Journal of Chromatography, 467 (1989) 149–157
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 237

COMBINED MONITORING OF UV ABSORBANCE AND FLUORESCENCE INTENSITY AS A DIAGNOSTIC CRITERION IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF NATURAL PHENOLIC ACIDS

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(First received February 29th, 1988; revised manuscript received December 29th, 1988)

SUMMARY

Under standardized conditions, the combined monitoring of UV absorbance (A_{280}) and fluorescence intensity (I_F) at 390 or 393 nm (on excitation at 300 or 340 nm, respectively) provides a diagnostic criterion for the identification of cinnamic and benzoic acid derivatives by reversed-phase high-performance liquid chromatographic seperations of natural phenolic acids. The method can easily be used to determine the concentrations of these compounds if 3,5-dinitrobenzoic acid is applied as an internal standard. When applied to a saponified rye kernel water extract, ferulic, *p*-coumaric, sinapic, syringic and vanillic acids were shown to be present.

INTRODUCTION

Reversed-phase high-performance liquid chromatographic (HPLC) separations of natural phenolic acids are traditionally monitored by measurement of the UV absorbance of the eluate[1–8]. The retention time of these components under standardized conditions is, however, not a reliable criterion for their identification because in complex chromatograms of natural extracts many substances of similar structures occur that can yield peaks with only slightly different or even the same retention times[8]. It is therefore advisable in many instances to verify the identity of a particular peak by applying a second chromatographic technique such as thin-layer chromatography (TLC)[1,2,7–13]. The recovery of samples run on analytical reversed-phase HPLC columns in order to perform this second analysis (*e.g.*, by TLC) is, however, laborious.

As cinnamic and *o*-hydroxybenzoic acid and their derivatives display fluorescence when exposed to light of the Wood wavelength (366 nm)[14] and as this property is exploited both in the study of cereals by fluorescence microscopy[9,15–19] and in the TLC analysis of phenolic acids, we decided to establish whether or not the joint monitoring of UV absorbance and fluorescence intensity could provide an additional and simple criterion for the identification of phenolic acids in our standardized HPLC

0021-9673/89/$03.50

separation of these natural substances. To the best of our knowledge, the use of fluorescence intensity as a monitoring technique for the HPLC separations of plant phenolic acids has not been reported before. This is understandable to a certain extent, as it is necessary to find suitable combinations of excitation and emission wavelengths such that at least a considerable proportion of the studied compounds display fluorescence.

It seemed to us that the joint measurement of the UV absorbances and fluorescence intensities of the separated compounds could offer an interesting diagnostic criterion, not only to allow for a distinction between the compounds that display fluorescence under the experimental conditions and those that do not, but also between different phenolic acids characterized by varying ratios between their UV absorbance and fluorescence intensity under standardized conditions.

In this paper it is shown that different excitation and fluorescence wavelength combinations can be chosen for the identification of natural phenolic acids via the above method. It is also demonstrated that 3,5-dinitrobenzoic acid can serve as an internal standard for quantitative work. The method was applied to the chromatography of the natural phenolic acids associated with rye water solubles.

Ferulic acid was previously found to be associated with rye water soluble pentosan material[12]. It is likely that ferulic acid associated with such pentosans is important for the oxidative gelation of these materials[20,21], which have a positive effect on the breadmaking properties of rye flour[21].

EXPERIMENTAL

Instrumentation

The HPLC system consisted of the following Shimadzu equipment: an SCL-6A system controller, two LC-60 chromatography pumps and an HPLC fluorescence monitor. The UV absorbance detector, equipped with a 280-nm filter, was a Pharmacia UV-2 dual-path monitor. The chromatographic separations were performed on an Alltech ROSIL C_{18} column (250 × 4.6 mm I.D., particle size 5 μm). We used a precolumn (10 × 4.6 mm I.D.) with the same stationary phase. All samples were injected with a Rheodyne 7125 injector (installed with a 20-μl loop) as solutions in glass-distilled methanol after double filtration on Schleicher & Schüll FP 030/70 disposable 0.45-μm filter holders. The eluate from the fluorescence detector was fed directly to the UV monitor and both the UV and the fluorescence signal were registered with a Pharmacia Rec-2 two-channel recorder.

All separations were achieved under isocratic conditions. Although the gradient mode allows for faster separations, we preferred to achieve separations devoid of baseline drift, a phenomenon encountered in the gradient elution of the compounds studied here[1,2,8].

Solvents and chemicals

The elution solvent (flow-rate 1.0 ml/min) was water–methanol–acetic acid (95:15:5, v/v/v). The water was deionized and glass distilled immediately before use. Methanol was glass distilled and glacial acetic acid was purchased from Merck (GR grade). The solvent was vacuum filtered through FP-450 47 mm 0.45 μm FP Vericel membrane filters purchased from Gelman and used without further degassing.

Caffeic, ferulic, *p*-coumaric, syringic, vanillic and 3,5-dinitrobenzoic acids were purchased from Fluka and sinapic and gallic acids were obtained from Aldrich. Protocatechuic acid, *p*-hydroxybenzoic acid and salicylic acid were obtained from Sigma, UCB and Eastman Kodak, respectively. The cinnamic acid derivatives were *trans* isomers.

All compounds were injected as solutions in methanol (60 ppm), except for vanillic acid and *p*-hydroxybenzoic acid, where other concentrations in the same solvent (30 and 240 ppm, respectively) were used. When injected alone they all gave single peaks.

Isolation of rye water solubles

The rye sample was of the Danko variety (1987 harvest), grown in Limburg Province, Belgium. The sample was the same as that used in a study of the relative monosaccharide compositions in milled rye products[22].

Rye kernels were cooked in ethanol (95%) for 60 min. After removal of the solvent by filtration and drying at room temperature, the kernels were milled using a D.D.D. President Mill (Ieper, Belgium). Whole rye meal (500 g) was extracted with 2000 ml of deionized water for 4 h at room temperature with continuous mechanical stirring. After centrifugation (30 min, 2000 *g*) the extract (1650 ml) was lyophilized (yield 39.84 g).

Isolation of phenolic acids in rye water solubles

The isolation was carried out in the dark whenever possible, as free cinnamic acids can rapidly undergo *trans–cis* isomerization in daylight[10,23,24].

Lyophilized water solubles (1.00 g) were saponified in 100 ml of 0.5 *M* sodium hydroxide solution for 90 min at 60°C under a nitrogen atmosphere. The saponified mixture was neutralized with concentrated hydrochloric acid. Two volumes of ethanol (95%) were added to one volume of extract in order to precipitate the pentosan material (overnight at 6°C). The precipitate was removed by centrifugation (10 min, 2000 *g*), the residue was washed with ethanol and the washings were added to the supernatant. The ethanol was removed from this solution by vacuum evaporation at 40°C. The pH of the water phase was then adjusted to 3.0 using 2.0 *M* hydrochloric acid. The phenolic acids were subsequently extracted with ethyl acetate (3 × 100 ml). The extract was dried over anhydrous sodium sulphate and evaporated (under vacuum at 40°C) to dryness. This extract was dissolved in 2.0 ml of methanol containing the internal standard 3,5-dinitrobenzoic acid (0.1 mg/ml). It was then filtered twice as above and analysed by HPLC as described above.

Quantitative data were obtained using the relative peak heights (peak height of the compound to be analysed / peak height of 3,5-dinitrobenzoic acid) in the chromatogram (A_{280}) and the relationship between the relative peak heights and the relative concentrations for each compound.

RESULTS

The structures of the compounds studied are shown in Fig. 1 and typical examples of chromatographic separations monitored either by UV absorbance at 280 nm (A_{280}) or by the fluorescence intensities at different filter combinations (I_F) are given

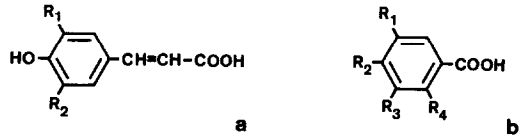

Fig. 1. Structural formulae of (a) cinnamic acid derivatives and (b) benzoic acid derivatives. (a) $R_1 = R_2 =$ H, *p*-coumaric acid; R_1 = H, R_2 = OH, caffeic acid; R_1 = OMe, R_2 = H, ferulic acid; $R_1 = R_2$ = OMe, sinapic acid. (b) $R_1 = R_2 = R_3$ = H, R_4 = OH, salicylic acid; $R_1 = R_3$ = OMe, R_2 = OH, R_4 = H, syringic acid; R_1 = OMe, R_2 = OH, $R_3 = R_4$ = H, vanillic acid; $R_1 = R_3 = R_4$ = H, R_2 = OH, *p*-hydroxybenzoic acid; $R_1 = R_2$ = OH, $R_3 = R_4$ = H, protocatechuic acid; $R_1 = R_2 = R_3$ = OH, R_4 = H, gallic acid; $R_1 = R_3 = NO_2$, $R_2 = R_4$ = H, 3,5-dinitrobenzoic acid.

in Fig. 2. Adequate baseline separations were obtained under the chromatographic conditions applied.

Typical retention times of the compounds and ratios between their UV absorbance and fluorescence intensities at different wavelength combinations are listed in

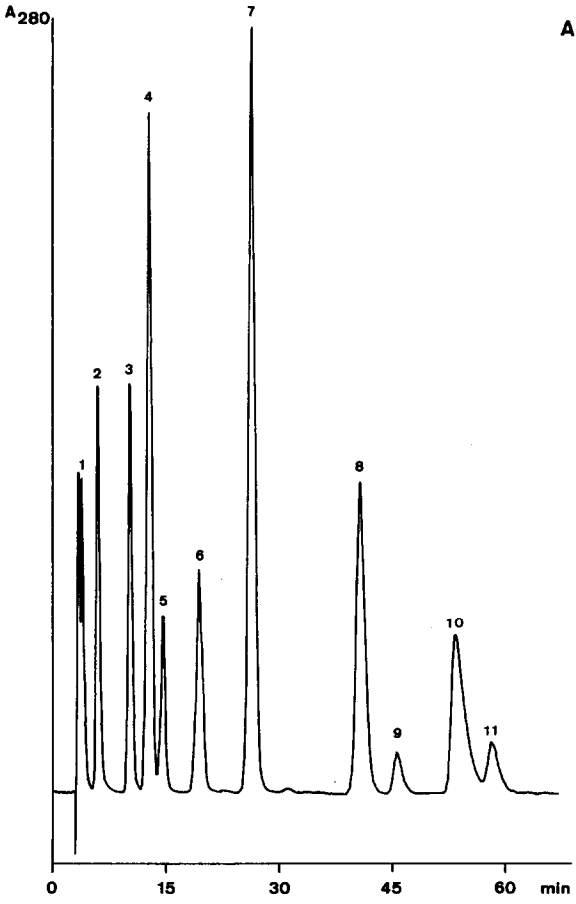

Fig. 2.

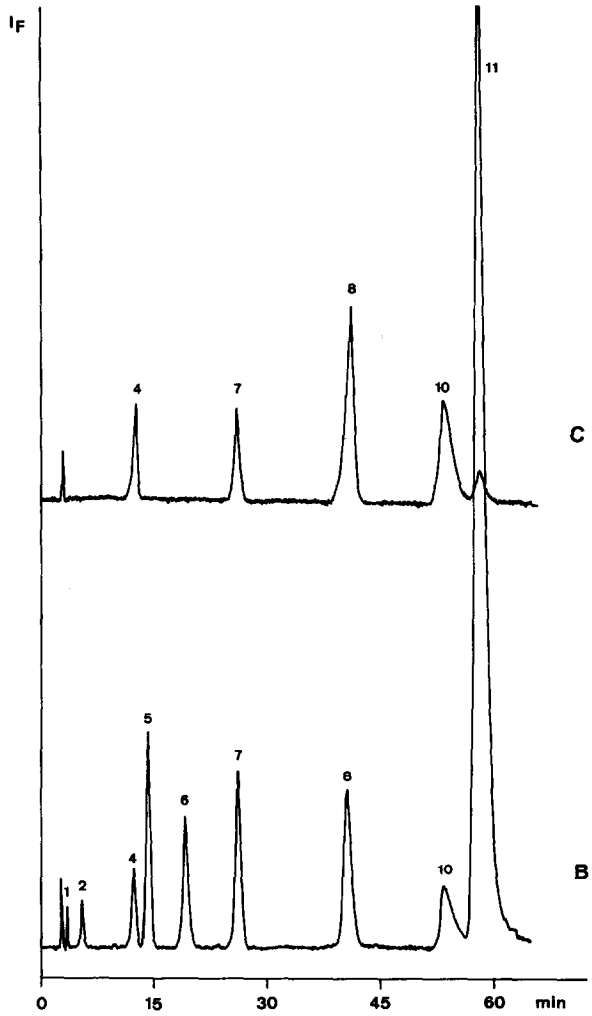

Fig. 2. HPLC separation of standard phenolic compounds on ROSIL C_{18} (250 × 4.6 mm I.D., 5 μm, with precolumn) with water–methanol–acetic acid (95:15:5, v/v/v) at 1.0 ml/min as eluent. An aliquot (20 μl) of solutions in methanol with concentrations as described in the text was chromatographed and the separations were monitored by recording (A) the A_{280} values (0.2 a.u.f.s.) or the fluorescence intensities (I_F) at (B) 390 and (C) 393 nm with excitation at 300 and 340 nm, respectively. Peaks: 1 = gallic acid; 2 = protocatechuic acid; 3 = *p*-hydroxybenzoic acid; 4 = caffeic acid; 5 = vanillic acid; 6 = syringic acid; 7 = *p*-coumaric acid; 8 = ferulic acid; 9 = 3,5-dinitrobenzoic acid; 10 = sinapic acid; 11 = salicylic acid.

Table I. These ratios are given in arbitrary units as they are based on peak heights registered under highly standardized conditions.

Of further interest is the fact that the *cis* isomers generated on UV irradation of cinnamic acid derivatives[10,23,24] do not display fluorescence under the experimental conditions applied here.

Phenolic acids associated with rye water solubles are vanillic, syringic, *p*-coumaric, ferulic and sinapic acids (Table II). This follows not only from the retention

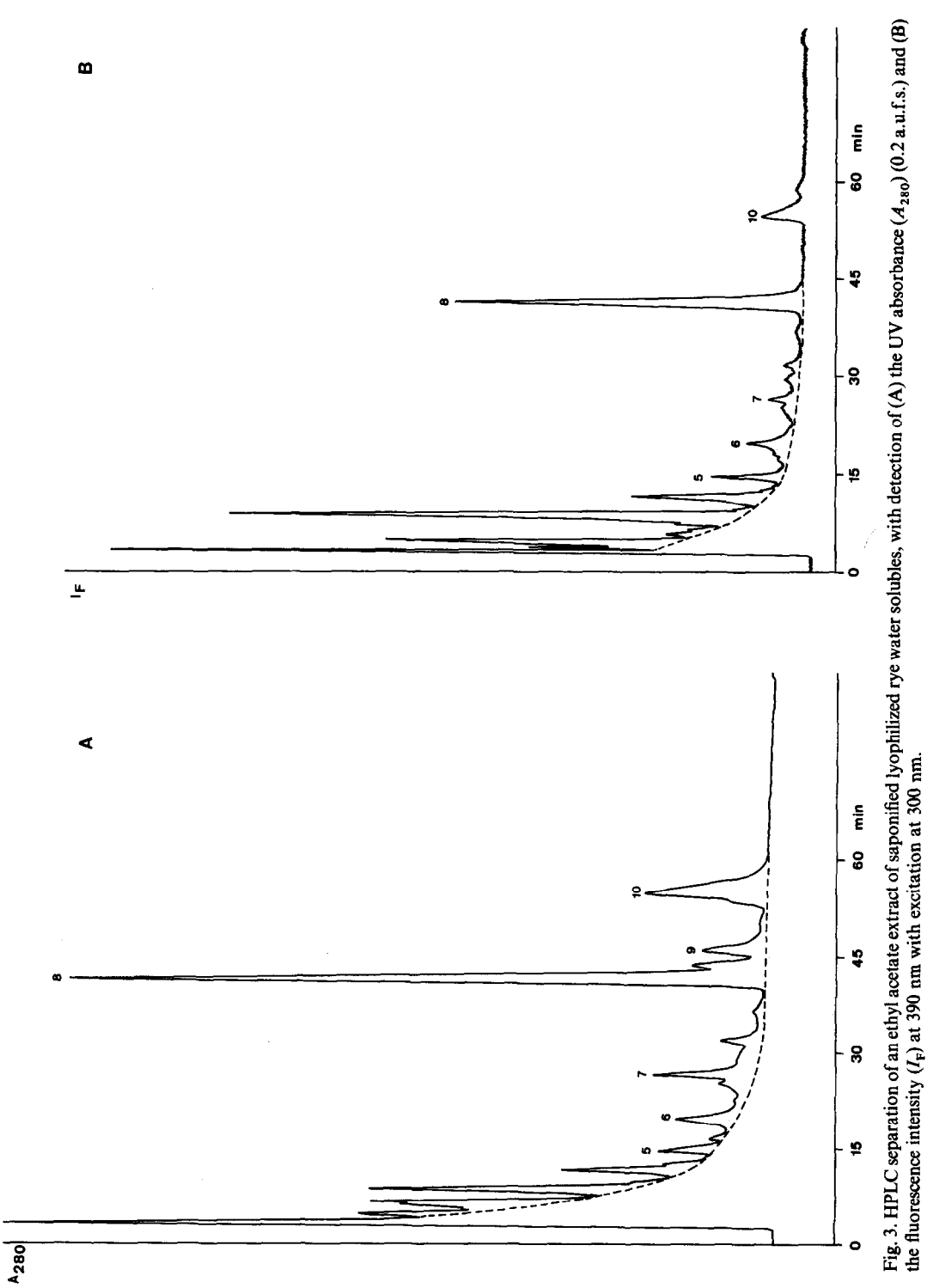

Fig. 3. HPLC separation of an ethyl acetate extract of saponified lyophilized rye water solubles, with detection of (A) the UV absorbance (A_{280}) (0.2 a.u.f.s.) and (B) the fluorescence intensity (I_F) at 390 nm with excitation at 300 nm.

TABLE I

TYPICAL RETENTION TIMES AND RATIOS (ARBITRARY UNITS) BETWEEN THE UV ABSORBANCE (A_{280}) AND THE FLUORESCENCE INTENSITIES (I_F) AT 393 AND 390 nm ON EXCITATION AT 340 AND 300 nm, RESPECTIVELY, OF DIFFERENT PHENOLIC COMPOUNDS SEPARATED AS DESCRIBED IN FIG. 2

N.f. = No fluorescence under the experimental conditions.

Compound	*Retention time (min)*	A_{280}/I_F	
		393 nm	*390 nm*
Gallic acid	4	N.f.	8.2
Protocatechuic acid	6	N.f.	8.2
p-Hydroxybenzoic acid	10	N.f.	N.f.
Caffeic acid	13	7.2	9.0
Vanillic acid	15	N.f.	0.9
Syringic acid	19.5	N.f.	1.7
p-Coumaric acid	26.5	9.4	4.3
Ferulic acid	41	1.6	2.1
3,5-Dinitrobenzoic acid	46	N.f.	N.f.
Sinapic acid	53.5	1.5	2.7
Salicylic acid	58.5	1.6	0.1

times of these compounds, but also from the ratios of the UV absorbance (280 nm) to the fluorescence intensity at 390 nm on excitation at 300 nm. With *p*-coumaric acid, the theoretical ratio (4.3) was not identical with that of the same compound observed in the natural extract (3.7). It is believed that this is mainly due to the presence of a minor component in the extract with a retention time slightly lower than that of *p*-coumaric acid. A typical chromatogram is shown in Fig. 3. The analytical data indicate that the most abundant phenolic acid is ferulic acid, as was to be expected[20,21,25]. To the best of our knowledge, the presence of these other phenolic acids in rye water solubles has not been reported before, although their occurrence in wheat, rice, oat and corn kernels is well known[25].

TABLE II

PHENOLIC ACID CONTENTS OF LYOPHILIZED RYE WATER SOLUBLES AND THE RATIOS BETWEEN THE UV ABSORBANCE (A_{280}) AND FLUORESCENCE INTENSITIES (I_F) AT 390 nm ON EXCITATION AT 300 nm, OBTAINED AS DESCRIBED IN FIG. 2

Phenolic acid	*Concentration (ppm)*	A_{280}/I_F
Vanillic acid	28	0.8
Syringic acid	26	1.5
p-Coumaric acid	21	3.7
Ferulic acid	332	2.0
Sinapic acid	18	2.8

DISCUSSION

When exposed to UV light of a particular wavelength, different fluorescent phenolic acids have different fluorescence spectra, as exhibited by differences in the wavelength and the intensity of maximum fluorescence. From this it follows that the chromatographic analysis of these phenolic acids by monitoring both the UV absorbance and the fluorescence intensity at, *e.g.* excitation and emission wavelength combinations of 300 and 390 nm or 340 and 393 nm will result in different ratios between the peak heights in the measurement of the UV absorbance and fluorescence intensity. These ratios can therefore be applied as diagnostic criteria for the identification of these phenolic compounds because we found (as was to be expected at low concentrations) that they are independent of the concentration of the chromatographed substance. In our experience gained during the identification of phenolic acids associated with rye arabinoxylans[13] and partly described here, the determination of the ratio between the peak heights of the UV absorbance and the fluorescence intensity at only one wavelenght combination suffices for the identification of unknown fluorescent phenolic acids. Further, the ratios obtained under highly standardized conditions can in many instances also be applied in order to verify whether or not a particular peak in a chromatogram results from a single substance or not.

ACKNOWLEDGEMENTS

One of us (J.A.D.) gratefully thanks the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek for a research position as "Bevoegdverklaard Navorser" and for "Krediet aan Navorsers" financial support. Helpful discussions by Professor P. Dondeyne and excellent technical assistance by Mr. Luc Van den Ende are gratefully acknowledged.

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