Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 354–361
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Analysis of flavonoids from propolis by on-line HPLC–electrospray mass spectrometry

Nicola Volpi [a,*], Gianluca Bergonzini [b]

[a] *Department of Biologia Animale, University of Modena and Reggio Emilia, Via Campi 213/d, 41100 Modena, Italy*
[b] *Docteur Nature, Modena, Italy*

Received 6 September 2005; received in revised form 31 March 2006; accepted 10 April 2006
Available online 9 June 2006

Abstract

In this paper, the qualitative and quantitative separation and determination of the polyphenolic component of propolis preparations in the form of ethanolic extract, usually used for commercial pharmaceutical preparations, has been investigated by means of on-line HPLC–ESI/MS technique. Propolis of different origin have been evaluated for their components and a specific fingerprint has been determined potentially useful for the quality control of extracts in pharmaceutical preparations.

The ethanolic extracts of propolis from Argentina, Italy and Spain shows approximately the same total ion chromatogram (TIC) profile due to the presence of the same molecular species, identified by the negative ESI–MS. On the contrary, the samples from Azerbaijan, China, Ethiopia and Kenya show a very peculiar TIC profiles. By using many purified flavonoids and calibration curves over a wide concentration range, from 0.05 (5 μg/ml) to 5 μg (500 μg/ml), an accurate assessment of the contents of several bioactive compounds in extract samples was performed. The propolis from Argentina, Italy and Spain show a great amount of pinocembrin (approximately 49%, 48% and 39% of the total identified flavonoids, respectively) and variable but similar percentages of the other species. On the contrary, the propolis from China, Azerbaijan and Ethiopia have a great amount of pinocembrin (approximately 63%, 46% and 62%, respectively) but no presence of genistein, kaempferol, apigenin and chrysin for the sample from China, genistein, kaempferol, acacetin and chrysin for the propolis from Azerbaijan, and no kaempferol and acacetin for the sample from Ethiopia. The ethanolic extract from propolis of Kenya has no identified flavonoid species but just a peak possessing a *m/z* of 253.0. Finally, an evaluation of the presence of total flavonoids for the various propolis samples was performed, with extracts from Argentina, Italy and Spain more rich in polyphenols than those from Azerbaijan, China, Ethiopia and Kenya.

The HPLC–ESI/MS under the experimental conditions illustrated represents a valuable method for the qualitative and quantitative assay of the most relevant components of propolis. On-line HPLC–ESI/MS analysis constitutes an alternative to obtain typical fingerprints of propolis and a reliable identification of a large number of propolis polyphenolic components.

Keywords: Propolis; Polyphenols; Flavonoids; HPLC; Electrospray ionization; Mass spectrometry

1. Introduction

Propolis is a resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants, mainly from the poplar (Populus) genus and, to a lesser extent, beech, horsechestnut, birch and conifer trees. Propolis has been used extensively in folk medicine for many years, and there is substantial evidence to indicate that propolis has antiseptic, antifungal, antibacterical, antiviral, anti-inflammatory and antioxidant properties [1]. Current applications of propolis include over-the-counter preparations, mainly based on ethanolic extracts, for cold syndrome (upper respiratory tract infections, common cold, flu-like infection) as well as dermatological preparations useful in wound healing, treatment of boils, acne, herpes simplex and genitalis, and neurodermatitis [1,2].

Abbreviations: API, atmospheric pressure ionization; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ESI, electrospray ionization; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; RP, reverse phase; SIM, selected ion monitoring; TIC, total ion chromatogram

* Corresponding author. Tel.: +39 059 2055543; fax: +39 059 2055548.
E-mail address: volpi@unimo.it (N. Volpi).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.04.017

Research of the polyphenols (flavonoids and related phenolic acids) has been prompted by their visible beneficial effects on health, for example their antimutagenic, anticarcenogenic, antiatherogenic effects. Primarily flavonoids aroused great interest after they had been found to have effects in inhibiting the copper-catalyzed oxidation of low-density lipoprotein, inhibiting platelet clotting and arachidonate metabolism, reducing liver injury from peroxidized oil, and having cancer-chemopreventative properties [3,4].

In spite of possible differences in composition due to propolis collecting bees that use resins from different plant sources, most propolis samples share considerable similarity in their overall chemical nature. Raw propolis is composed of 50% resin, composed of flavonoids and related phenolic acids and known as the polyphenolic fraction, 30% wax, 10% essential oils, 5% pollen and 5% various organic compounds [5]. Propolis cannot be used as raw material, and it must be purified by extraction with solvents. This process should remove the inert material and preserve the polyphenolic fraction. A multi-step extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components [5]. These last compounds are considered to contribute more to the visible healing effects than the other propolis constituents. Flavonoids and phenolic acids, especially caffeates, are known for their antibacterial, antiviral and antioxidant action [6].

Several methods have been developed to analyse the polyphenols in various matrices: thin-layer chromatography, gas chromatography, high-performance liquid chromatography (HPLC), HPLC–mass spectrometry [7], and capillary electrophoresis (CE) [8,9] are the most powerful analytical separation methods. In particular, the advent of fast atom bombardment (FAB), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) combined with tandem mass spectrometry (MS/MS) has permitted ready study of the flavonoids, their ion chemistry, and the determination of flavonoids in low concentrations in several extracts [7,10]. Furthermore, liquid chromatography (LC)–MS technique is able to separate each other single components in complex mixtures and to perform their identification and quantification [7,10].

Due to these several advantages, LC–MS has gained widespread interest as a favourable technique for the determination of pharmacologically interesting compounds in biological matrices, such as wood pulp [10], plants [11], shoots [12], fibres [13], various extracts [14–17], human urine [18].

However, very few studies have been reported on the characterization of flavonoids from propolis by LC–MS [11,19] with no application to the quality of different propolis extracts. This paper aims to gain new insight into the qualitative and quantitative separation and determination of the polyphenolic component of propolis preparations in the form of ethanolic extract, usually used for commercial pharmaceutical preparations, by means of the HPLC–ESI/MS technique. Furthermore, propolis of different origin have been evaluated for their components and a specific fingerprint has been determined potentially useful for the quality control of propolis extracts in pharmaceutical preparations.

2. Materials and methods

2.1. Materials

Various flavonoids (see Scheme 1 for the identified species in propolis extracts by means of HPLC–ESI/MS), acacetin, apigenin, baicalein, catechin, chrysin, galangin, genistein, kaempferol, luteolin, myricetin, naringenin, pinocembrin, poncirin, quercetin and vanillin, were purchased by Sigma. Samples were prepared by dissolving the standard in ethanol at a concentration of 1 mg/ml and diluting 1:10, 1:50 and 1:100 to obtain a final concentration of 0.1, 0.02 and 0.01 mg/ml, respectively. In order to test the linearity of the selected ion monitoring (SIM) response under the negative ion mode, the different polyphenol solutions prepared as reported above were injected at increasing concentration, from 0.05 (5 μg/ml) to 5 μg (500 μg/ml). The calibration graphs were constructed by plotting the SIM response of flavonoids against their concentration. The limit of detection was estimated as the signal-to-noise ratio = 3 [7] and calculated at 0.025 μg (2.5 μg/ml).

Accuracy and precision were determined by repeating six times the HPLC quantitative evaluation for all standards. The values were reported as means and standard deviation (S.D.). Furthermore, the coefficient of variation (CV%) for each standard was determined.

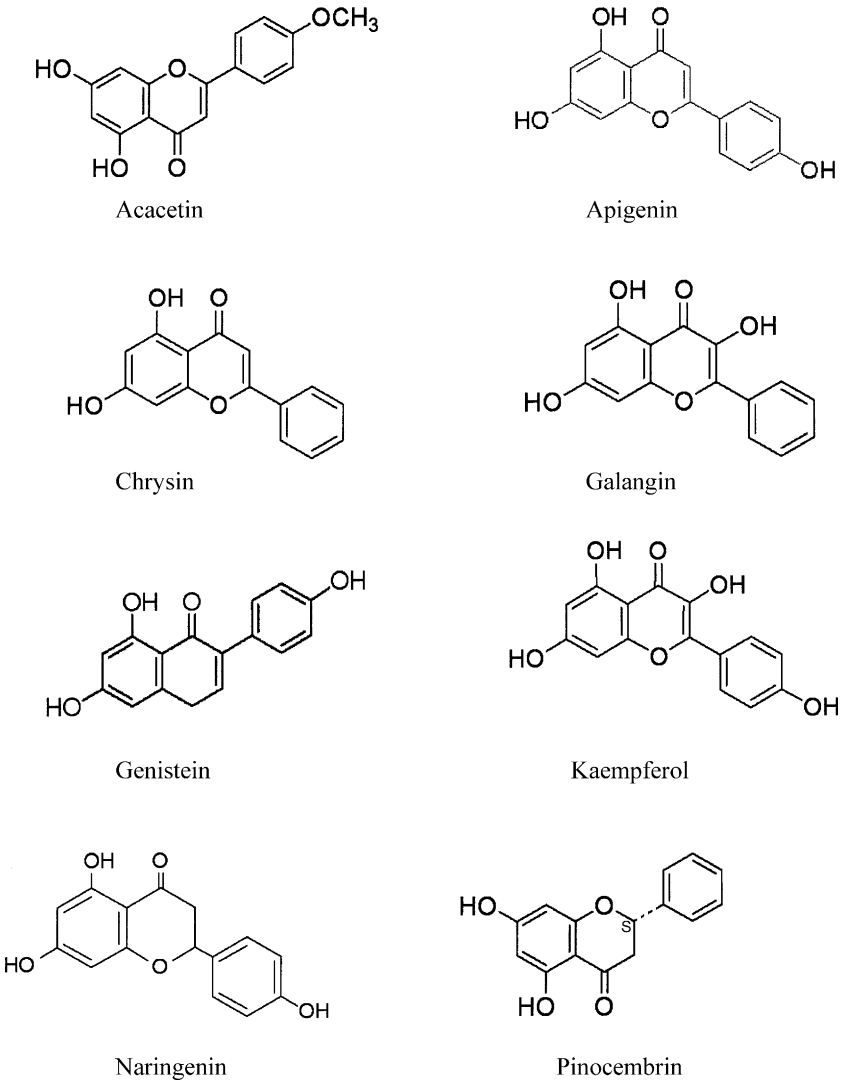
The precision of the HPLC/MS method with SIM under negative mode was also performed by injecting a propolis extract (from Argentina) six times. The standard deviation (S.D.) and coefficient of variation (CV%) values for the identified flavonoids were determined.

The different propolis samples were from Argentina, Azerbaijan, China, Ethiopia, Kenya, Italy and Spain. Preparations were performed in the form of ethanolic extracts used to prepare various products such as oral sprays and syrups, at a concentration of 1 mg/ml. Samples of propolis were extracted by means of ethanol (10 mg per 10 ml ethanol) under continuous mixing at room temperature for 6 h. After extraction and centrifugation at 10,000 rpm for 10 min, the ethanolic preparations were directly used for the HPLC–ESI/MS analysis.

2.2. HPLC–ESI/MS

The high-performance liquid chromatography equipment was from Jasco (pump mod. PU-1580, Rheodyne injector equipped with a 10 μl loop, software Jasco-Borwin rel. 1.5). The flavonoids from propolis were separated by using a 150 mm × 4.6 mm stainless-steel column Synergi 4 μm Fusion-RP (C18) 80Ä. The eluents were (A) 0.25% acetic acid and (B) methanol. Separations were performed at room temperature by solvent gradient elution from 0 min at 50% A/50% B to 60 min at 100% B at a flow rate of 0.5 ml/min.

An Agilent 1100 VL series mass spectrometer (Agilent Technologies Inc.) was used on-line with HPLC equipment. The electrospray interface was set in negative ionization mode with the capillary voltage at 3500 V and a source of temperature of 350 °C in full scan spectra (200–2200 Da, 10 full scans/s). Nitrogen was used as a drying (9 l/min) and nebulizing gas (11 p.s.i.).



Scheme 1. Structure of the separated and identified flavonoids by means of HPLC/ESI–MS. The structures of polyphenols were from SciFinder Scholar Database, ver. 2002.1, by 2002 American Chemical Society.

Software versions were 4.0 LC/MSD trap control 4.2 and Data Analysis 2.2 (Agilent Technologies Inc.).

3. Results and discussion

Fig. 1, from A to G, illustrates the total ion chromatogram (TIC) of the propolis extracts under negative ion mode. As evident, the ethanolic extracts of propolis from Argentina (Fig. 1A), Italy (Fig. 1F) and Spain (Fig. 1G) shows approximately the same TIC profile due to the presence of the same molecular species, also considering that the migration times for the different molecular species changed depending on the column conditions according to a percentage calculated to be lower than approximately 10%. In fact, for each peak (identified in Fig. 1A–G with 1–17), the negative ESI–MS was able to identify the same ion species (Table 1). Fig. 2 shows as example the negative ESI–MS spectra of peaks 2, 3, 4, 5 and 7. Furthermore, in Fig. 2 is also illustrated the negative ESI–MS spectrum of the peak 8, showing the three main ions at *m/z* 253.1, 283.1 and 313.1, while Fig. 2B–D shows the spectra of the peaks 10, 11 and 14, respectively. By using many purified flavonoids, we were able to identify several species in the propolis extracts by performing coelution with standards and by comparing the retention time and the ESI/MS spectrum of each standard with those of each peak separated by on-line HPLC–ESI/MS. The results are illustrated in Table 1. Other ions with higher ratio *m/z* were found to be present for example in peaks 11 and 14 (see Fig. 2). However, we can exclude that these ions could be precursors of the main ions at *m/z* 283.1 and 327.1 (peak 11) and 253.1 and 341.1 (peak 14) by means of MS/MS experiments (not shown).

From a qualitative point of view, by considering the peaks from 1 to 17 detected by HPLC–ESI/MS, the propolis samples from Argentina (Fig. 1A), Italy (Fig. 1F) and Spain (Fig. 1G) appear quite similar, while the samples from China (Fig. 1C), Azerbaijan (Fig. 1B), Ethiopia (Fig. 1D) and Kenya (Fig. 1E) show a very peculiar TIC profiles. This aspect is more evident by considering the quantitative evaluation of the TIC species from 1 to 17 (see below).

For LC/MS with SIM under the negative ion mode, we selected the $[M-H]^-$ ion peak to quantify identified flavonoids in propolis extracts. Under this negative mode, at the energy level of 100%, the drying temperature at 350 °C, the $[M-H]^-$ were observed as the major ion peaks for identified flavonoids illustrated in Table 1, thereby allowing these ions to be selected for use in the quantitative analysis. We found this method to be very sensitive and accurate in achieving a linearity over a wide concentration range, from 0.05 (5 μg/ml) to 5 μg (500 μg/ml) (Table 2), and as a consequence quantification was based on the LC/MS peak areas and standard curves of known flavonoids were used for calculation (Table 2).

Accurate assessment of the contents of bioactive compounds in extract samples requires the validation of certain analytical parameters such as precision, recovery, linearity and limit of detection. The accuracy and precision of this HPLC/MS method with SIM under negative mode were determined by repeating six times the HPLC quantitative evaluation for all standards. The coefficient of variation (CV%) for each standard was determined at different points of concentration and it was found to be always lower than 10%. The precision was also performed by injecting a propolis extract (from Argentina) six times. The coefficient of variation (CV%) for the identified flavonoids was found to be lower than 10%, suggesting that the method is suitable for quantitative and routine analysis. The recovery was validated by spiking from ethanolic extract of propolis (from Argentina) one sample (chrysin) with known concentrations and then calculating recovery rate. Results showed that approximately 90% of the theoretical amounts of chrysin were recovered. The calibration curves (Table 2) of this method were constructed by injecting the standard solution across eight different concentrations (from 0.05 to 5 μg). The coefficients of correlation for the known flavonoids were found to be greater than 0.996 (Table 2). Furthermore, the limit of detection for the standards was determined at 0.025 μg (2.5 μg/ml). These validation studies show that the recommended method is reliable and sensitive allowing for the quantitative analysis of propolis extracts.

The propolis from Argentina, Italy and Spain show a great amount of pinocembrin (approximately 49%, 48% and 39% of the total identified flavonoids, respectively) and variable but similar percentages of the other species. On the contrary, the propolis from China, Azerbaijan and Ethiopia have a great amount of pinocembrin (approximately 63%, 46% and 62%, respectively) but no presence of genistein, kaempferol, apigenin and chrysin

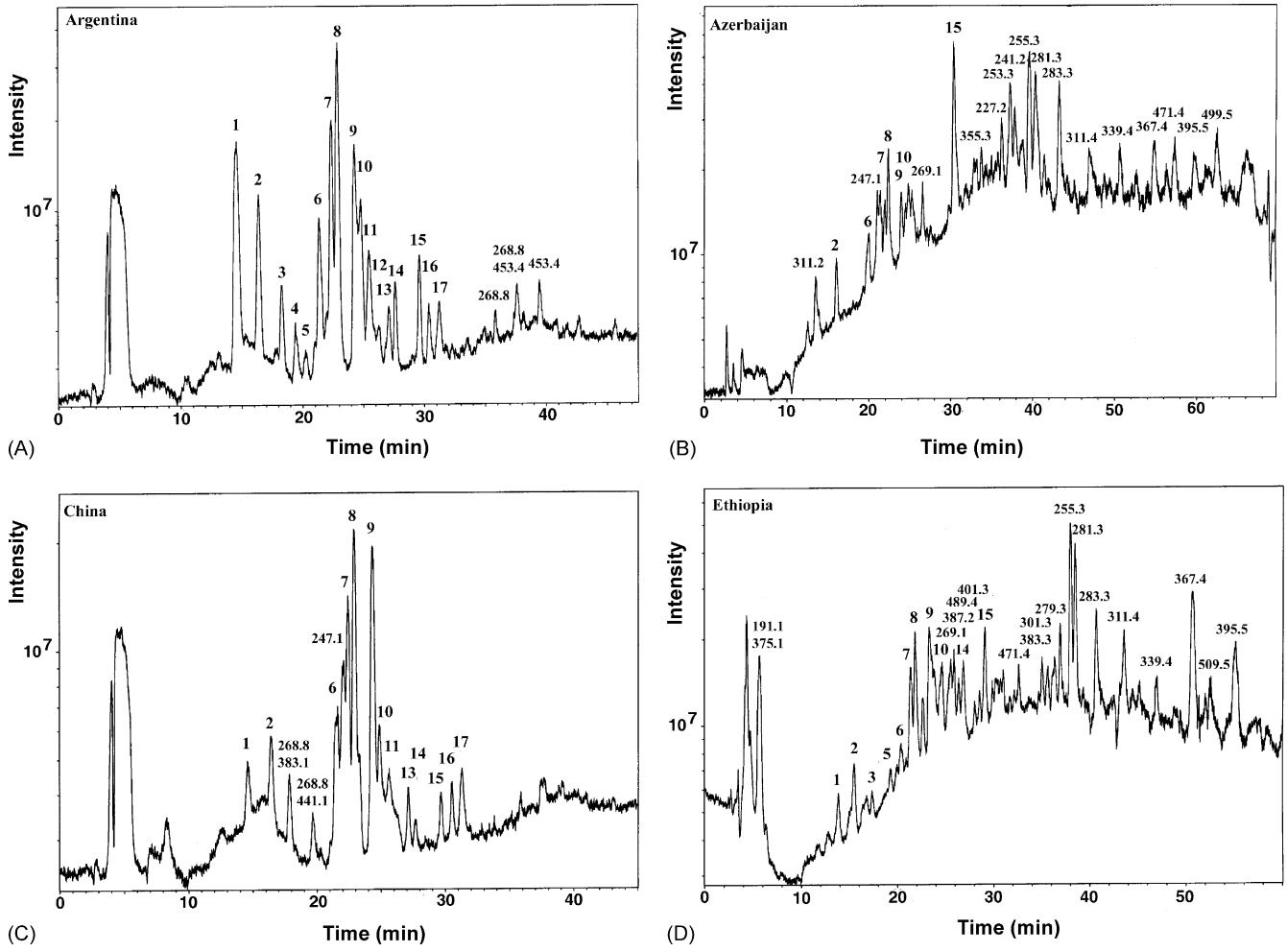


Fig. 1. Total ion chromatograms (TIC) of ethanolic extracts of propolis of various origin. For the identification of peaks signed from 1 to 17 see Table 1. Peaks having a retention time greater than approximately 35 min or lower than about 15 min are identified by their mass values.

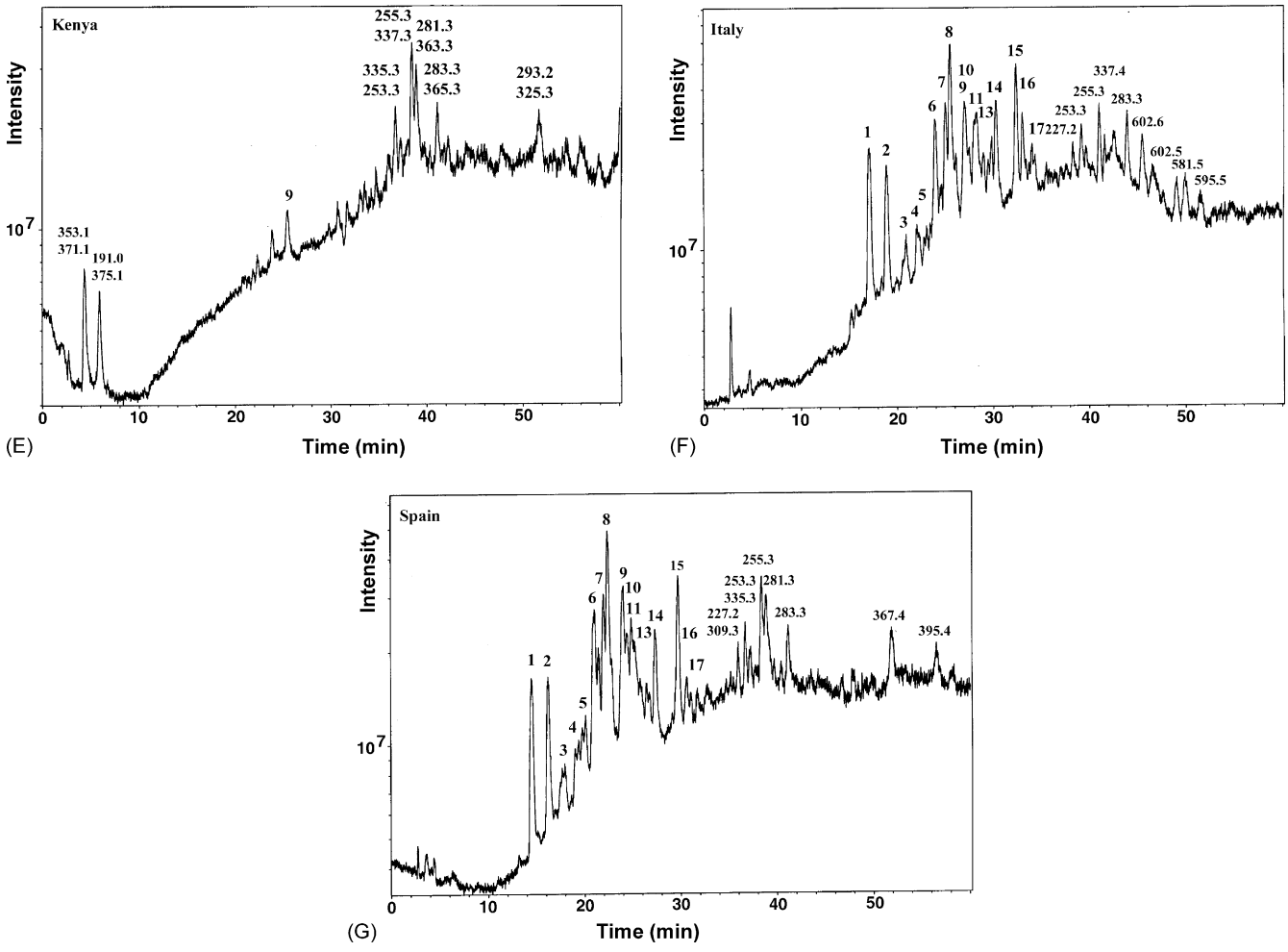


Fig. 1. (*Continued*).

for the sample from China, genistein, kaempferol, acacetin and chrysin for the propolis from Azerbaijan, and no kaempferol and acacetin for the sample from Ethiopia (Table 3). The ethanolic extract from propolis of Kenya has no identified flavonoids species but just the peak 9 at *m/z* 253.0 (see Fig. 1E). Furthermore, by using the calibration curves for identified flavonoids and a semiquantitative approach for the unknown peaks, in particular peaks number 1, 6, 8, 9, 12, 13, 15, 16 and 17 in Fig. 1 from A to G and Table 1, we were able to give an evaluation of the presence of total flavonoids for the various propolis samples, with extracts from Argentina, Italy and Spain more rich in polyphenols than those from Azerbaijan, China, Ethiopia and Kenya.

As evident from the TIC profiles of propolis extracts (Fig. 1 from A to G), several peaks having retention times greater than approximately 35 min were detected. The identity of these peaks is different depending on the propolis samples as evaluated by their mass values. However, due to their HPLC behaviour as they are strongly retained by the hydrophobic column, we assume that these molecular species are indeed hydrophobic compounds, in particular wax or essential oils, or probable, acylated, methylated or prenylated derivatives [7]. Furthermore, samples from Azerbaijan (Fig. 1B), Ethiopia (Fig. 1D) and Kenya (Fig. 1E) show the presence of molecular species (identified in the figures by their mass values) having retention times lower than about 15 min (the propolis from China shows unidentified species at retention times between 17 and 20 min), probably very polar molecules possibly glycosylated [7].

The samples of propolis used in this study were utilized with no pretreatment as the different commercial preparations are in the form of ethanolic extracts and are used to prepare various products such as oral sprays and syrups. As a consequence, a rapid qualitative and quantitative HPLC–ESI/MS separation of these products is of interest and this technique can be applied to separate and quantify polyphenols in propolis extracts used in medicine. As already reported in a previous work by using capillary zone electrophoresis [9], the ethanol alone in the extraction solvent is unable to extract the most polar component of polyphenols, contrary to the acqueous-ethanolic solvent. As a consequence, the most polar flavonoids, such as caffeic acid, are not detected in these preparations.

By performing a micellar electrokinetic capillary chromatography separation of propolis alcoholic extracts, Hilhorst et al. [20] and Fontana et al. [21] found pinocembrin, chrysin and galangin to be the flavonoids at the highest concentration, in good agreement with the present study. Furthermore, Bankova

et al. [22,23] also found that pinocembrin, galangin and chrysin are the main flavonoids in other propolis samples. In another study conducted by means of HPLC, several propolis extracts were analysed for their flavonoid component, and the most abundant species were found to be galangin, pinocembrin, chrysin, quercetin, kaempferol and naringenin, yet differing in the content of specific components [5]. Furthermore, several non-identified molecular species were detected for the ethanolic propolis extract [5] probably derivatives of the most representative polyphenols.

Gas chromatography/MS is not widely used in flavonoid analysis owing to the limited volatility of flavonoids. Since the development of atmospheric pressure ionization (API) sources, LC/MS coupling became more efficient and easy to use, making it by far the most popular technique for on-line flavonoid analysis nowadays. LC/MS is rarely used for full structure characterization, but it provides the molecular mass of the different constituents [7]. Additionally, it can be used to determine the occurrence of previously identified compounds, and so minimizes the effort lost in their isolation. It is also employed for quantitative analysis or is suited to the identification of labile compounds in solution, such as acylated flavonoids. The efficiency of different API sources, i.e., ESI, APCI and atmospheric pressure photoionization (APPI), has been compared by Rauha et al. [24]. The highest sensitivity is obtained using ESI in the negative ion. Both APCI and ESI (see [7] for review) appear to be favored for the analysis of flavonoids with the eluent system usually consisting of an acidified aqueous solvent and acetonitrile or methanol. Acidification provides a better retention and separation on the C_8- and C_{18}-RP columns which are almost

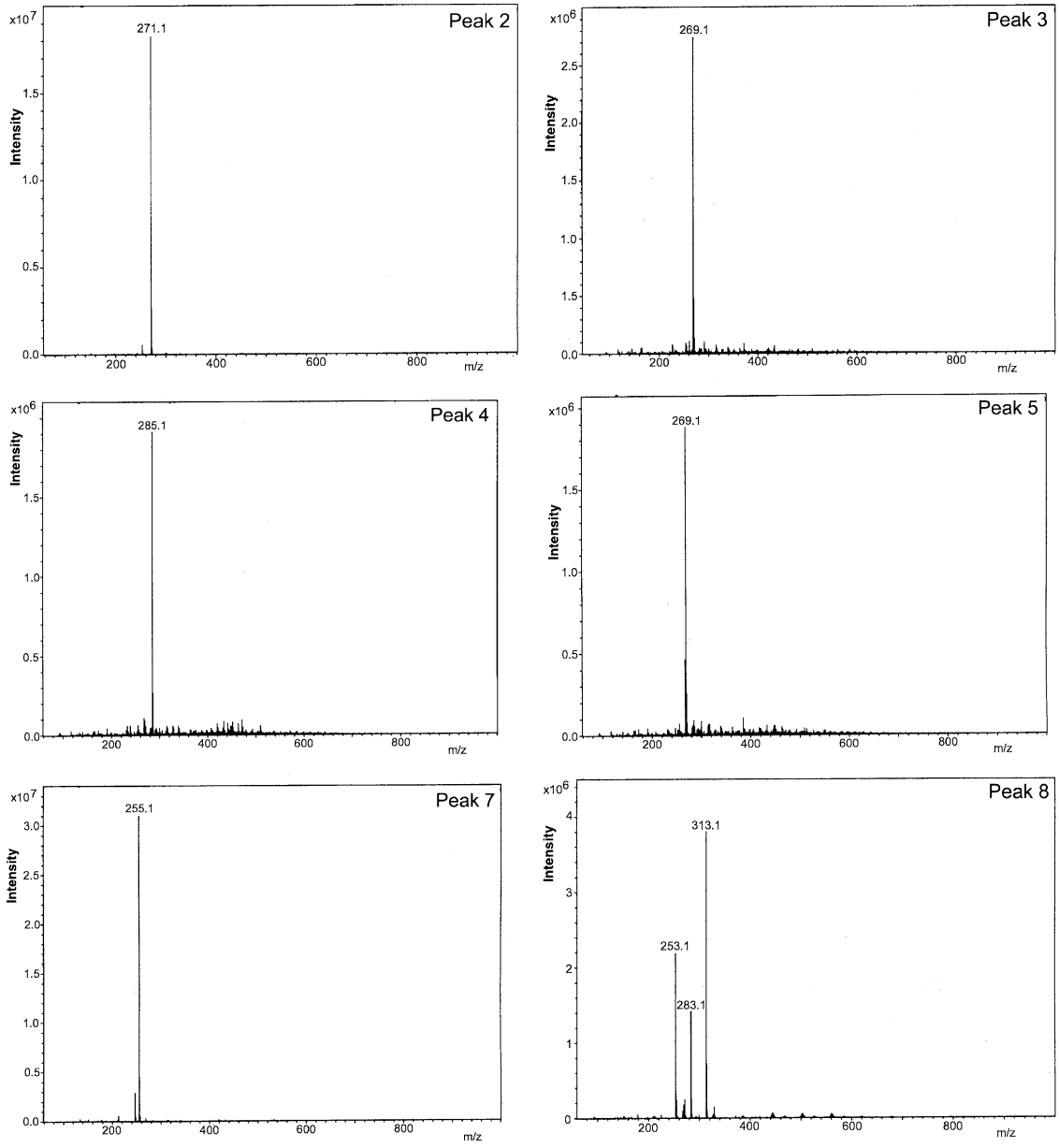


Fig. 2. The ESI–MS spectrum in the negative mode of the peaks 2, 3, 4, 5, 7, 8, 10, 11 and 14 separated by HPLC and detected in the total ion chromatograms of Fig. 1.

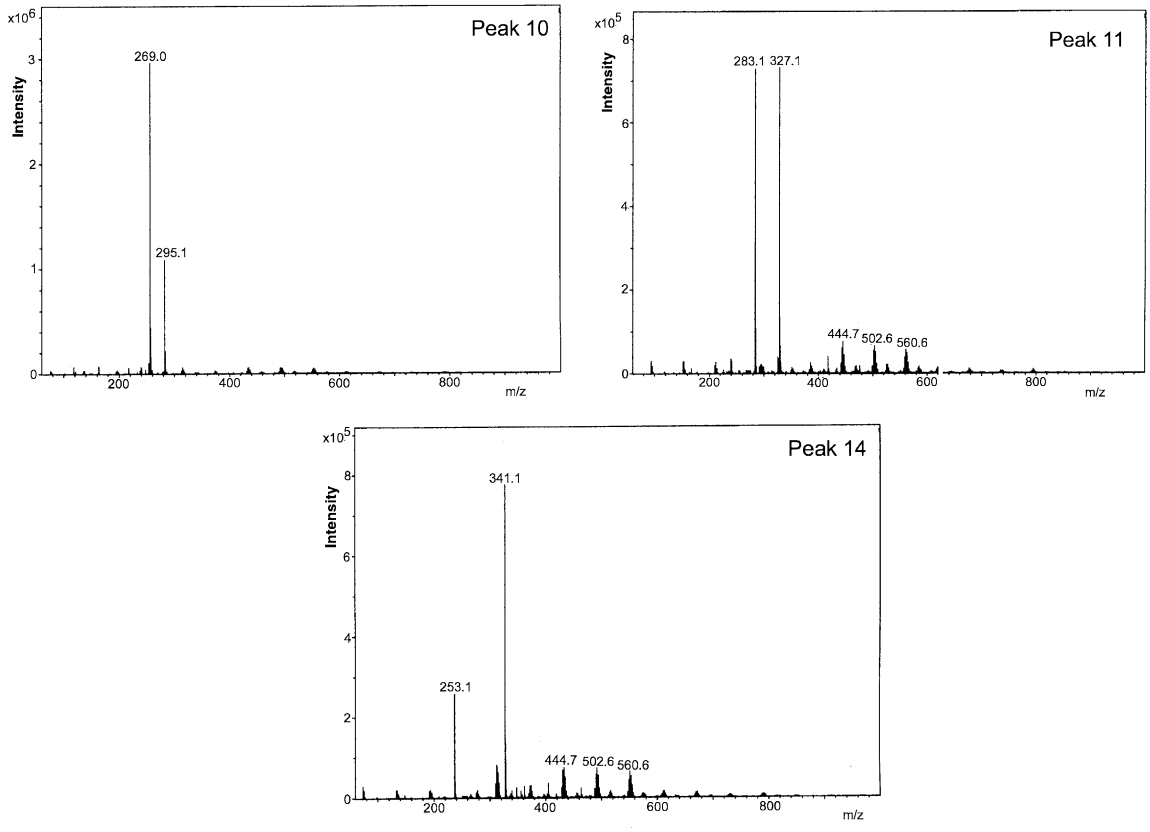


Fig. 2. (*Continued*).

Table 1
LC–ESI/MS data for ethanolic propolis extracts (see Fig. 1 from A to G)

Peak	Molecular species	Exact mass	Major ions *m/z* for $[M-H]^-$	HPLC retention time (min)
1	nd		285.1	14.6
2	Naringenin	272.1	271.1	16.4
3	Genistein	270.1	269.1	18.2
4	Kaempferol	286.1	285.1	19.4
5	Apigenin	270.1	269.1	20.2
6	nd		269.1	21.3
7	Pinocembrin	256.1	255.1	22.4
8	nd		253.1	22.9
	nd		283.1	
	nd		313.1	
9	nd		253.0	24.2
10	Galangin	270.0	269.0	24.8
	nd		295.1	
11	Acacetin	284.1	283.1	25.4
	nd		327.1	
12	nd		417.2	26.3
	nd		475.2	
13	nd		268.8	27.1
	nd		279.1	
14	Chrysin	254.1	253.1	27.6
	nd		341.1	
15	nd		355.2	29.6
16	nd		268.8	30.4
	nd		355.3	
	nd		403.1	
17	nd		268.8	31.2

nd: unidentified.

exclusively employed. Formic, acetic and trifluoroacetic acid and ammonium acetate and formate are volatile and thus compatible with LC/MS systems. Acetic acid was found to have weak ion-pairing capacity and it slightly increases the ESI efficiency [7].

The negative ion mode provides the highest sensitivity and results in limited fragmentation [25,26], making it most suited to infer the molecular mass of the separated flavonoids, especially in cases where concentrations are low. The peak at the highest *m/z* ratio is not always the molecular ion species ($[M-H]^-$ in the negative mode), because adducts with solvent and/or acid molecules and also molecular complexes can be generated [7]. However, an increase in cone voltage reduces the incidence of both adduct and complex formation [27].

Table 2
Equations of calibration curves and the coefficients of correlation calculated for flavonoid standards at a concentration ranging from 0.05 (5 μg/ml) to 5 μg (500 μg/ml)

Flavonoid standard	Calibration curves	R^2
Naringenin	$y = 2490.6x - 18.0$	0.999
Genistein	$y = 2337.9x + 110.0$	0.996
Kaempferol	$y = 2430.6x + 45.9$	0.999
Apigenin	$y = 2557.6x + 11.9$	0.999
Pinocembrin	$y = 2590.0x - 104.0$	0.998
Galangin	$y = 2579.7x - 72.1$	0.999
Acacetin	$y = 2499.8x - 34.6$	0.996
Chrysin	$y = 2617.4x - 53.0$	0.997

Table 3
μg (μg/ml) ± standard deviation (S.D.) of identified flavonoids in propolis extracts of different origin

Molecular species	Argentina	China	Italy	Spain	Azerbaijan	Ethiopia	Kenya
Naringenin	0.51 (51) ± 0.043	0.21 (21) ± 0.017	0.57 (57) ± 0.051	0.48 (48) ± 0.037	0.39 (39) ± 0.035	0.12 (12) ± 0.001	nd
Genistein	0.21 (21) ± 0.015	nd	0.03 (3) ± 0.003	0.05 (5) ± 0.004	nd	0.12 (12) ± 0.001	nd
Kaempferol	0.05 (5) ± 0.004	nd	0.10 (10) ± 0.009	0.16 (16) ± 0.013	nd	nd	nd
Apigenin	0.09 (9) ± 0.006	nd	0.13 (13) ± 0.010	0.17 (17) ± 0.012	0.12 (12) ± 0.012	0.12 (12) ± 0.001	nd
Pinocembrin	1.65 (165) ± 0.160	1.12 (112) ± 0.090	1.66 (166) ± 0.120	1.34 (134) ± 0.121	0.79 (79) ± 0.066	1.10 (110) ± 0.095	nd
Galangin	0.72 (72) ± 0.064	0.38 (38) ± 0.027	0.40 (40) ± 0.040	0.83 (83) ± 0.071	0.43 (43) ± 0.034	0.22 (22) ± 0.022	nd
Acacetin	0.09 (9) ± 0.006	0.07 (7) ± 0.007	0.31 (31) ± 0.025	0.31 (31) ± 0.022	nd	nd	nd
Chrysin	0.05 (5) ± 0.004	nd	0.26 (26) ± 0.006	0.12 (12) ± 0.011	nd	0.10 (10) ± 0.009	nd

nd: not detected.

Structural information can also be obtained from the chromatographic retention times. For the C_{18}- or C_8-RP columns generally used, the more polar compounds are eluted first. Thus, retention times are inversely correlated with increasing glycosylation, whereas acylation, methylation or prenylation have the opposite effect [7]. Furthermore, an on-column limit of detection of around 10 ng is attainable for LC/MS in the TIC mode, whereas a limit of detection <1 ng can be achieved in the SIM mode using negative ionization [7]. In this study we evaluated a limit of detection at 25 ng.

4. Conclusions

At our knowledge, this is the first paper describing the on-line HPLC–ESI/MS analysis in the negative mode of propolis extracts of various origin performed by a solvent generally used to prepare various pharmaceutical products. On the basis of the results of this study, it may be concluded that HPLC–ESI/MS under the experimental conditions illustrated represents a valuable method for the qualitative and quantitative assay of the most relevant components of propolis. On-line HPLC–ESI/MS analysis constitutes an alternative to obtain typical fingerprints of propolis and a reliable identification of a large number of propolis polyphenolic components.

References

[1] S. Castaldo, F. Capasso, Fitoterapia 73 (2002) S1–S6.
[2] A.H. Banskota, Y. Tezuka, S. Kadota, Phytother. Res. 15 (2001) 561–571.
[3] V. Barak, S. Birkenfeld, T. Halperin, I. Kalickman, Isr. Med. Assoc. J. 4 (2002) 919–922.
[4] P. Cos, P. Rajan, I. Vedernikova, M. Calomme, L. Pieters, A.J. Vlietinck, K. Augustyns, A. Haemers, D. Vanden Berghe, Free Radic. Res. 36 (2002) 711–716.
[5] P.G. Pietta, C. Gardana, A.M. Pietta, Fitoterapia 73 (2002) S7–S20.
[6] B.H. Havsteen, Pharmacol. Ther. 96 (2002) 67–202.
[7] F. Cuyckens, M. Claeys, J. Mass Spectrom. 39 (2004) 1–15.
[8] T. Watanabe, S. Terabe, J. Chromatogr. A 880 (2000) 311–322.
[9] N. Volpi, Electrophoresis 25 (2004) 1872–1878.
[10] R.J. Hughes, T.R. Croley, C.D. Metcalfe, R.E. March, Int. J. Mass Spectrom. 210/211 (2001) 371–385.
[11] P. Mauri, P. Pietta, J. Pharm. Biomed. Anal. 23 (2000) 61–68.
[12] M. Wang, Y. Tadmor, Q.L. Wu, C.K. Chin, S.A. Garrison, J.E. Simon, J. Agric. Food Chem. 51 (2003) 6132–6136.
[13] R.W. Owen, R. Haubner, W.E. Hull, G. Erben, B. Spiegelhalder, H. Bartsch, B. Haber, Food Chem. Toxicol. 41 (2003) 1727–1738.
[14] J.F. Stevens, A.W. Taylor, M.L. Deinzer, J. Chromatogr. A 832 (1999) 97–107.
[15] A. Raffaelli, G. Moneti, V. Mercati, E. Toja, J. Chromatogr. A 777 (1997) 223–231.
[16] X. He, L. Lin, L. Lian, J. Chromatogr. A 755 (1996) 127–132.
[17] N. Chaves, J.J. Rýos, C. Gutierrez, J.C. Escudero, J.M. Olýas, J. Chromatogr. A 799 (1998) 111–115.
[18] S.E. Nielsen, R. Freese, C. Cornett, L.O. Dragsted, Anal. Chem. 72 (2000) 1503–1509.
[19] K. Midorikawa, A.H. Banskota, Y. Tezuka, T. Nagaoka, K. Matsushige, D. Message, A.A. Huertas, S. Kadota, Phytochem. Anal. 12 (2001) 366–373.
[20] M.J. Hilhorst, G.W. Somsen, G.J. de Jong, HRC-J. High Res. Chromatogr. 21 (1998) 608–612.
[21] J.D. Fontana, M. Passos, M.H.R. dos Santos, C.K. Fontana, B.H. Oliveira, L. Schause, R. Pontarolo, M.A. Barbirato, M.A. Ruggiero, F.M. Lancas, Chromatographia 52 (2000) 147–151.
[22] V. Bankova, R. Christoy, G. Stoev, S. Popov, J. Chromatogr. 607 (1992) 150–153.
[23] V. Bankova, S. Popov, N.L. Marekov, J. Chromatogr. 242 (1982) 135–143.
[24] J.P. Rauha, H. Vuorela, R. Kostiainen, J. Mass Spectrom. 36 (2001) 1269–1280.
[25] M. Careri, L. Elviri, A. Mangia, Mass Spectrom. 13 (1999) 2399–2405.
[26] N. Fabre, I. Rustan, E. de Hoffmann, J. Quetin-Leclercq, J. Am. Soc. Mass Spectrom. 12 (2001) 707–715.
[27] Q. Tian, D. Li, B.S. Patil, Phytochem. Anal. 13 (2002) 251–260.