Journal of Chromatography, 240 (1982) 397–404
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,694

SEPARATION AND QUANTIFICATION OF BASIC HYDROXYCINNAMIC AMIDES AND HYDROXYCINNAMIC ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MICHEL PONCHET*
Station de Botanique et de Pathologie Végétale, I.N.R.A., B.P. 78, 06602 Antibes (France)
JOSETTE MARTIN-TANGUY
Station de Physiopathalogie Végétale, I.N.R.A., B.V. 1540, 21034 Dijon (France)
and
ALAIN POUPET, ANTOINE MARAIS and DANIEL BECK
Station de Physiopathologie Végétale, I.N.R.A., B.V. 1540, 21034 Dijon (France)
(Received December 29th, 1981)

SUMMARY

A method for the separation of basic hydroxycinnamic amides and hydroxycinnamic acids by high-performance liquid chromatography is described. N-*p*-Coumaryl-, N-caffeyl- and N-ferulylputrescine, N-*p*-coumaryl-, N-caffeyl- and N-ferulylspermidine and *p*-coumaric, caffeic, ferulic and sinapic acids were chromatographed on a μBondapak C_{18} reversed-phase column (particle size 9 μm) with different methanol–water gradients as the mobile phase. It is possible with this high-resolution and reproducible method to assay biological samples containing more than 10^{-5} *M* of hydroxycinnamic amides, using either *p*-coumaric or ferulic acid as the internal standard: this is demonstrated for tobacco extracts.

INTRODUCTION

Hydroxycinnamic amides (HCA) have been observed in higher plants. HCA are hydroxycinnamic acid (HC)–amine conjugates with an amide bond between them, and they occur in basic (water-soluble) and neutral (water-insoluble) forms. In the basic form, only aliphatic amines (di- and polyamines) are linked with HC.

Numerous basic HCA have been found in plant tissues (ferulylputrescine[1–5], caffeylputrescine[4–10], *p*-coumarylputrescine[4] and caffeylspermidine[10]), but more recently several workers have reported that the different basic HCA are closely related to the flowering process and to sexual organogenesis in different botanical species[11], in *Nicotiana tabacum* sp.[12–14], in *Zea mays*[15] and in *Araceae* species[16].

Moreover, results obtained in the study of the interaction between tobacco and tobacco mosaic virus (TMV) strongly suggest that basic HCA have an antiviral effect and are synthesized after virus infection as a protective mechanism[17,18]; in addition, virus particles are nearly absent from meristems, sex organs and seeds[19], where basic

0021-9673/82/0000–0000/$02.75

HCA accumulate in large amounts. Some HCA derivatives, *viz.*, glycocinnamoylspermidines, have been considered as a new class of antibiotics[20].

Previously reported procedures for the separation of basic HCA, such as differential extraction, thin-layer chromatography, paper chromatography and the use of cation-exchange resins, were not suitable for rapid assays because of their low resolution, long duration and lack of sensitivity.

The development of rapid and reproducible techniques for the separation, quantification and identification of these metabolites is essential in order to elucidate the molecular mechanism of their action.

In this paper we report an efficient method using reversed-phase high-performance liquid chromatography (HPLC), which allows the rapid and reproducible separation of basic HCA.

EXPERIMENTAL

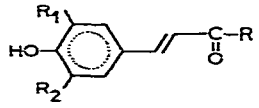
Apparatus

All experiments were performed on a Model 200/6/4/GM solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), using a U6K injector (Waters Assoc.). The column used was reversed-phase μBondapak C_{18} (particle size 9 μm; 30 cm × 3.9 mm I.D.; Waters Assoc.).

To protect the analytical column, a small and easily replaceable precolumn (2.3

TABLE I

STRUCTURES OF HYDROXYCINNAMIC ACIDS AND HYDROXYCINNAMIC ACID DERIVATIVES



Compound	*R*	R_1	R_2	*Supplier*
p-Coumaric acid	OH	H	H	Sigma (St. Louis, MO, U.S.A.)
Caffeic acid	OH	OH	H	
Ferulic acid	OH	OCH_3	H	
Sinapic acid	OH	OCH_3	OCH_3	
p-Coumarylputrescine	$HN(CH_2)_4NH_2$	H	H	Rhône-Poulenc
Caffeylputrescine	$HN(CH_2)_4NH_2$	OH	H	
Ferulylputrescine	$HN(CH_2)_4NH_2$	OCH_3	H	
p-Coumarylspermidine	$HN((CH_2)_7NH)NH_2$	H	H	—*
Caffeylspermidine	$HN((CH_2)_7NH)NH_2$	OH	H	—**
Ferulylspermidine	$HN((CH_2)_7NH)NH_2$	OCH_3	H	—*
Chlorogenic acid	$OC_7O_5H_{11}$	OH	H	Fluka (Buchs, Switzerland)
p-Coumarylquinic acid	$OC_7O_5H_{11}$	H	H	—***

* Purified from mature *Zea mays* seeds

** Purified from *Nicotiana tabacum* var. *Samsun* pistils.

*** Purified from apple fruits.

cm × 4 mm I.D.) filled with μBondapak C_{18} Porasil (Waters Assoc.) was used. Basic HCA and HC were detected on a Schoeffel spectrophotometer (monitored at 310 nm).

Recording and integration were carried out on ICAP 50-EI 510 (Delsi, France). Samples were injected with the use of a 25-μl Precision Sampling syringe (Precision Sampling Co., Baton Rouge, LA, U.S.A.) and chromatography was performed at ambient temperature (flow-rate 2 ml/min).

Reagents and standards

Methanol and acetic acid (R.P. Normapur) were obtained from Prolabo (Paris, France). Pic A (tetrabutylammonium phosphate) was purchased from Waters Assoc. The water used was deionized, redistilled twice and stored in dark containers.

The solvents were filtered under vacuum through a 5-μm Millipore Mitex LS type filter and degassed before use.

Details of the standards are given in Table I. Hydroxycinnamylspermidines were purified from plant tissues and synthetic hydroxycinnamylputrescines were given by Rhône-Poulenc Industries, France.

Preparation of standards

Concentrated standard stock solutions were prepared by direct weighing and dissolution in methanol (HC) or methanol–water (1:1) (HC derivatives), then stored at −20°C.

The concentration of the working standards generally ranged from 10^{-4} to $5 \cdot 10^{-4}$ *M* (for an injection volume of 15 μl), whereas those of stock solutions were 10^{-2} *M*; working standards were obtained from the stock solutions by dilution with methanol–water (1:1).

HPLC procedure

The gradient system consisted of absolute methanol (solvent A) and water–1% acetic acid–1.5% Pic A (solvent B), and the mobile phase obtained was run at a constant flow-rate. Acetic acid and Pic A (as ion-pairing reagent) were necessary to reduce retention times and to bring significant improvements in peak shape and resolution. This is because HCA have basic (amine) and acidic (phenolic) functions, which interfere with the free silicic acid poles of the packing material, to which no C_{18} carbon chain was added.

Linearity

Linearity was tested by injecting various volumes of standard solutions.

Sample preparation

Freshly harvested plant tissues (10 g fresh weight) were homogenized in methanol (100 ml) in a Sorwall Omnimixer, then filtered. The pellet was washed twice with methanol (100 ml), then discarded.

The filtered methanol extract was evaporated to 5 ml at 40°C under vacuum, diluted with water (50 ml) and treated with ethyl acetate (three 50-ml volumes) to remove neutral substances. The ethyl acetate fraction was dicarded. The aqueous fraction was concentrated to dryness and dissolved in methanol–water (1:1) (5 ml/g fresh material weight).

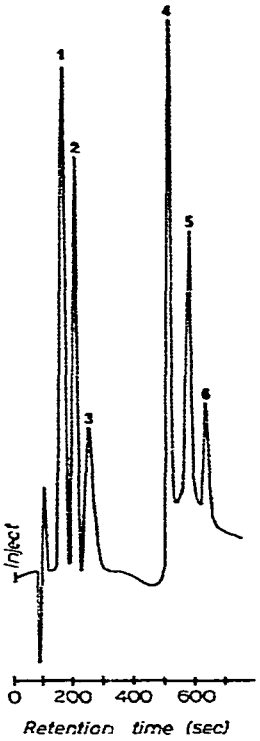


Fig. 1. Chromatogram of a mixture of six basic HCA. Column: μBondapak C_{18}. 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1% acetic acid, 1.5% Pic. A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated with A–B (10:90) for 10 min before injection. Peaks: 1 = caffeylputrescine; 2 = *p*-coumarylputrescine; 3 = ferulylputrescine; 4 = caffeylspermidine; 5 = paracoumarylspermidine; 6 = ferulylspermidine.

The solution was filtered through a 5-μm Mitex LS Millipore filter before injection.

RESULTS

Separation of basic HCA

Preliminary studies, using isocratic conditions with various proportions of water in methanol, showed that putrescine and spermidine derivatives required two different mobile phases to be chromatographed well. Moreover, for each group, isocratic conditions resulted in good resolution but the last peak (*i.e.*, ferulic acid derivative) spread over 2 min and did not allow reproducible quantitation. To obtain a good peak shape, a gradient (with an increasing proportion of methanol) was necessary and a convex gradient gave a more efficient resolution than a linear one.

The presence of Pic A as an ion-pairing reagent brought significant improvements: it reduced the retention time by almost half and it gave excellent symmetrical pointed peaks, whereas omission of Pic A caused trailing peaks.

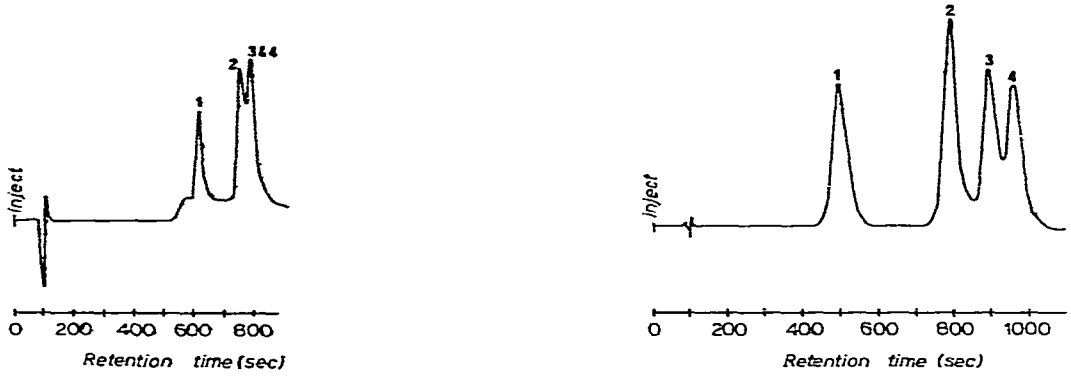


Fig. 2. Chromatogram of a mixture of HC. Column: μBondapak C_{18}, 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1% acetic acid, 1.5% Pic A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated with A–B (10:90) for 10 min before injection. Peaks: 1 = caffeic acid; 2 = paracoumaric acid; 3 = ferulic acid; 4 = sinapic acid.

Fig. 3. Chromatogram of a mixture of HC. Conditions and peaks as in Fig. 2, except mobile phase: solvent A (methanol)–solvent B (water with 2% acetic acid, no Pic A), convex gradient A–B (20:80) to A–B (30:70) for 10 min. The column was equilibrated with A–B (20:80) for 10 min before injection.

The best results were obtained with the solvent programme convex gradient 10% solvent A to 35% solvent A (in solvent B) for 5 min. Under these conditions, the separation of a mixture of basic HCA is effective, as shown in Fig. 1. Basic HCA were separated in the following order: caffeyl-, paracoumaryl-, feruloylputrescine, caffeyl-, *p*-coumaryl- and feruloylspermidine.

Using a detector sensitivity of 0.02 a.u.f.s., the minimum detectable amount of HCA was about 10 ng. Linearity was satisfactory up to 50 ng. Increasing the sensitivity would permit the detection of smaller amounts, but such an improvement would require additional precautions with respect to solvent purity and other conditions because rapid gradients are very detrimental to baseline stability when high sensitivity is employed.

The recovery was about 95% for the different compounds when the same sample was injected several times. The retention times were constant, the variations not exceeding 1%.

Separation of HC

Incomplete separation of HC occurred under the conditions outlined above (Fig. 2) and caffeic acid had the same retention time as feruloylspermidine. As free HC have not been found in plant tissues, either *p*-coumaric, ferulic or sinapic acid could be used as the internal standard for HCA assay.

When the gradient conditions were modified, as specified in Fig. 3, the separation of HC could be achieved in the following order: caffeic, *p*-coumaric, ferulic and sinapic acids (Fig. 3). The separation of HC could be helpful in the analysis of hydrolysed fractions containing HC derivatives, especially HCA.

Basic HCA separation was developed for the study of these compounds in tobacco extracts (HCA accumulated in ovaries during flowering and HCA formed in

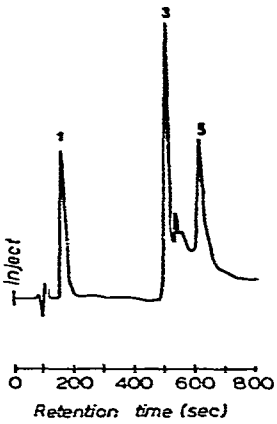


Fig. 4. Chromatogram of an extract of *Nicotiana tabacum* c.v. *Samsun* n.n. pistils (from non-bloomed flowers). Column: μBondapak C_{18}, 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1% acetic acid, 1.5% Pic A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated for 10 min before injection. Peaks: 1 = caffeylputrescine; 2 = ferulylputrescine; 3 = caffeylspermidine; 4 = unknown; 5 = caffeyl-3-quinic acid (chlorogenic acid); 6 = *p*-coumarylquinic acid.

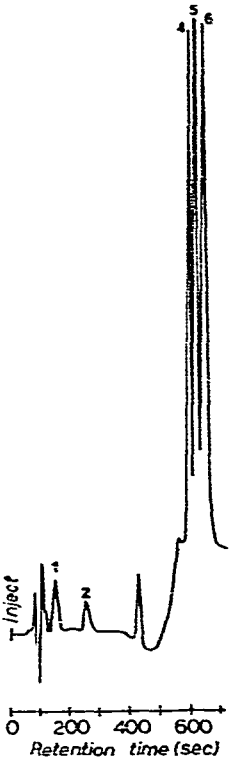


Fig. 5. Chromatogram of an extract of TMV-infected vegetative *Nicotiana tabacum* c.v. *Xanthi* n.c. leaves (48 h after inoculation). Conditions and peaks as in Fig. 4.

leaves during the hypersensitive reaction of *Nicotiana tabacum* c.v. *Xanthi* n.c. to TMV).

Basic HCA in non-purified tobacco extracts

All of the conditions were as in Fig. 1.

Fig. 4 shows the separation profile obtained from the analysis of *Nicotiana tabacum* c.v. *Samsun* n.n. pistils. Caffeylputrescine and caffeylspermidine were the main basic HCA accumulated in this sex organ, with concentrations close to 0.9 mg/g fresh material weight for caffeylputrescine and 1.5 mg/g fresh material weight for caffeylspermidine.

Fig. 5 illustrates the separation of basic HCA synthesized in the leaves of TMV-infected non-flowering *Nicotiana tabacum* c.v. *Xanthi* n.c. at the beginning of infection (48 h after inoculation with an inoculum of 0.1 mg/ml TMV). Ferulylputrescine and caffeylputrescine were formed and reached concentrations of about 0.1 mg/g fresh material weight. All of these compounds were absent from the fully extended vegetative leaves under normal growing conditions and from the control leaves inoculated with water.

Peak 5 (620 sec, Figs. 4 and 5) contained caffeylquinic acid (chlorogenic acid) and peak 6 (650 sec, fig. 5) *p*-coumarylquinic acid. Chlorogenic acid appeared in the peak area of ferulylspermidine and interfered with the *p*-coumarylspermidine peak area, so that sample purification was necessary to improve the separation and the quantitation of these compounds. The use of Amberlite CG-50 (H^+) resin (Serva, Heidelberg, G.F.R.) allows the separation of quinic esters from basic HCA and is thus the most suitable technique. The identity of peaks 5 and 6 was based on results obtained both by injection of individual standards and of a pair of standards (varying the concentration of one standard in the mixture each time).

Table II summarizes retention times of the various HC and HC derivatives (for chromatographic conditions, see Fig. 1).

DISCUSSION

This paper demonstrates the ability of a C_{18} reversed-phase column to separate basic HCA and HC by HPLC. HPLC provides a highly sensitive (the minimum detectable amount of HCA is *ca.* 10 ng, or *ca.* 50 pmole), rapid and reproducible method for the determination and quantitation of basic HCA.

This technique allows the separation of these compounds from non-purified plant extracts or simply purified extracts [prior clean-up with Amberlite CG-50 (H^+) resin]. Detection of peaks at 310 nm is very helpful in eliminating UV absorption of all co-extractable compounds able to interfere at lower wavelengths (254 or 270 nm); for example, aromatic amines (tyramine, dopamine, tryptamine) have almost the same retention time as caffeylputrescine but do not absorb at 310 nm.

HC such as *p*-coumaric and ferulic acids can be used as internal standards for quantitation of basic HCA.

The proposed method promises to be very useful for the study of these compounds with respect to the flowering process and to virus resistance.

TABLE II

RETENTION TIMES OF THE DIFFERENT HC AND HC DERIVATIVES

Column: μBondapak C_{18} (9 μm), 30 cm × 3.9 mm I.D. Flow-rate: 2 ml/min. Wavelength: 310 nm. Mobile phase: Water with 1% acetic acid and 1.5% Pic A–methanol, 90:10 to 65:35 for 5 min.

Compound	*Retention time (sec. ± 5 sec)*
Caffeylputrescine	160
p-Coumarylputrescine	200
Feruloylputrescine	255
Caffeylspermidine	515
p-Coumarylspermidine	585
Caffeyl-3-quinic acid (chlorogenic acid)	620
Caffeic acid	620
Feruloylspermidine	640
p-Coumarylquinic acid	650
p-Coumaric acid	750
Ferulic acid	780
Sinapic acid	785

ACKNOWLEDGEMENTS

The authors thank Rhône-Poulenc Industries for supplying hydroxycinnamylputrescines, and Mr. Barbes (Waters Assoc.) for assistance.

REFERENCES

1 A. A. Ryabinin and E. M. Il'ina, *Dokl. Akad. Nauk SSSR*, 67 (1949) 513–516.
2 A. A. Ryabinin and E. M. Il'ina, *Chem. Abstr.*, 44 (1950) 1455.
3 T. A. Wheaton and I. Stewart, *Nature (London)*, 206 (1965) 620–621.
4 S. Mizusaki, Y. Tanabe, M. Noguchi and E. Tamaki, *Phytochemistry*, 10 (1971) 1347–1350.
5 J. Berlin, *Phytochemistry*, 20 (1981) 53–55.
6 A. Hollerbach and G. Spiteller, *Monatsh. Chem.*, 101 (1970) 141–156.
7 S. Mizusaki, Y. Tanabe and M. Noguchi, *Agr. Biol. Chem.*, 34 (1970) 972–973.
8 J. G. Buta and R. R. Izac, *Phytochemistry*, 11 (1972) 1188–1189.
9 E. I. Mbadiwe, *Phytochemistry*, 12 (1973) 2546.
10 J. Deletang, *Ann. Tab.*, 11 (1974) 124–130.
11 J. Martin-Tanguy, F. Cabanne, E. Perdrizet and C. Martin, *Phytochemistry*, 17 (1978) 1927–1928.
12 F. Cabanne, J. Martin-Tanguy, E. Perdrizet, J. C. Vallee, L. Grenet, J. Prevost and C. Martin, *C.R. Acad. Sci., Ser. D*, 282 (1976) 1959–1962.
13 F. Cabanne, J. Martin-Tanguy and C. Martin, *Physiol. Veg.*, 15 (1977) 429–443.
14 F. Cabanne, *Thèse Doct. d'Etat*, Univ. Dijon, (1980) 191 pp.
15 J. Martin-Tanguy, A. Deshayes, E. Perdrizet and C. Martin, *Febs Lett.*, 108 (1979) 176–178.
16 M. Ponchet, *Thèse Doct. Ing.*, I.N.A., Paris-Grignon, 1981, pp. 179–228.
17 J. Martin-Tanguy, C. Martin and M. Gallet, *C.R. Acad. Sci., Ser. D*, 276 (1973) 1433–1435.
18 J. Martin-Tanguy, C. Martin, M. Gallet and R. Vernoy, *C.R. Acad. Sci., Ser. D*, 282 (1976) 2231–2234.
19 C. Grison and C. Martin, *Ann. Epiphyt.*, (1960) 1–6.
20 G. A. Ellestad, D. B. Cosulich, R. W. Broschard, J. H. Martin, M. P. Kunstmann, G. O. Morton, J. E. Lancaster, W. Fulmor and F. M. Lovel, *J. Amer. Chem. Soc.*, 100 (1978) 2515–2524.