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ANALYTICAL AND PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HYDROXYCINNAMIC ACID ESTERS

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SUMMARY

Two methods are described for the determination of 3-, 4- and 5-isomers of hydroxycinnamoylquinic acids and hydroxycinnamoylglucoses in plant extracts. The analyses are performed on LiChrosorb RP-18 using gradient elution with 2% acetic acid–methanol and on LiChrosorb-Diol using gradient elution with methylene chloride–methanol + phosphoric acid. They allow the determination of hydroxycinnamic acid esters in complex plant extracts.

The isolation of sinapoylglucose and 5-caffeoylquinic acid from garden cress and Brussels sprouts shows that HPLC on reversed-phase material is suitable for the isolation on a semi-preparative scale of the naturally occurring hydroxycinnamic acid compounds from plant extracts.

INTRODUCTION

In recent years, several high-performance liquid chromatographic (HPLC) methods for the determination of caffeic and other hydroxycinnamic acid derivatives which are important with regard to plant physiology and food technology have been published[1–10]. Reversed-phase (RP) materials were the stationary phases predominantly used. One method with the use of LiChrosorb-Diol was also described[5]. None of the reversed-phase methods prevailed as optimal. Although the use of an acid modifier in addition to the eluent is generally considered necessary for good separations, the other important parameters, *e.g.*, dimensions of the column, type and particle size of the reversed-phase material, eluent and modifier, flow-rate and wavelength of detection, differ widely (Table I).

The esters of hydroxycinnamic acids with glucose or quinic acid are of particular importance because of their ubiquitous and quantitative occurrence in plant materials[11]. As there are a number of chemically and chromatographically similar substances in plant extracts, it seems important to confirm the results obtained by RP-HPLC by using another method of a similar quality, *e.g.*, capillary GC[12]. The application of a Diol-HPLC method, as described by Nagels *et al.*[5], is preferable, as there is no need for additional apparatus. For this reason, an HPLC method for the

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TABLE I

HPLC METHODS FOR THE SEPARATION OF HYDROXYCINNAMIC ACID ESTERS

Compounds	*Column*	*Eluent*	*Flow-rate (ml/min)*	*Detection (nm)*	*Ref.*
Caffeoylquinic acids	C_{18}, 10 μm; 300 × 4 mm I.D.	0.1 *N* KH_2PO_4–methanol	1	350	1
Caffeoylquinic acids	C_{18}, 10 μm; 100 × 4 mm I.D.	Water–methanol (80:20), H_3PO_4 to pH 3	2.5	328	2
Hydroxycinnamoylglucoses and hydroxycinnamoylquinic acids	C_{18}, 5 μm	Water–acetic acid–methanol	2	312	3
Hydroxycinnamoylglucoses	C_{18}, 10 μm, 250 × 4.6 mm I.D.	0.001 *N* H_2SO_4–methanol	4	280	4
Hydroxybenzoylglucoses, hydroxycinnamoylglucoses and hydroxycinnamoylquinic acids	C_8, 10 μm; 250 × 4.6 mm I.D.	10 m*M* H_3PO_4–methanol	4	280	5
Hydroxybenzoylglucoses, hydroxycinnamoylglucoses and hydroxycinnamoylquinic acids	Diol, 10 μm; 250 × 4.6 mm I.D.	CH_2Cl_2–acetic acid–1-propanol	4	280	5
p-Coumaroylglucoses and chlorogenic acids	C_{18}	Water–0.1% H_3PO_4–methanol	–	360	6
Hydroxycinnamoyltartaric acids	C_{18}, 10 μm; 250 × 4.6 mm I.D.	Water–acetonitrile, H_3PO_4 to pH 2.6	2.5	320	7
Sinapic acid esters	C_{18}, 5 μm; 250 × 4 mm I.D.	Water–5% acetic acid–acetonitrile	1	330	8
Caffeic acid esters	C_{18}, 10 μm; 300 × 3.9 mm I.D.	Methanol–0.033 *M* PO_4^{3-} acetic acid (40:60:0.4); pH 3	0.8	313	9
Hydroxycinnamoylglucoses	C_{18}, 250 × 4.6 mm I.D.	8% Acetonitrile in 0.5% formic acid	1.8	320	10

separation of the quinic acid and glucose esters of the *trans*-hydroxycinnamic acids was elaborated.

In the past, the isolation of hydroxycinnamic acid derivatives from plant materials was generally effected by preparative paper chromatography and column chromatography on Sephadex; preparative HPLC was used only in few instances[5,10]. With two examples we shall demonstrate here not only that preparative HPLC is suitable for relatively simple problems of isolation, such as the purification of synthesized compounds, but also that it can be used to isolate naturally occurring compounds from complex plant extracts.

For easier comparison with previously published papers, we have chosen the older but still applicable nomenclature instead of IUPAC nomenclature.

EXPERIMENTAL

Preparation of plant extracts

A 20-g amount of plant material was communited in 200 ml of methanol in a mixer and homogenized using an Ultra Turrax. The plant pulp was filtered through a G4 glass frit and the residue was washed with hot methanol. The methanol extract was evaporated to about 50 ml *in vacuo*, cleared of chlorophyll by extraction with light petroleum (b.p. 60–80°C) and then evaporated to about 5 ml. The extract was made up to 25 ml in a measuring flask with methanol (for Diol-HPLC) or water (for RP-HPLC) and filtered through a 2-μm filter before injection. This method of preparation is applicable for determinations of parts per million amounts.

Analytical HPLC

The experiments were performed with a Pye Unicam gradient HPLC chromatograph (Philips, Kassel, G.F.R.) consisting of an LC-XPD pump, an LC-XPD gradient programmer and an LC UV detector. A Rheodyne 7125 injection valve with a 10-μl sample loop and a 3390 A reporting integrator (Hewlett Packard, Frankfurt, G.F.R.) were used.

For the reversed-phase analyses a 250 × 4 mm I.D. stainless-steel column of LiChrosorb RP-18, 5 μm (Merck, Darmstadt, G.F.R.) was used. Gradient elution was carried out with solvent A (2% acetic acid) and solvent B (methanol), from A to 40% B in A in 80 min, at a flow-rate of 1 ml/min, and with detection at 320 nm and degassing with helium.

The Diol analyses were performed with a 250 × 4.6 mm steel column of LiChrosorb-Diol, 5 μm. Gradient elution: solvent A: methylene chloride; solvent B: methanol + 0.3% phosphoric acid; from 2% to 40% B in A in 60 min, flow-rate; 1.3 ml/min.

Preparative HPLC

For preparative experiments, a Pye Unicam HPLC instrument (Philips) equipped with a preparative pump head (0.028–27.972 ml/min), an LC UV detector with a flow cell, prep., and a photocell filter was used. A Rheodyne 7125 injection valve with a 2-ml sample loop were used. The apparatus was supplemented by a computing integrator (Pye Unicam) connected with a fraction collector (Isco Model 2200; Instrumentation Specialities, U.S.A.).

RESULTS AND DISCUSSION

Separation of hydroxycinnamic acid esters on LiChrosorb RP-18

From consideration of the methods known so far, we developed an optimized procedure for the hydroxycinnamic acid esters by HPLC. The experiments were started with mixtures of water with methanol or acetonitrile. Methanol seemed preferable to acetonitrile. The addition of acetic acid proved to be essential, as the compounds were eluted more sharply and the resolution was improved. The addition of phosphate in the form of phosphoric acid or as a buffer solution did not improve the separation. The best chromatograms were obtained with 250 × 4 mm I.D. columns with RP-18 (5 μm) as the stationary phase. The separations were less favourable with RP-8 and 7- or 10-μm stationary phases.

Using a methanol–2% acetic acid gradient the glucose and quinic acid esters were separated in an optimal way in the following sequence (Fig. 1 and Table II): (a) 1, caffeic acid esters; 2, *p*-coumaric acid esters; 3, ferulic acid esters; 4, sinapic acid esters; (b) 1, 5-hydroxycinnamoylquinic acids; 2, hydroxycinnamoylglucoses; 3, 4-hydroxycinnamoylquinic acids; 4, 3-hydroxycinnamoylquinic acids; (c) *cis* before *trans*, 5- and 4-hydroxycinnamoylquinic acids; *trans* before *cis*, 3-hydroxycinnamoylquinic acids, hydroxycinnamoylglucoses. *trans*-5-Feruloylquinic acids and *cis*-*p*-coumaroylglucose as well as *trans*-4-*p*-coumaroylquinic acid and *cis*-sinapoylglucose were not separated. For the retention times of the separated esters a variation of $\bar{s}(t_R^{RP}) = 2.7$ min was noted.

This HPLC method is also suitable for the separation of the free hydroxybenzoic acids and hydroxycinnamic acids. Most of the flavonoids present in plant ex-

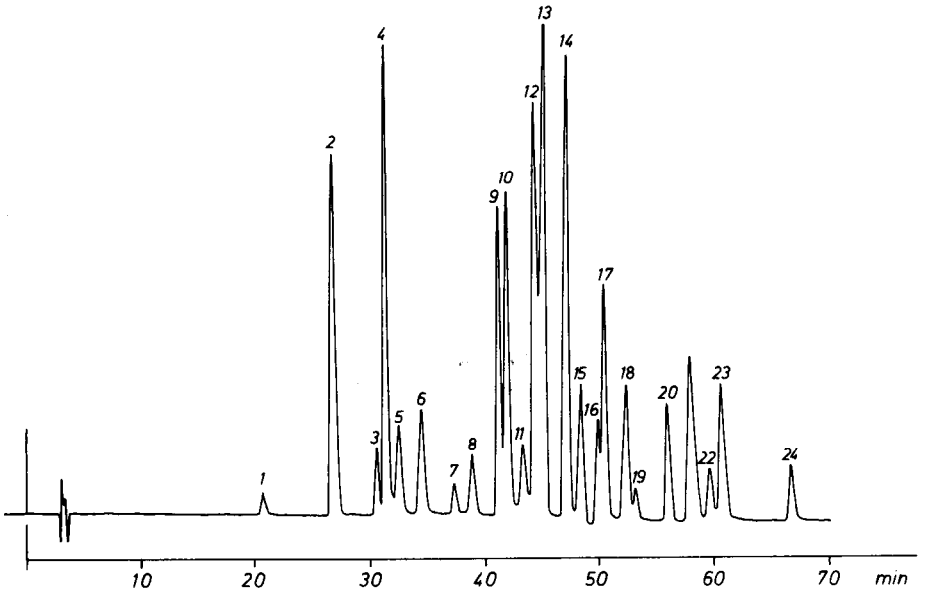

Fig. 1. Separation of hydroxycinnamic acid esters. Column: LiChrosorb RP-18, 5 μm (250 × 4 mm I.D.). Detection: 320 nm. Flow-rate: 1 ml/min. Gradient elution: solvent A = 2% acetic acid, solvent B = methanol; from A to 40% B in A in 80 min. Peaks as in Table II.

TABLE II

CAPACITY FACTORS (k'), SEPARATION FACTORS (α) AND RESOLUTION (R) USING LICHROSORB RP-18

*Peak No.**	*Compound*	k'	α	R
1	*cis*-5-Caffeoylquinic acid	5.8		
2	*trans*-5-Caffeoylquinic acid	7.9	1.34	7.00
3	*cis*-5-*p*-Coumaroylquinic acid	9.1	1.12	4.00
4	*trans*-Caffeoylglucose	9.4	1.03	1.00
5	*cis*-Caffeoylglucose	9.8	1.04	1.33
6	*trans*-5-*p*-Coumaroylquinic acid	10.4	1.07	2.33
7	*cis*-4-Caffeoylquinic acid	11.4	1.10	3.33
8	*cis*-5-Feruloylquinic acid	11.9	1.04	1.67
9	*trans*-*p*-Coumaroylglucose	12.7	1.07	2.67
10	*trans*-5-Feruloylquinic acid	12.9	1.02	1.00
10	*cis*-*p*-Coumaroylglucose	12.9	1.00	0
11	*cis*-4-*p*-Coumaroylquinic acid	13.4	1.03	1.33
12	*trans*-4-Caffeoylquinic acid	13.7	1.02	1.67
13	*trans*-3-Caffeoylquinic acid	14.0	1.02	1.00
14	*trans*-Feruloylglucose	14.6	1.04	2.00
15	*cis*-Feruloylglucose	15.1	1.03	1.67
16	*cis*-3-Caffeoylquinic acid	15.6	1.03	1.33
17	*trans*-Sinapoylglucose	15.8	1.01	0.67
18	*trans*-4-*p*-Coumaroylquinic acid	16.4	1.04	2.00
18	*cis*-Sinapoylglucose	16.4	1.00	0
19	*cis*-4-Feruloylquinic acid	16.7	1.02	1.00
20	*trans*-3-*p*-Coumaroylquinic acid	17.6	1.05	3.00
21	*trans*-4-Feruloylquinic acid	18.3	1.04	2.33
22	*cis*-3-*p*-Coumaroylquinic acid	18.8	1.03	1.67
23	*trans*-3-Feruloylquinic acid	19.2	1.02	1.33
24	*cis*-3-Feruloylquinic acid	21.2	1.10	6.67

* See Fig. 1.

tracts were eluted later than the hydroxycinnamic acid compounds. The flavonoids can easily be recognized by their absorption maximum at 350 nm (hydroxycinnamic acid derivatives *ca.* 320 nm).

Separation of hydroxycinnamic acid esters on LiChrosorb-Diol

On LiChrosorb-Diol as the stationary phase using methylene chloride–methanol + phosphoric acid as the eluent the *trans*-hydroxycinnamic acid esters were eluted in the following sequence (Fig. 2 and Table III): (a) 1, sinapic acid esters; 2, ferulic acid esters; 3, *p*-coumaric acid esters; 4, caffeic acid esters; (b) 1, 3-hydroxycinnamoylquinic acids; 2, 4-hydroxycinnamoylquinic acids; 3, 5-hydroxycinnamoylquinic acids; 4, hydroxycinnamoylglucoses. For the retention times a variation of $\bar{s}$ (t_R^{Diol}) = 0.6 min was noted.

Compared with the reversed-phase separations, the hydroxycinnamic acid esters are eluted in a reversed sequence and the glucose esters appear later than the quinic acid esters.

In the selection of the eluents, the miscibility of the components A and B proved to be of particular importance. Because of its better miscibility with methylene

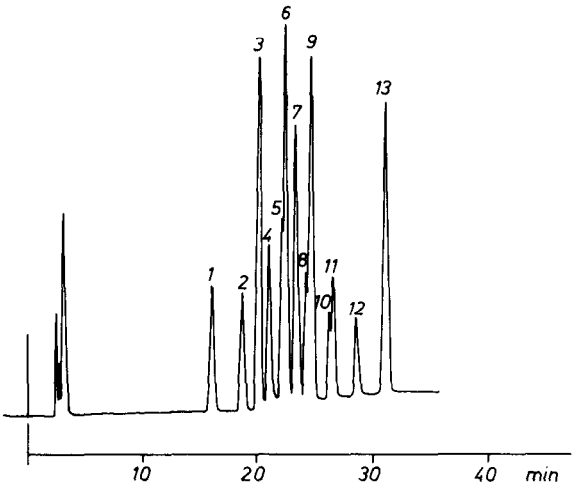

Fig. 2. Separation of *trans*-hydroxycinnamic acid esters. Column: LiChrosorb-Diol, 5 μm (250 × 4.6 mm I.D.). Detection: 320 nm. Flow-rate: 1.3 ml/min. Gradient elution: solvent A = methylene chloride, solvent B = methanol + 0.3% phosphoric acid; from 2% to 40% B in A in 60 min. Peaks as in Table III.

chloride, methanol was preferred to propanol. On addition of phosphoric acid the compounds were eluted more sharply and the resolution was improved. This method is well suited for the identification of hydroxycinnamic acid esters. As a baseline drift caused by the gradient may occur, quantitative determinations, especially of smaller amounts of hydroxycinnamic acid esters, should preferably be carried out with the reversed-phase method.

Quantitative determinations

Because of the variation of the retention times, the identifications of the hy-

TABLE III

CAPACITY FACTORS (k'), SEPARATION FACTORS (α) AND RESOLUTION (R) USING LICHROSORB-DIOL

*Peak No.**	*Compound*	k'	α	R
1	3-Feruloylquinic acid	5.7		
2	4-Feruloylquinic acid	6.9	1.21	3.60
3	3-*p*-Coumaroylquinic acid	7.4	1.07	1.60
4	5-Feruloylquinic acid	7.8	1.05	1.20
5	Sinapoylglucose	8.1	1.05	1.11
6	4-*p*-Coumaroylquinic acid	8.3	1.02	0.40
7	Feruloylglucose	8.7	1.05	1.40
8	3-Caffeoylquinic acid	9.1	1.05	1.40
9	5-*p*-Coumaroylquinic acid	9.3	1.01	0.40
10	4-Caffeoylquinic acid	9.9	1.08	2.22
11	*p*-Coumaroylglucose	10.1	1.02	0.52
12	5-Caffeoylquinic acid	10.9	1.08	2.56
13	Caffeoylglucose	11.9	1.10	3.32

* See Fig. 2.

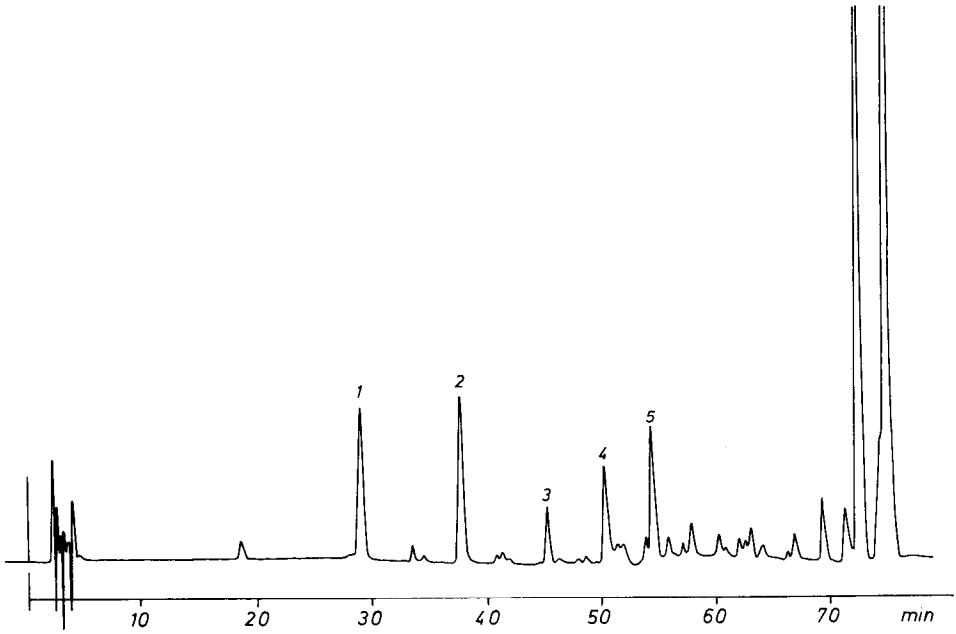

Fig. 3. Chromatogram of Chinese cabbage obtained using a LiChrosorb RP-18 column. Conditions as in Fig. 1. 1 = *trans*-5-Caffeoylquinic acid; 2 = *trans*-5-*p*-coumaroylquinic acid; 3 = *trans*-5-feruloylquinic acid; 4 = *trans*-feruloylglucose; 5 = *trans*-sinapoylglucose.

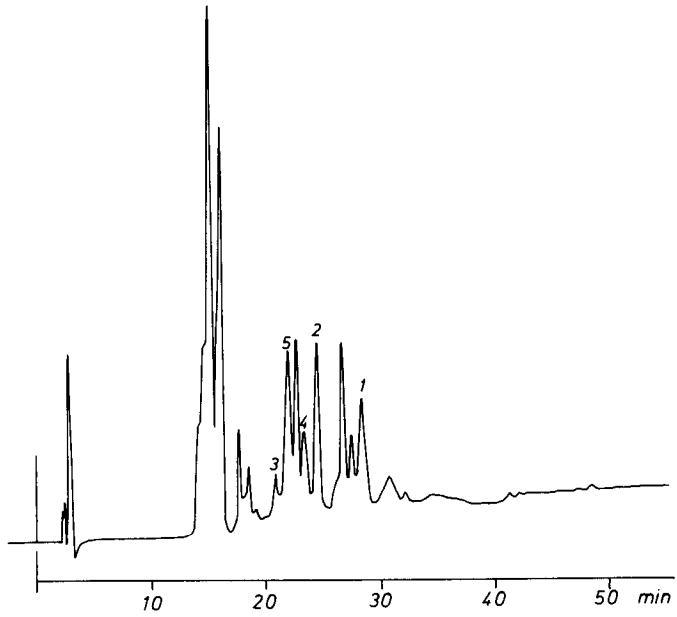

Fig. 4. Chromatogram of Chinese cabbage obtained using a LiChrosorb-Diol column. Conditions as in Fig. 2. Peaks as in Fig. 3.

droxycinnamic acid esters were performed by means of co-chromatography (addition of esters to the extract).

The quantitative determinations of the esters in different species of vegetables were carried out by using a calibration graph for the hydroxycinnamic acid in question. This method of calculation was necessary because only small amounts of compounds were available for comparison. Determination using the calibration graphs for the free acids is possible, as the UV spectra of the hydroxycinnamic acids and their esters differ only slightly. Determinations of chlorogenic acid by using the calibration graph for caffeic acid showed that the error of calculation can be neglected in comparison with the range of variations of the ester concentrations in plant material. In this paper, the chromatograms of Chinese cabbage are presented as examples (Figs. 3 and 4). The results of the analyses of the vegetables will be reported elsewhere[13].

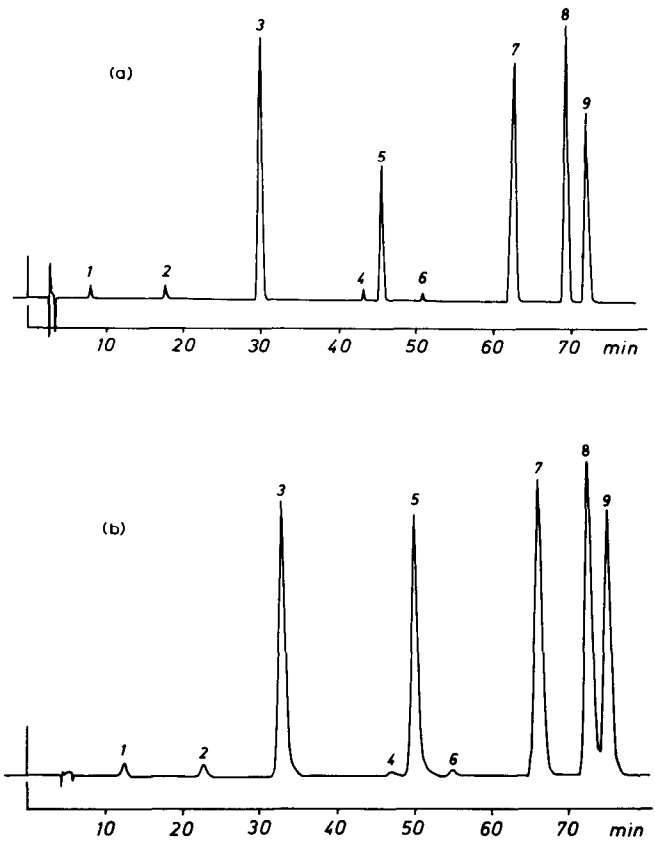

Fig. 5. (a) Chromatogram of hydroxybenzoic and hydroxycinnamic acids on the analytical scale obtained using a LiChrosorb RP-18 column. Conditions as in Fig. 1. 1 = Gallic acid; 2 = protocatechuic acid; 3 = gentisic acid; 4 = vanillic acid; 5 = caffeic acid; 6 = syringic acid; 7 = *p*-coumaric acid; 8 = ferulic acid; 9 = sinapic acid. (b) Chromatogram of hydroxybenzoic and hydroxycinnamic acids on the semi-preparative scale obtained using a LiChrosorb RP-18 column. Column: LiChrosorb RP-18, 10 μm (250 × 16 mm I.D.). Detection: 320 nm. Flow-rate:10 ml/min. Gradient elution: solvent A = 2% acetic acid, solvent B = methanol; from A to 35% B in A in 80 min. Peaks as in (a).

Preparative HPLC

Determinations of the efficiency of HPLC on the preparative scale indicated that separations obtained with a 250 × 16 mm I.D. column (Chrompack, Middelburg, The Netherlands) are of similar good quality to analytical separations. By means of chromatography of the free hydroxybenzoic acids and hydroxycinnamic acids it could be demonstrated that it is even possible to work with gradient elution on the preparative scale (Fig. 5a and b).

This makes possible the isolation of different compounds from a plant extract in one operation. To obtain pure compounds, it is necessary to clean the enriched fractions by isocratic elution. This can easily be managed after establishing a suitable eluent, *e.g.*, on the basis of the peak position in the gradient profile.

In this way, 5-caffeoylquinic acid and sinapoylglucose were isolated from Brussels sprouts. The substances were isolated with gradient elution (from 2% acetic acid to 35% methanol in 2% acetic acid in 80 min at a flow-rate of 10 ml/min) and cleaned by isocratic elution (12% methanol in 2% acetic acid at a flow-rate of 10 ml/min for 5-caffeoylquinic acid; 18% methanol in 2% acetic acid at a flow-rate of 10 ml/min for sinapoylglucose).

The purity of the substances obtained was checked by analytical HPLC. Their identity was verified by comparing them with authentic compounds, through hydrolyses and definition of the fragments by mass spectrometry.

From a methanolic extract of 800 g of garden cress, 200 mg of sinapoylglucose could be isolated by isocratic elution with 18% methanol in 2% acetic acid. The fractions were evaporated and lyophilized. After crystallization from hot water and washing with ice-cooled water, sinapoylglucose could be obtained in the form of fine, yellow needles (m.p. 128°C).

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