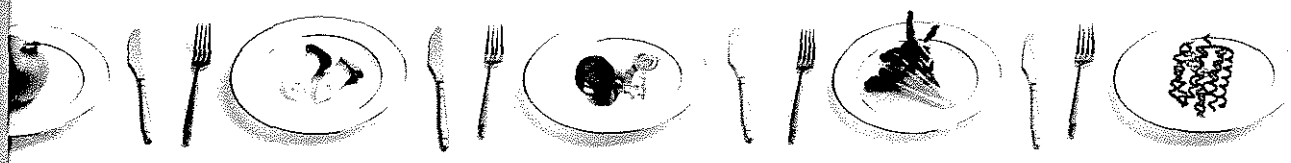

EuroFoodChem XV

FOOD FOR THE FUTURE

- the contribution of chemistry
to improvement of food quality

Proceeding 2

Copenhagen, Denmark
5-8 July 2009

M132:
Characterization of New Phenolic Derivatives in Portuguese Propolis by Electrospray Mass Spectrometry

Soraia I. Falcão[1,2*], Miguel Vilas-Boas[1], Cristina Freire[2], Maria R.M. Domingues[3], Susana M. Cardoso[1]

[1]*CIMO - Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Sta. Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*

[2]*Departamento de Química, Faculdade de Ciências do Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal*

[3]*Centro de espectrometria de massa, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal;* *sfalcao@ipb.pt

KEYWORDS

propolis, phenolic compounds, mass spectrometry, electrospray ionization

SUMMARY

This research outlines an extensive characterization of the phenolic composition of a propolis sample from the northeast of Portugal. For that, an ethanolic extract was prepared, fractionated by HPLC and the identification of the phenolic compounds was done by electrospray mass spectrometry in the negative mode. This technical approach allowed the identification of 37 phenolic compounds in the Portuguese propolis sample, including seven that were described for the first time. Two of these new compounds had $[M-H]^-$ ions at *m/z* 403, and the others had $[M-H]^-$ ions at *m/z* 433, *m/z* 461, *m/z* 417, *m/z* 475, and *m/z* 565. In general, the molecular weight of these compounds was higher than the common phenolic compounds of propolis and their fragmentation pattern suggested that they belong to the flavonoid family probably linked with a phenylpropanoic acid moiety in the position C5 (*m/z* 403, 433, 461, 475) and C3 (*m/z* 403, 417), corresponding to pinocembrin and pinobanksin derivatives. The ion at *m/z* 565 seems to be a *p*-coumaric ester derivative dimer.

INTRODUCTION

Propolis is a chemically complex resinous substance collected by honeybees (*Apis mellifera*) from tree buds, comprising plant exudates, secreted substances from bee metabolism, pollen and waxes (Marcucci, 1995). This product is widely used in traditional medicine (Marcucci, 1995) and it has recently gained popularity as a health food supplement. Currently, it is extensively used in foods and beverages, and it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes and cancer (Burdock, 1998).

The chemical composition of propolis depends strongly on the plant sources available to bees at different locations (Popova *et al.*, 2004). In temperate zones, bud excudates from *Populus* species and their hybrids are the main sources of the bee glue (Bankova *et al.*, 2000). In tropical areas where these plants are not native, bees find other plant sources for this resin such as *Clusia* in Cuba and *Baccharis* in Brazil (Park *et al.*, 2002).

The most frequently reported phenolic components in propolis samples originated from Europe, North America and Asia are flavonoids and phenolic acid esters (Marcucci, 1995), whereas prenylated derivatives of *p*-coumaric acids are abundant in Brazilian propolis (Park *et al.*, 2002).

This work aims to study the phenolic composition of a Northeast Portuguese propolis sample by electrospray mass spectrometry. New phenolic derivatives were found and characterized in propolis for the first time.

MATERIALS AND METHODS

Sample. The propolis sample was collected in the fall of 2007 from *Apis mellifera* hives of the Northeast of Portugal. It was obtained after the honey extraction, by scratching the hive walls and frames, following by the removal of debris of wood and bees. This propolis sample was then stored at -20 ºC until analysis.

Extraction of phenolic compounds. The propolis was grounded and homogenized. The sample was extracted with 80% of ethanol/water (1/10, w/v) at 70ºC for 1h, the resulting mixture was filtered and the residue was re-extract in the same conditions. The filtrated solutions were combined, concentrated, frozen at -20ºC and freeze-dried.

HPLC. The propolis extract was analyzed and fractionated by reversed-phase high-performance liquid chromatography (HPLC), according to the method of Gardana *et al.* (2007) with some modifications. The HPLC analysis was performed on a RP-C18 column 250 mm× 4 mm id, 5µm bead diameter and its temperature was maintained at 30ºC with a flow rate of 1 mL/min. The mobile phase comprised (A) 0,1% formic acid in water and (B) 0,1% formic acid in acetonitrile, which were previously degassed and filtrated. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min, then returning to the initial conditions.

Mass spectrometry analysis by ESI-MS and ESI-MS^n. The HPLC fractions were dissolved in methanol and directly injected into the ESI source by means of a syringe pump, at a flow rate of 8 µL min^{-1}. Typical ESI conditions were: nitrogen sheath gas 30 psi, spray voltage 4.7 kV, capillary temperature 350°C, capillary voltage -7.0 V and tube lens voltage -71.8 V. CID-MS/MS and MS^n experiments were performed on mass-selected precursor ions using standard isolation and excitation configuration.

RESULTS AND DISCUSSIONS

As shown in Fig.1, the HPLC analysis of the ethanolic extract obtained from the Portuguese propolis sample allowed the collection of thirty five resolved fractions, that where further studied by electrospray mass spectrometry in the negative ion mode. This experimental approach allowed the identification of 37 phenolic compounds which included phenolic acids and flavonoids already reported in propolis, and also new compounds. The most abundant HPLC-UV fractions contained phenolics that are frequently reported in propolis from the temperate zones, including the pinocembrin (*m/z* 255, fraction 17), chrysin (*m/z* 253 fraction 19) and pinobanksin-3-*O*-acetate (*m/z* 313 fraction 20).

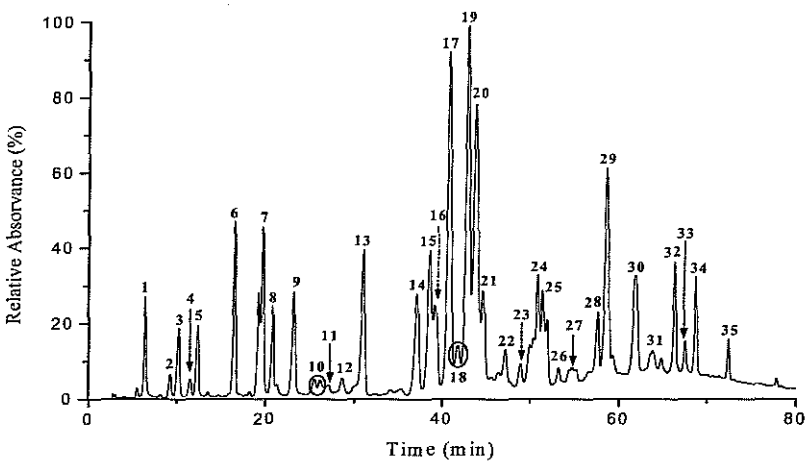

Fig.1. Chromatographic profile at 280 nm for the ethanolic extract of the Portuguese propolis sample.

The new phenolic compounds corresponded to the $[M-H]^-$ ions at *m/z* 403, *m/z* 433, *m/z* 461 (fraction 25), *m/z* 417 (fraction 28), *m/z* 475 (fraction 29), *m/z* 403 and *m/z* 565 (fraction 31). The results obtained by MS and MS/MS analysis are shown in Table 1.

Table 1. MS data obtained for the new phenolic compounds identified in the ethanolic extract of the Portuguese propolis sample.

Fraction number	$[M-H]^-$	Main Fragments ESI$^-$MS[a]
25	403	293, 255, 385, 267, 281
	433	309, 401, 323, 415, 255
	461	351, 443, 401, 291, 419, 253
28	417	267, 281, 402, 385
29	475	415, 433, 400, 253
31	403	253, 283, 271, 297
	565	283, 269, 417, 455

[a]Ordered by decreasing intensities.

The ions at *m/z* 403 and 433 in fraction 25 derive from pinocembrin (structures I and II in Fig.2), as their MS^2 spectra showed the fragment ion at *m/z* 255, which corresponds to the $[M-H]^-$ ion of pinocembrin. Moreover, the ions at *m/z* 403 (fraction 31), 417, 461 and 475 showed fragment ions corresponding to the $[M-H]^-$ ion of chrysin or its methylated derivative (ions at *m/z* 253 and 267, respectively). Thus, attending that chrysin is the main fragment ion of the esterified pinobanksins, these compounds were considered to be derivatives of pinobanksin (structures III, IV, V and VI in Fig.2). Also, the fragmentation pathways of these new phenolic compounds suggested that their flavonoid structures are linked with phenylpropanoic acid moiety on position C5, C7 or C3. The fragmentation pathway of the compound at *m/z* 565 suggested the presence of a *p*-coumaric ester derivative dimer (structure VII in Fig.2).

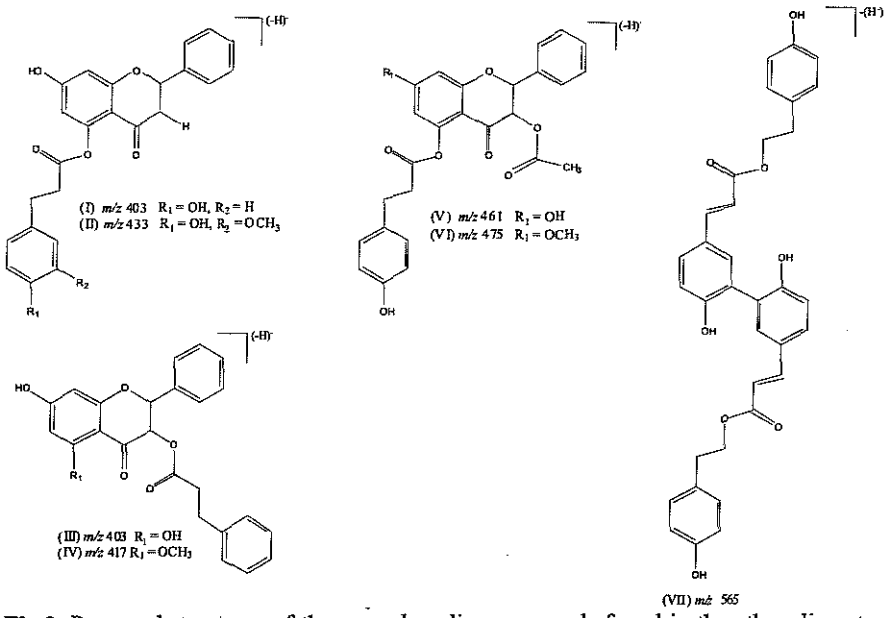

Fig.2. Proposed structures of the new phenolic compounds found in the ethanolic extract of the Portuguese propolis sample.

CONCLUSIONS

The ESI-MS and MS^n analysis of the ethanolic extract obtained from the Portuguese propolis, after reversed phased HPLC fractionation, allow to conclude that this sample is rich in the common flavonoids typical of propolis from temperate zones. Still, new phenolic derivatives were also detected.

ACKNOWLEDGMENTS

Soraia I. Falcão thanks FCT for a PhD grant (SFRH/BD/44855/2008).

REFERENCES

Bankova, V.S., S.L. De Castro and M.C. Marcucci, 2000: Propolis: Chemical composition, biological properties and therapeutic activity. Apidologie 31, 3-15.

Burdock, G.A., 1998: Review of the biological properties and toxicity of bee propolis (propolis). Food Chem. Toxicol. 36, 347–363.

Gardana, C., M. Scaglianti, P. Pietta and P. Simonetti, 2007: Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography tandem mass spectrometry. J. Pharma. Biomed. Anal. *45*, 390-399.

Marcucci, M.C., 1995: Propolis: Chemical composition, biological properties and therapeutic activity. Apidologie 26, 83–99.

Park Y.K., S.M. Alencar and C.L. Aguiar, 2002: Botanical origin and chemical composition of Brazilian propolis. J. Agric. Food Chem. 50, 2502–2506.

Popova M., V. Bankova, D. Butovska, V. Petkov, B. Nikolova-Damyanova, A.G. Sabatini, G.L. Marcazzan and S. Bogdanov, 2004: Validated methods for the quantification of biologically active constituents of poplar-type propolis. Phytochem. Anal. 15, 235–240.