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Journal of Chromatography A, 730 (1996) 25–29

JOURNAL OF CHROMATOGRAPHY A

Solid-phase extraction and reversed-phase high-performance liquid chromatography of free phenolic acids in some *Echinacea* species

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Abstract

A new method is described combining solid-phase extraction (SPE) and reversed-phase high-performance liquid chromatography (RP-HPLC) for the isolation, purification as well as qualitative and quantitative determination of free phenolic acids in six *Echinacea* species. Plant extracts were purified and phenolic acids isolated on octadecyl and quaternary amine Bakerbond SPE columns; final eluates were analysed by RP-HPLC. Significant differences in the composition and amount of phenolic acids within *Echinacea* genus have been shown. The method can be used for quick screening analysis of the content of phenolic acids in plant material.

Keywords: Sample preparation; *Echinacea*; Phenolic acids

1. Introduction

Phenolic acids constitute a large group of naturally occurring organic compounds showing a broad spectrum of pharmacological activity. Traditional preparative techniques, including the dissolving of the sample followed by liquid–liquid extraction or column chromatography on different sorbents, are well known procedures applied for isolation and purification of phenolics [1,2]. However, extraction of phenolic acids from plant material and their further purification for HPLC analysis is usually a complex procedure because of the presence of various nonpolar ballast compounds in biological extracts (e.g. chlorophyll, waxes, sterols, oils etc.) which can cause damage to analytical columns and interfere with the process of chromatographic determination. Hence, solid-phase extraction (SPE) [3] has become a popular procedure which is used for isolation, purification and preconcentration of organic compounds present in biological material as an alternative to the methods described above.

In this study SPE was used for isolation of free phenolic acids from aerial parts of six *Echinacea* species. This genus is of great interest in human therapy as it possesses active compounds showing bacteriostatic, virusstatic and immunostimulating activity [4,5].

Most phenolic compounds interact with anion-exchange sorbents, however, in scientific literature for the last 25 years we found only a few papers dealing with the separation of phenolics (not phenolic acids) on anion-exchange columns, especially as a preparative technique [6–10].

In order to obtain pure fractions of phenolic

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0021-9673/96/$15.00
SSDI 0021-9673(95)00877-2

acids from plant material, two combined SPE procedures based on reversed-phase chromatography with nonpolar bonded sorbents and ion-exchange chromatography with charged sorbents were applied. Eluates after SPE were qualitatively and quantitatively analysed by RP-HPLC.

Although there are reports in which the gradient mode of RP-HPLC analysis is applied for phenolic acids [11–14], all the separations carried out in this experiment were achieved under isocratic conditions to avoid baseline drift and ensure the most precise quantitative determination of compounds examined.

2. Experimental

2.1. Apparatus

The HPLC analysis was performed using a Hewlett-Packard (Palo Alto, CA, USA) Model 1050 liquid chromatograph equipped with a 20-μl sample injector (Rheodyne, Cotati, CA, USA) and a variable-wavelength UV–Vis detector. The chromatograms were recorded at 254 nm with a 3396A reporting integrator (Hewlett-Packard). A stainless-steel column (200 × 4.6 mm I.D.) packed with ODS Hypersil (5 μm) was used.

2.2. Mobile phase

As a mobile phase methanol–water (25:75, v/v) with 1% (v/v) addition of acetic acid was used, at a flow-rate of 1 cm^3/min and at ambient temperature.

2.3. Chemicals

All reagents used (methanol, acetic acid, phosphoric acid, sodium bicarbonate, sodium hydroxide) were of chromatographic grade (Merck, Darmstadt, Germany) and in all experiments bidistilled water was used.

Phenolic acids were purchased from Sigma (St. Louis, MO, USA). A 1-mg amount of each standard was dissolved in 10 ml of methanol and applied to the column. Samples of 10 μl were injected.

2.4. Plant material

Aerial parts of six *Echinacea* species: *E. purpurea*, *E. angustifolia*, *E. montana*, *E. multiflora*, *E. commutata*, *E. umbellata* were collected in the Pharmacognostic Garden of the Department of Pharmacognosy, Medical Academy of Gdańsk (Poland) in summer 1993. They were air-dried at room temperature and immediately milled to tiny particles (0.5 mm) just before extraction.

2.5. Extraction and sample preparation

Milled aerial parts of the six plants mentioned above (5 g of each) were extracted with methanol in a Soxhlet apparatus for 6 h. Methanolic extracts were evaporated to dryness in a rotary vacuum evaporator at a temperature not higher than 50°C and the residues were dissolved in 30% (v/v) methanol (10 ml).

2.6. SPE procedure

After preliminary conditioning of six microcolumns (octadecyl, 500 mg, J.T. Baker, Phillipsburg, NJ, USA) with methanol (10 ml), filtered methanolic (30%) plant extracts were passed through the columns inserted into a vacuum manifold processor (system Baker SPE-12G, J.T. Baker, Phillipsburg, NJ, USA). This way nonpolar ballast compounds were retained.

The aromatic carboxylic acids are much stronger acids than the phenols; this is the basis for their separation. In our experiment on aqueous solution of sodium bicarbonate was used as a reagent which when applied in strictly determined concentration ensured the proper value of pH (about 7.0–7.2) for sample application, because phenolic acids as weakly acidic compounds ($pK_a \sim 4$–5) are extracted most effectively by anion-exchange sorbents about 2 pH units above their pK_a.

Hence, for isolation of phenolic acid fractions, the next six microcolumns (quaternary amine,

500 mg, J.T. Baker, Phillipsburg, NJ, USA) were washed with 10 ml of methanol and conditioned with water (10 ml) and a 0.15% aqueous solution of sodium bicarbonate (20 ml).

The eluates from octadecyl microcolumns were adjusted to pH 7.0–7.2 with a 5% aqueous solution of sodium bicarbonate, diluted with water to obtain 15% concentration of methanol, and transferred to the wet quaternary amine columns; the samples were passed through the beds of sorbent under reduced pressure. Columns were further washed with 2 ml of 0.15% sodium bicarbonate buffer to remove matrix impurities. Finally, phenolic acids retained on the sorbent beds were eluted with 0.2 *M* H_3PO_4–methanol (1:1, v/v); 5 ml of the mixture was used for each sample. The collected eluates were subsequently adjusted to pH 3 with 1 *M* NaOH and analysed directly by RP-HPLC.

The identification and quantitative determination of the compounds examined were accomplished by a comparison of retention times, peak heights and areas with the standard solutions of phenolic acids.

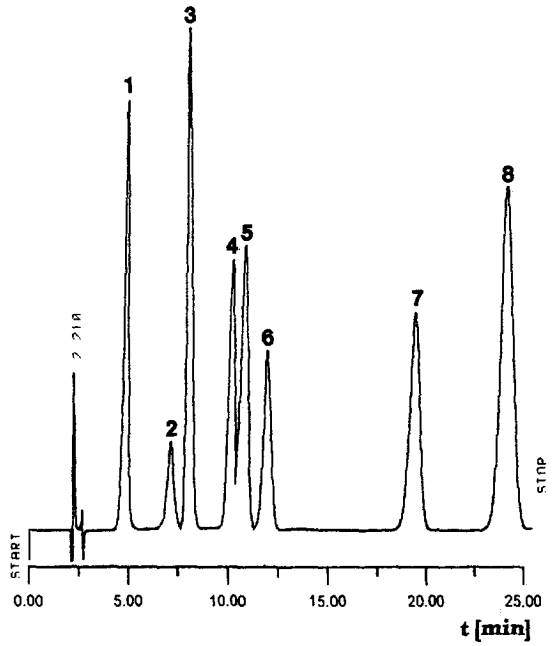

Fig. 1. Experimental chromatogram of the test mixture of phenolic acids. Peaks: 1 = protocatechuic; 2 = chlorogenic; 3 = *p*-hydroxybenzoic; 4 = vanillic; 5 = caffeic; 6 = syryngic; 7 = *p*-coumaric; 8 = ferulic. HPLC analysis carried out on ODS Hypersil (200 × 4.6 mm I.D., 5 μm) column under isocratic conditions; mobile phase, methanol–water–acetic acid (25:75:1, v/v); flow-rate, 1 cm^3/min; detection, 254 nm.

2.7. Recovery tests

To establish the efficiency of the applied SPE method, recovery tests were performed. Methanolic (30%) solutions (10 ml) of 1 mg caffeic and *p*-hydroxybenzoic acids (as the representatives of the derivatives of cinnamic and benzoic acids) were submitted to the whole procedure as de-

Table 1
Quantitative results (mean ± S.D., $n = 5$) of the content of phenolic acids in plants (μg/g of dry mass) obtained by application of combined SPE and RP-HPLC method

Phenolic acid no.[a]	*Echinacea angustifolia*	*Echinacea purpurea*	*Echinacea montana*	*Echinacea multiflora*	*Echinacea commutata*	*Echinacea umbellata*
1	33.4 ± 0.7	3.6 ± 0.2	10.6 ± 0.5	40.0 ± 1.8	360.0 ± 26.1	8.3 ± 0.3
2	25.8 ± 3.1	125.0 ± 11.4	24.5 ± 0.9	106.7 ± 9.2	416.0 ± 16.0	16.3 ± 0.6
3	17.2 ± 0.6	1.2 ± 0.1	9.00 ± 0.3	56.9 ± 0.9	156.2 ± 11.2	8.4 ± 1.5
4	27.4 ± 0.9	—	11.5 ± 0.7	43.3 ± 2.1	211.6 ± 12.9	9.9 ± 0.7
5	—	320.2 ± 14.6	49.7 ± 0.4	167.3 ± 6.0	80.3 ± 7.7	15.2 ± 1.5
6	—	—	37.5 ± 1.8	51.9 ± 2.6	65.1 ± 8.0	15.0 ± 2.4
7	30.9 ± 7.1	179.0 ± 2.1	—	29.6 ± 6.3	97.1 ± 7.5	—
8	54.9 ± 0.9	240.8 ± 1.4	—	—	—	—
Total	189.6	869.8	142.8	495.7	1386.3	73.1

[a] Numbers of phenolic acids as in Fig. 1.

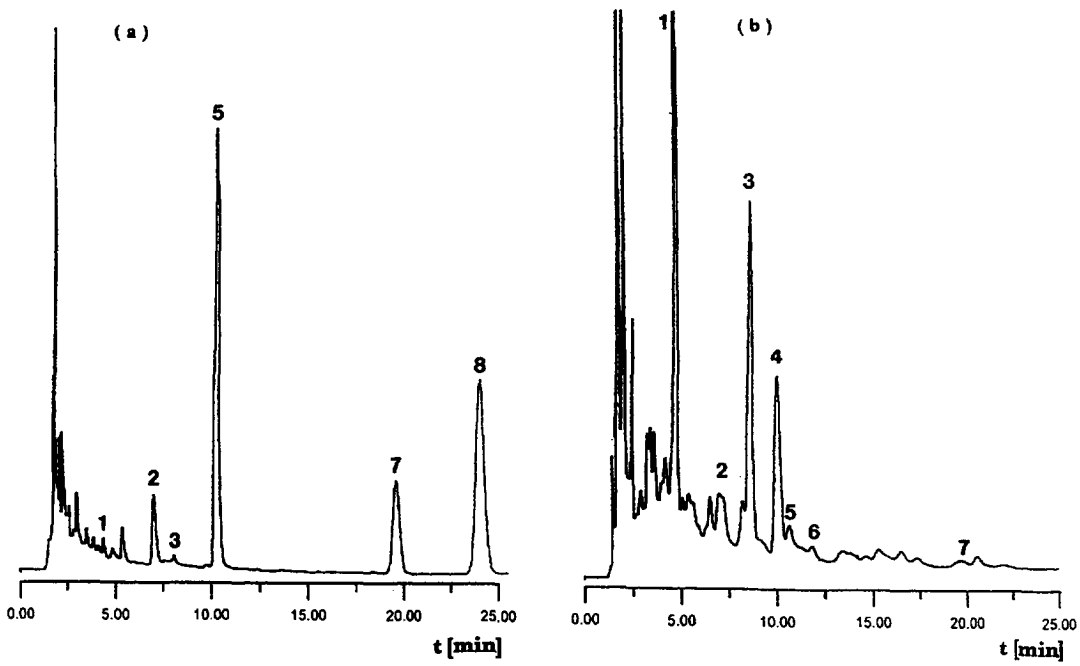

Fig. 2. Chromatograms of two of the richest fractions of phenolic acids from (a) *E. purpurea*, (b) *E. commutata*. Conditions of HPLC analysis and numbers of compounds as in Fig. 1.

scribed. A percentage recovery of 98.5 ± 0.5% for these compounds was obtained.

2.8. Breakthrough volume

The breakthrough volume for phenolic acids on the quaternary amine columns was determined. Methanolic (15%) aliquots of 10 mg *p*-hydroxybenzoic and caffeic acids (20 ml) adjusted to pH 7.0–7.2 with 5% aqueous solution of sodium bicarbonate were passed separately through conditioned quaternary amine columns.

Fractions (1 ml) were collected and analysed quantitatively, after adjustment to pH 3 with 1 *M* NaOH, by RP-HPLC. The breakthrough volumes for compounds examined were respectively: 7 ml for caffeic acid and 10 ml for *p*-hydroxybenzoic acid. The results obtained prove that better sorption of the more polar phenolic acids is obtained on the quaternary amine sorbent.

The two-step SPE "off-line" procedure carried out on octadecyl and quaternary amine Bakerbond microcolumns permits effective elimination of nonpolar ballast compounds which are retained on the first C_{18} sorbent used, as well as isolation of free phenolic acids on the anion-exchange cartridge.

Further advantages of this method are: high reproducibility (low values of S.D. for quantitative results, see Table 1) and high recovery of analytes. Very small amounts of plant material (1–5 g of dry mass) are needed and the method is characterized by the simplicity of the analytical procedure and the relatively low cost of the reagents and equipment used. The selectively performed SPE isolation of free phenolic acids from crude methanolic extracts enables direct qualitative and quantitative HPLC determination of these compounds in plant material.

Hence, the method described can be especially recommended for quick screening analysis of the content of phenolic acids in biological samples. Such a procedure can be useful in the scientific field of pharmaceutics technology in the search for new sources of natural drugs.

In our experiment, owing to the method men-

tioned above, significant differences in the composition and amount of free phenolic acids in the *Echinacea* genus have been shown (Table 1). Eight compounds, interesting from the point of view of their pharmacological properties, were identified and quantitatively determined in the plants examined (Figs. 1 and 2).

Acknowledgements

The authors wish to express their gratitude to J.T. Baker Inc. for the financial support of the work and Prof. E. Soczewiński (Dept. of Inorganic and Analytical Chemistry, Medical Academy of Lublin, Poland) for the critical review of the manuscript.

References

[1] F. Drawert, V. Lessing and G. Leupold, Chem. Mikrobiol. Technol. Lebensm., 5 (1977) 65.
[2] C.F. Van Sumere, in P.M. Dey and J.B. Harborne (Editors), Methods in Plant Biochemistry, Vol. 1, Plant Phenolics, Academic Press, London, 1989, Ch. 3, p. 39.
[3] M. Zief, Solid Phase Extraction for Sample Preparation, J.T. Baker, Phillipsburg, NJ, 1988.
[4] R. Bauer, P. Remiger, K. Jurcic and H. Wagner, Z. Phytother., 10 (1989) 43.
[5] A. Proksch and H. Wagner, Phytochemistry, 26 (1987) 1989.
[6] T.H. Lam and M. Shaw, Biochem. Biophys. Res. Commun., 39 (1970) 965.
[7] L. Nagels, W. Van Dongen and F. Parmentier, Arch. Int. Physiol. Biochem., 87 (1979) 585.
[8] B.F. Nilsson and O. Samuelson, J. Chromatogr., 198 (1980) 267.
[9] P. Jandera, J. Churacek, J. Caslavsky and D. Szabo, Chromatographia, 14 (1981) 100.
[10] K.P. Naikwadi, S. Rokushika and H. Hatano, Anal. Chem., 56 (1984) 1525.
[11] K. Vande Casteele, H. Geiger and C.F. Van Sumere, J. Chromatogr., 258 (1983) 111.
[12] J.M. Andersen and W.B. Pedersen, J. Chromatogr., 259 (1983) 131.
[13] E. Delage, G. Bohuon, A. Baron and J.F. Drilleau, J. Chromatogr., 555 (1991) 125.
[14] T.H. Dzido and H.D. Smolarz, J. Chromatogr. A, 679 (1994) 59.