS-06, Siemsen, Brusque/SC, Brazil) and extracted three times using dichloromethane as a solvent, over periods of 1.5 h each, at a ratio of 5:1 v/w (solvent:plant material) in an oscillating shaker at room temperature. The extract was filtered through a Büchner funnel and then through an analytical funnel containing cotton and sodium sulphate. Finally, the extract was concentrated under vacuum in a rotary evaporator (Buchi RE 215, Flawil, Switzerland).

Sample preparation

Oil samples (10 μL of each) were dissolved in 1 mL of methanol with 0.1% formic acid. Aliquots of 10 μL were withdrawn from these solutions and further diluted in 1 mL of methanol with 0.1% formic acid.

ESI(+)–MS and ESI(+)–MS/MS

The solutions were directly infused into the ESI ion source of the mass spectrometer. The total time for acquisition was set at 1 min. ESI–MS and ESI–MS/MS spectra were acquired in the positive mode in a Q-TOF Micromass spectrometer (Manchester, UK) with an ESI–QqTOF configuration with *m/z* resolution of ca. 6000 and ca. 10–30 ppm accuracy. The operating conditions were as follows: capillary voltage, 3.0 kV; source temperature, 80 °C; desolvation temperature, 80 °C; and cone voltage, 35 V. Diluted samples were infused by an automatic injection pump (Harvard Apparatus) with a continuous flow of 10 μL/min. The full scan spectra were acquired in the range of *m/z* 100–1000 and ESI–MS/MS spectra were all acquired in the Q-TOF mass spectrometer at collision energies of 10–30 eV from *m/z* 50 up to the *m/z* of the ion under study.

EASI(+)–MS

All the experiments were performed in a HCT Ultra ETD II Mass Spectrometer (Bruker Daltonics, Germany) which had its ESI source removed and coupled with a homemade EASI source. The EASI–MS spectra were acquired over the 50–1000 *m/z* range and performed in the positive ion mode. A seed cut was imprinted onto a paper surface (brown Kraft envelope paper) or the seed was placed directly in the ionisation source. The methanol flow rate was 20 μL/min, N_2 was used as the nebulising gas at 3 L/min and seed or paper-entrance angle was ~30°. Mass spectra were accumulated over 60 s.

Multivariate statistical analysis

The MS data sets from the samples were organised in a matrix using the XS (Extended Statistics) module of the MarkerLynx (Waters, USA) software. The data were truncated at 0.1 Da of peak separation and a threshold of 10 counts of intensity and then exported to Pirouette. The analysis of the data by priciplal components analysis (PCA) was conducted using the Pirouette 3.11 (InfoMetrix Inc., Bothell, WA,USA) software with the centre mean scaling method.

Results and discussion

Characterisation of the *P. pubescens* oil

The profiles of a fresh (natural) oil sample, a dichloromethane extract obtained directly from the fresh seed and a sample of a commercial

oil of unknown quality and origin, were initially evaluated via ESI(+)–MS (Fig. 1) so as to test and develop a proper methodology for the direct analysis of the *P. pubescens* oil. For direct infusion ESI (+)–MS, the samples were diluted in methanol:water (1:1, v/v), then acidified with formic acid to a final concentration of ca. 10 μg/mL.

Note in Fig. 1 that the ESI(+)–MS indeed offers typical MS fingerprints for the *P. pubescens* oil obtained directly from a fresh fruit (a certified sample from Sergipe State in Brazil) with great similarity between samples of the crude fresh oil and that obtained from the dichloromethane extract. The sample of commercial oil of unknown quality and origin displays also some similarities but is in general quite distinct and, as discussed below, a different geographical origin seems to be the major source of this dissimilarity. To better compare the diterpene profiles, Fig. 2 shows an expansion of the ESI(+)–MS data in the range from *m/z* 295 to 450. In this range, several ions are detected corresponding to a series of linear and cyclic diterpenes (Scheme 1) that are characteristic for the 'sucupira' species.

The ions of *m/z* 307, 325, 345, 361, 363, 373, 401, 403, 405, 417, 447 and 463 likely correspond to protonated moleculs of diterpenes that have already been reported for the *P. pubescens* oil (Arriaga *et al.*, 2000). To confirm these assignments, ions were subjected to ESI(+) MS/MS and HRESI(+)–MS experiments for structural investigation (Table 1).

Figure 3 shows a representative ESI(+)–MS/MS for the ion of *m/z* 307, which is attributed to 14,15-epoxy-geranylgeraniol (Table 1). The dissociation observed for this protonated molecule is indeed indicative of 14,15-epoxy-geranylgeraniol, and Scheme 2 proposes routes leading to major fragment ions. Note that the $C_{20}H_{34}O_2$ composition of this ion (Table 1) has also been indicated by high accuracy mass measurements with an error of 7.5 ppm (Table 1).

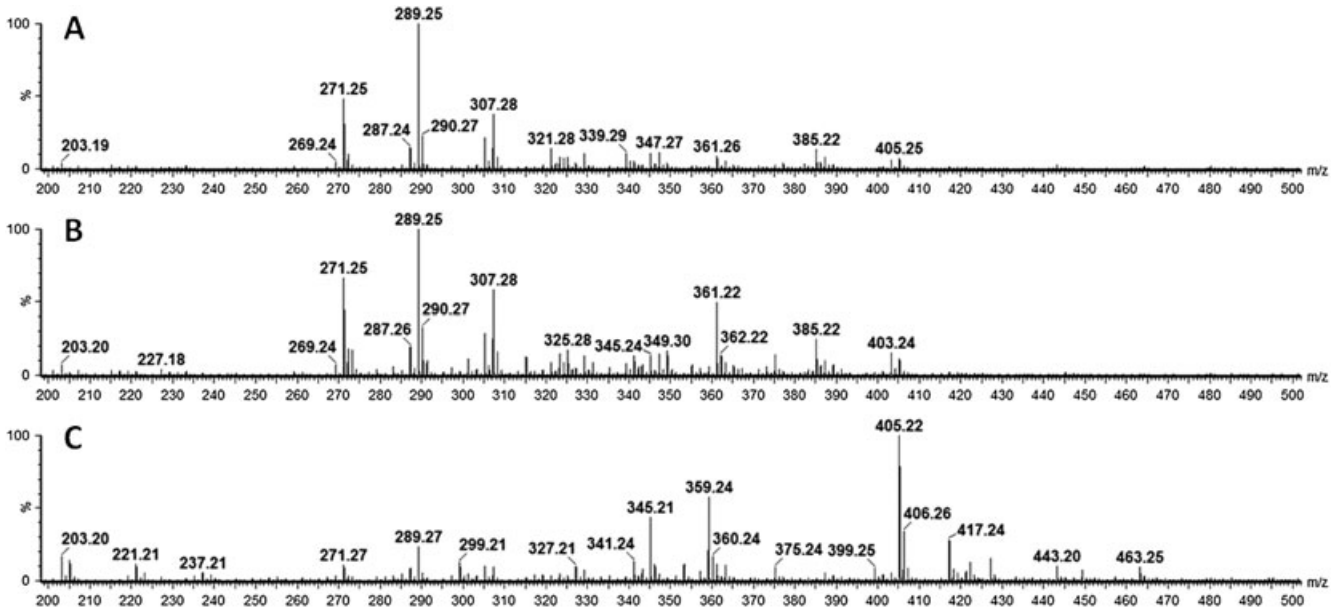


Figure 2. Expansion of the ESI(+)–MS of methanol solutions of (A) *Pterodon pubescens* oil sampled in the wild (natural), (B) the oil from a dichloromethane extract and (C) a sample of a commercial oil. The spectra are shown in the *m/z* range in which characteristic linear and cyclic diterpenes (Scheme 1) are detected.

Table 1. HRESI(+)–MS data of *Pterodon pubescens* diterpenes

Diterpene	Molecular formula	$[M+H]^+$ Calculated *m/z*	$[M+H]^+$ Experimental *m/z*	Error (ppm)
14,15-epoxy-geranylgeraniol	$C_{20}H_{34}O_2$	307.2637	307.2614	±7.5
15-dihidroxygeranylgeraniol	$C_{20}H_{36}O_3$	325.2743	325.2782	±12.0
1	$C_{22}H_{32}O_3$	345.2430	345.2397	±9.5
2	$C_{24}H_{34}O_5$	403.2484	403.2422	±15.4
4	$C_{25}H_{34}O_8$	463.2332	463.2298	±7.3
6	$C_{24}H_{32}O_5$	401.2328	401.2404	±5.2
7	$C_{24}H_{32}O_6$	417.2277	417.2317	±9.5
8	$C_{22}H_{28}O_5$	373.2015	373.2000	±4.0
9	$C_{21}H_{30}O_5$	363.2172	363.2193	±5.7
11, 12	$C_{23}H_{32}O_6$	405.2277	405.2235	±10.4
13	$C_{25}H_{34}O_7$	447.2383	447.2401	±4.0
15	$C_{21}H_{32}O_4$	361.2379	361.2411	±8.9

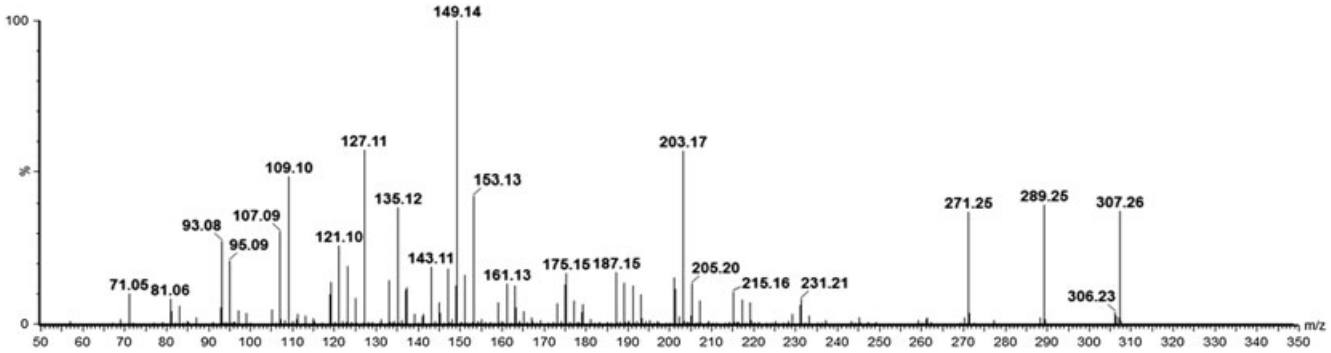
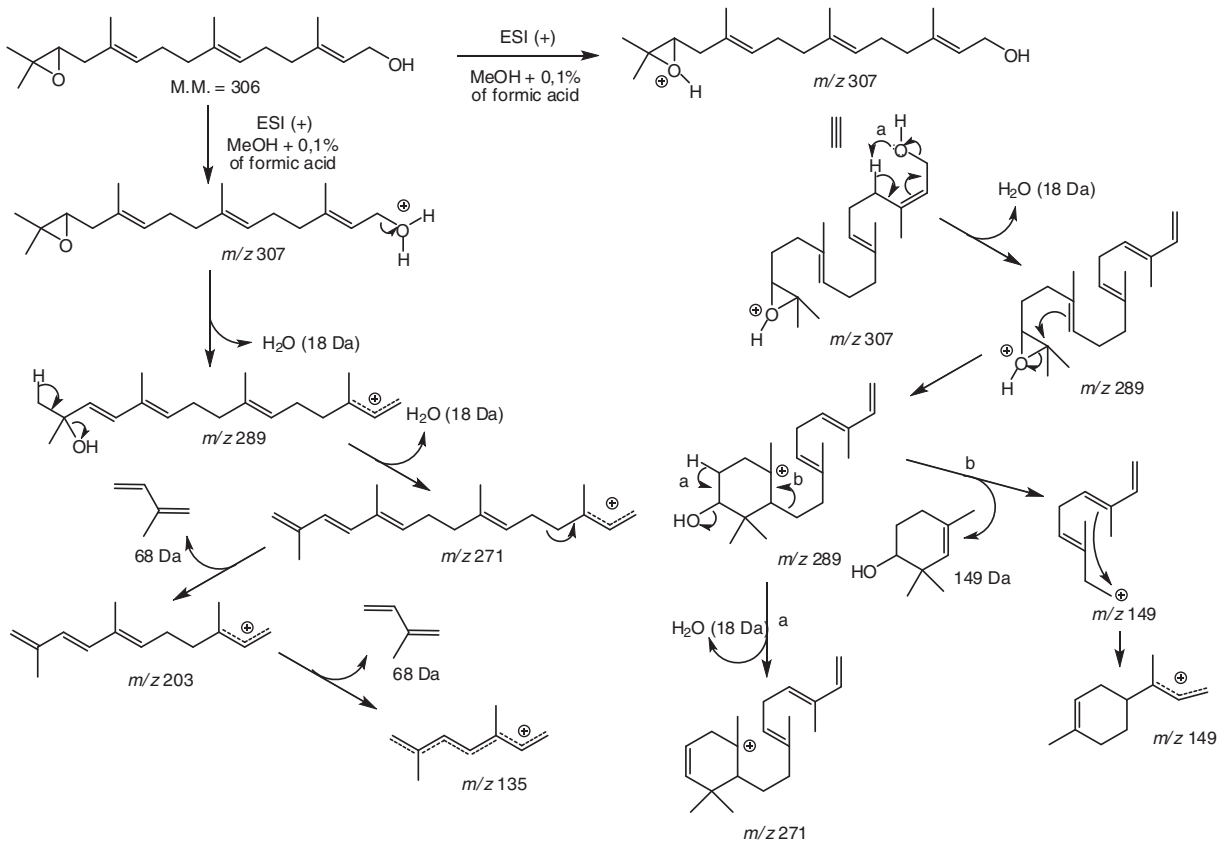


Figure 3. The ESI(+)–MS/MS spectrum of the ion of *m/z* 307.



Scheme 2. Proposed dissociation pathways of the *m/z* 307 ion, which has been attributed to protonated 14,15-epoxy-geranylgeraniol.

The ion of *m/z* 325, when subjected to ESI(+)–MS/MS (not shown), showed comparable dissociation chemistry as that of the ion of *m/z* 307. Based on such a profile and the high accuracy mass measurement (Table 1), this ion was tentatively attributed to protonated 14,15-dihydroxygeranylgeraniol (Table 1). In Fig. 1A and B, the linear diterpenes 14,15-epoxy-geranylgeraniol and 14,15-dihydroxygeranylgeraniol are also detected via the ions of *m/z* 613 and 649, which correspond to the dimeric species $[2\,M+H]^+$, as well as the ion of *m/z* 631, which corresponds to $[M+M'+H]^+$.

The ion of *m/z* 403 (Fig. 4B) was attributed to diterpene 4 based on the consecutive losses of molecules of acetic acid (60 Da) and water (Scheme 3). The ion of *m/z* 361 was attributed to the protonated molecule of diterpene 15 (Scheme 1), which displayed a rather unique and simple ESI(+)–MS/MS (Fig. 4A) with the predominance of a fragment ion of *m/z* 301, as well as a second fragment ion of *m/z* 283 of medium abundance. Loss of an acetic acid (60 Da) seems to lead to the ion of *m/z* 301 with subsequent loss of a water molecule (18 Da) leading to the ion of *m/z* 283. The other protonated molecules attributed to diterpenes (Table 1) were also subjected to ESI(+)–MS/MS experiments displaying similar dissociation routes as proposed in Scheme 3.

Certification of origin

We next tested the ability of direct infusion ESI(+)–MS to screen for the origin of the *P. pubescens* oil. Dichloromethane extracts

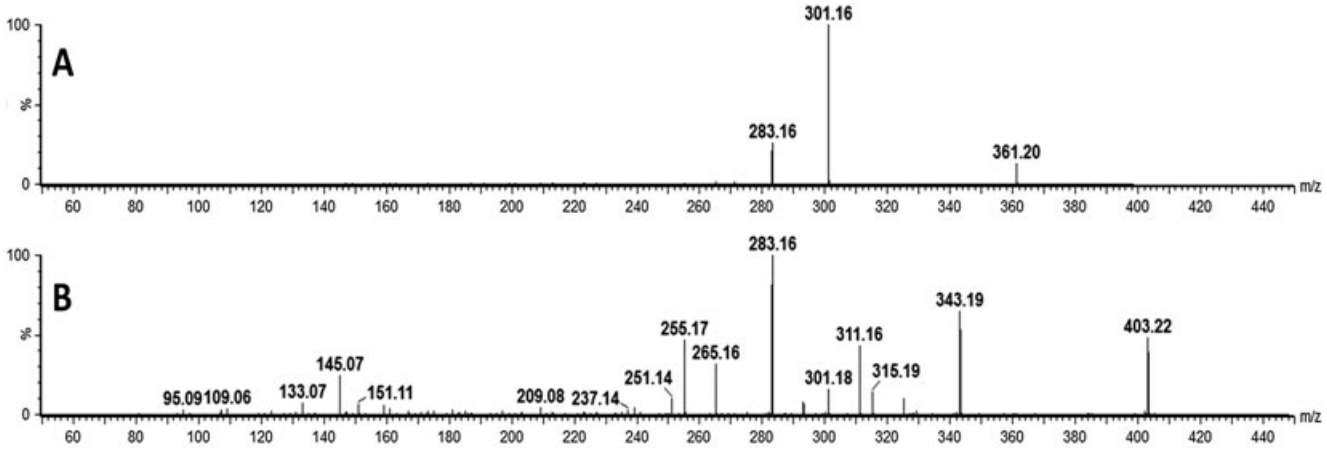
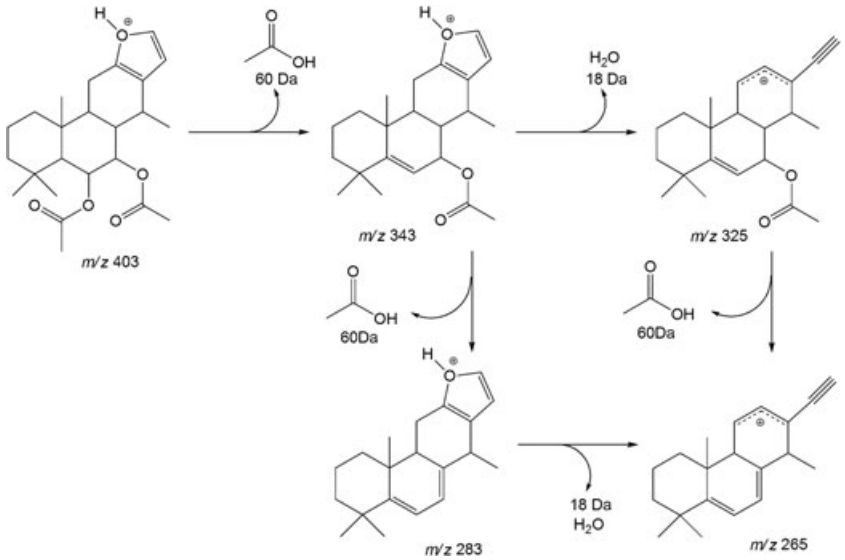


Figure 4. The ESI(+)–MS/MS spectra of (a) the ion of *m/z* 361 and (b) the ion of *m/z* 403.



Scheme 3. Proposed fragmentation of the *m/z* 403 ion, attributed to protonated diterpene **4**.

were obtained from certified seeds obtained from five different geographical areas (different states in Brazil) and their ESI(+)–MS data were obtained after dilution in acidified methanol in the typical *m/z* 200–460 range (Fig. 5). Note that the profiles of Fig. 5 display characteristic changes in relative abundances that seems to characterise the geographical origin of the oils. The profiles of the samples from the states of São Paulo, Bahia and Minas Gerais were the most similar (Figs 5C, 5D and 5E), showing essentially the same ions with small variations in abundances. The ESI(+)–MS profiles of samples from the states of Mato Grosso and Sergipe (Figs 5A and 5B) were the most contrasting, differing mainly by the low abundance of the ion of *m/z* 405, and in the predominance of the ions of *m/z* 289 for Mato Grosso and *m/z* 285 for Sergipe. Another unique feature of both spectra seems to be the relative high abundance of the ion of *m/z* 361and this ion, when Fig. 5D and 5E are compared to Figs 2A and 2B, seems to be a characteristic marker for their dichloromethane extracts.

To test statistically the performance of ESI(+)–MS fingerprinting to certify the geographical origin for *the P. pubescens* oil, PCA of the mean-centred data treatment was performed (Fig. 6). Three samples of dichloromethane extracts for each state were analysed. The results showed that PCA extracted three major principal components. The plot of PC1 versus PC2 versus PC3 accounted for ca. 99% of data variance (PC1 = 62.87 %, PC2 = 32.62 %, PC3 = 3.46 %) and showed that all oils from the different states in Brazil were clearly grouped. By the loadings analysis, which provides the most significant *m/z* values for separating the five groups, some ions with higher potential for biomarkers (the diterpenes forming the ions of *m/z* 289, 345, 361, 403 and 417) were determined to have contributed mostly to the major differences providing group distinction (Fig. 6B). Not only the presence or absence of

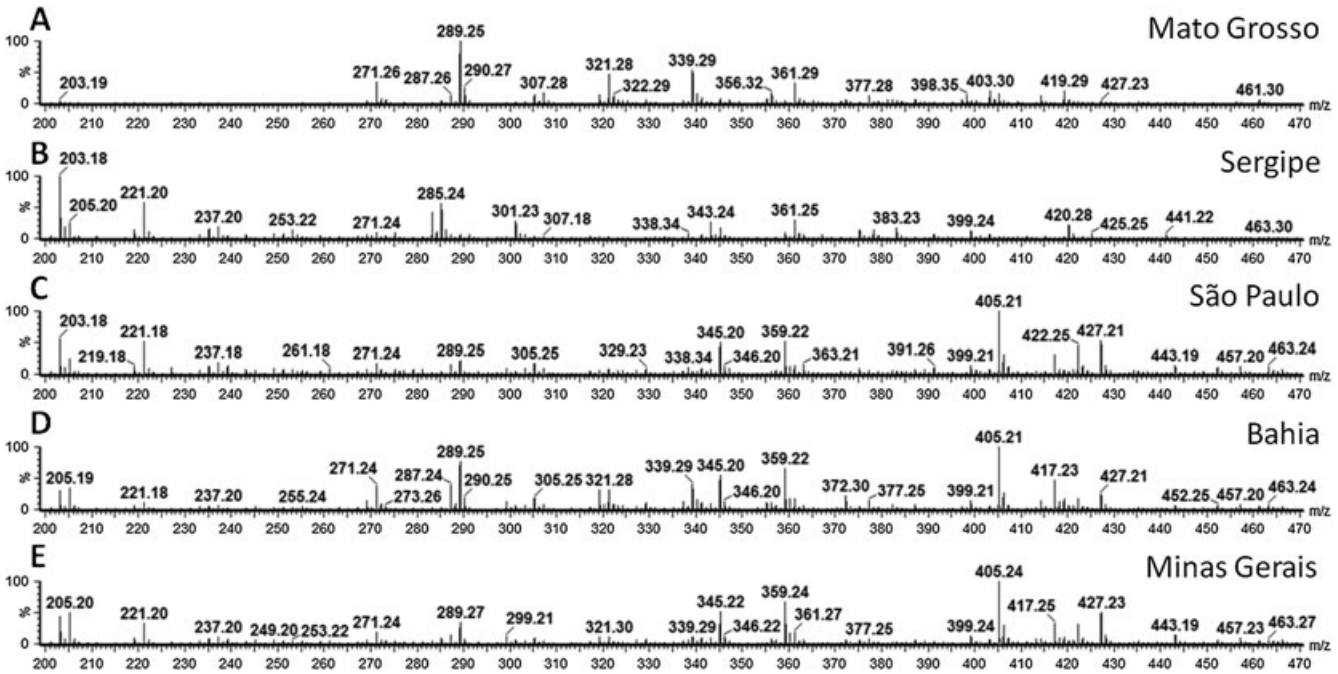


Figure 5. Characteristic ESI(+)–MS spectra (fingerprints) of acidified methanolic solutions of the certified dichloromethane extracts of *Pterodon pubescens* oils from different states in Brazil.

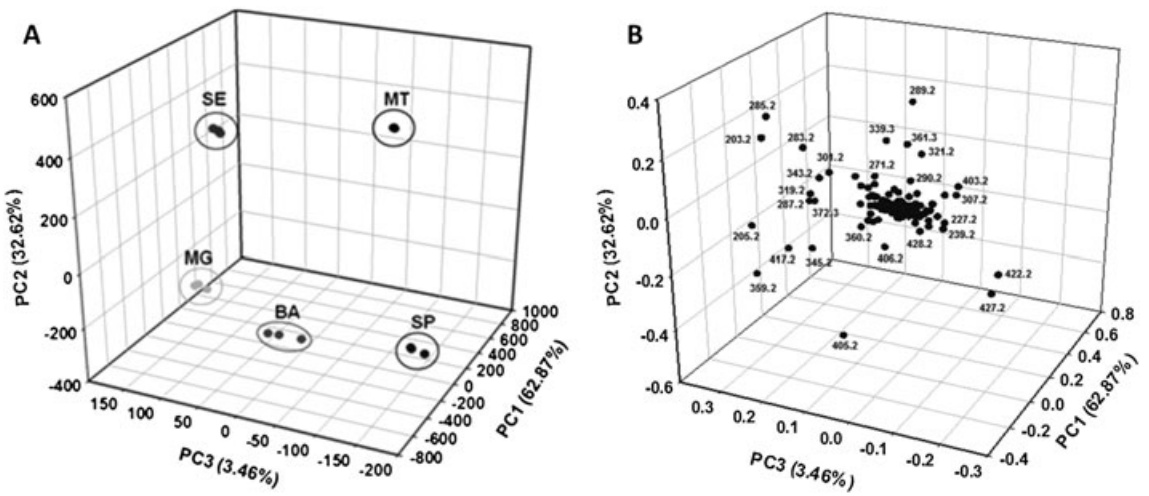


Figure 6. (A) Scatter plot and (B) loadings of PC1 versus PC2 versus PC3 for the ESI(+)–MS data of *Pterodon pubescens* oils from different states in Brazil.

specific ions, but also their relative intensity in the spectra provided separation between sample groups. The model demonstrates also the close similarity between samples from the states of São Paulo, Minas Gerais and Bahia, as well as the most contrasting profiles for the Sergipe and Mato Grosso samples.

When the spectra of Fig. 2 are compared with the spectra of Fig. 5, it is clear that the oil used as reference from the Sergipe State displays, as now expected, a profile quite similar to that of Fig. 5B (Sergipe), most particularly when the two dichloromethane extracts are compared (Fig. 2B versus Fig. 5B). The commercial oil (Fig. 2C) of unknown quality and origin displays a profile very similar to those for the oils from the São Paulo, Minas Gerais and Bahia states (Fig. 5), but PCA analysis indicates closest similarity to the oil from Bahia State.

EASI(+)–MS

Analysis of the *P. pubescens* oil via EASI(+)–MS fingerprinting was also tested for the fully direct, instantaneous classification of the oil. Figure 7 shows actual pictures of the analysis whereas Fig. 8 shows typical EASI(+)–MS obtained from the oil imprinted on a paper surface and directly from the seed surface after sectioning. This procedure is simpler and more direct than the ESI–MS analysis since it requires no oil extraction or sample preparation being applied to the oil directly imprinted to a paper surface.

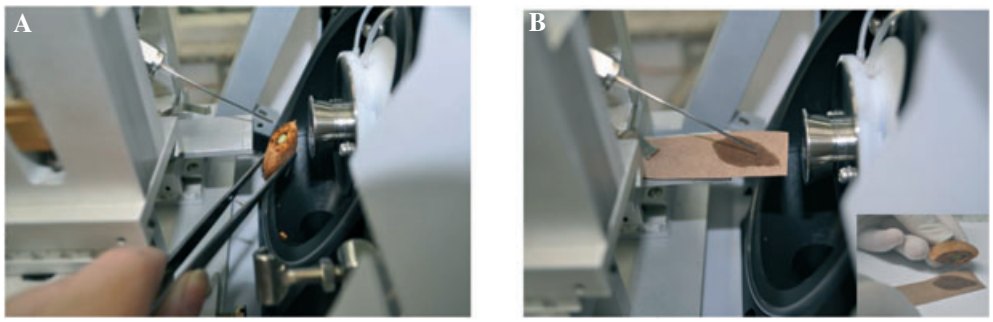


Figure 7. Photographs of the EASI(+)–MS analysis of *Pterodon pubescens* oil. (A) Directly from the seed surface. (B) From the oil imprinted on a paper surface. The insert shows imprinting of the oil onto the paper surface directly from the seed.

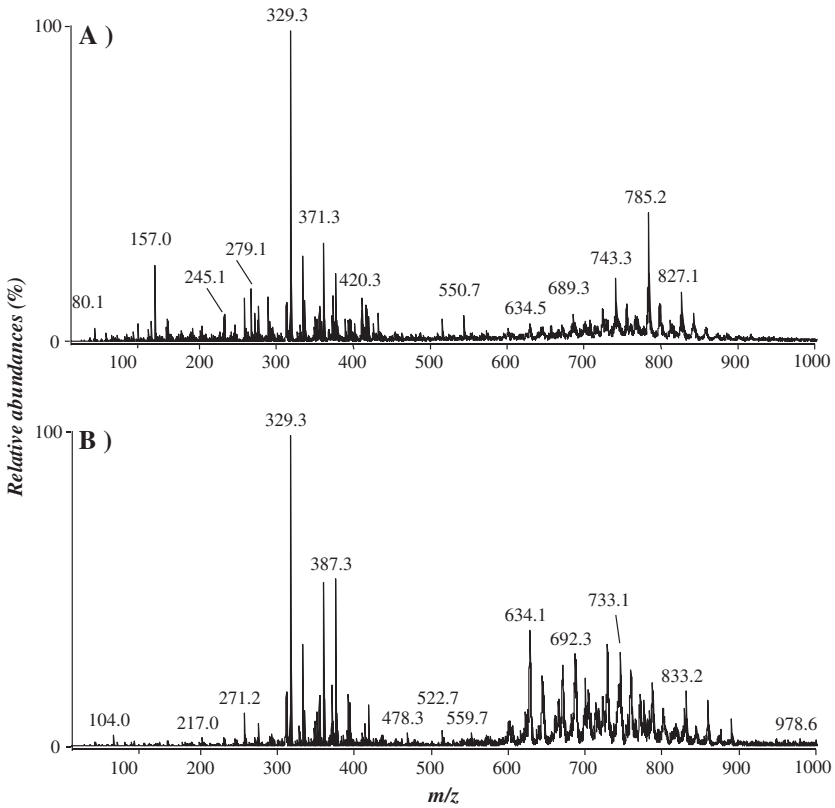


Figure 8. The EASI(+)–MS spectra (fingerprint) of the crude *Pterodon pubescens* oil performed from the oil (A) imprinted on a paper surface and (B) directly from the seed.

This imprinting process is similar to that recently applied for the EASI–MS analysis of meats and fats (Porcari *et al*., in press). When the ESI(+)–MS and EASI(+)–MS data are compared, the major difference found is related to the ionised forms via which the (di) terpenes are detected. In ESI(+)–MS (Figs 1 and 2), the terpernes are detected mainly as protonated molecules $[M + H]^+$ of *m/z* 289 and 307, for instance, whereas via EASI(+)–MS (Fig. 8) the terpenes are also detected as $[M + H]^+$ but mainly as $[M + Na]^+$ and $[M + K]^+$ adducts such as for instance that of *m/z* 329. This diversity of cationised forms for EASI(+)–MS complicates attribution of constituents, as compared to direct infusion ESI(+)–MS analysis, but offers a reliable method for the very fast and nearly instantaneous characterisation of the *P. pubescens* oil, either directly from its seed of after simple and fast imprinting on a paper surface. Note that the paper imprinting process seems quite appropriate for remote oil analysis due to the easy mailing of the sample.

Summary

Direct mass spectrometry analysis, either via direct infusion ESI (+)–MS after solvent extraction and/or dilution in a proper solvent or via ambient desorption/ionisation via EASI(+)–MS performed directly on the seed surface or on a paper surface imprinted with the oil, therefore has been shown to provide typical (di)terpene profiles for the oils. These direct MS techniques therefore offer simple and effective methods to characterise the *P. pubescens* oil, being applicable to its quality control and certification of authenticity and origin, as demonstrated herein by

the analysis of a commercialised oil sample of unknown quality and origin.

Acknowledgements

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Instituto Nacional de Materiais Complexos (INOMAT-FINEP) for financial assistance.

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