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Note

Separation of aromatic choline esters by high-performance liquid chromatography

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Choline esters are constituents of all living cells, and aromatic choline esters occur in appreciable amounts in seeds of several glucosinolate containing plants[1,2]. Sinapine, the choline ester of sinapic acid, is the best known, but six different aromatic choline esters have been described as plant products and others await identification[2,3].

The occurrence of a high concentration of phenolic choline esters in Cruciferae seeds seems to reduce the quality of this valuable oil and protein source[4–6]. However, only sinapine has been the subject of both quality and metabolic studies[7], probably owing to a lack of available methods of analysis, and much more information on this subject is required.

The methods of analysis previously used for the investigations of aromatic choline esters in plants or products thereof are usually adapted specifically for sinapine determination. They are based on differential extraction and spectrophotometric determination[8] or thin-layer chromatography[9]. The analysis of choline and some derivatives thereof by gas chromatography–mass spectrometry has also been described[10].

The development of a simple, fast and reproducible method for the determination of individual aromatic choline esters is essential for the above and other reasons. This paper describes such a method based on reversed-phase ion-pair high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

Heptanesulphonic acid was obtained from Fluka (Buchs, Switzerland); all other reagents were of analytical-reagent grade from E. Merck (Darmstadt, G.F.R.). The HPLC solvents were filtered under vacuum through a 0.5-μm Millipore FH filter or a 0.45-μm Millipore HA filter and degassed before use.

Compounds investigated

Tables I and II give the formulae and names of the aromatic choline esters studied. Sinapine and its glucoside (Nos. 21 and 22 in Table II) were isolated from

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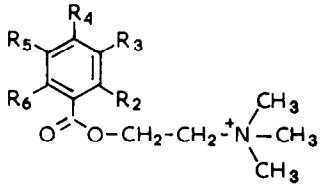
TABLE I

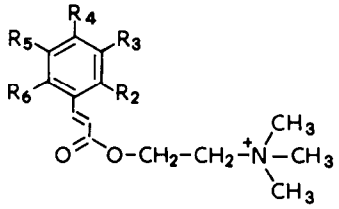
BENZOYLCHOLINE DERIVATIVES INVESTIGATED



No.	R_2	R_3	R_4	R_5	R_6	*Name*
1	H	H	HO	H	H	4-Hydroxybenzoylcholine
2	H	HO	H	H	H	3-Hydroxybenzoylcholine
3	HO	H	H	H	H	2-Hydroxybenzoylcholine
4	HO	HO	H	H	H	2,3-Dihydroxybenzoylcholine
5	HO	H	H	HO	H	2,5-Dihydroxybenzoylcholine
6	H	HO	HO	H	H	3,4-Dihydroxybenzoylcholine
7	H	HO	H	HO	H	3,5-Dihydroxybenzoylcholine
8	H	CH_3O	HO	H	H	4-Hydroxy-3-methoxybenzoylcholine
9	H	HO	CH_3O	H	H	3-Hydroxy-4-methoxybenzoylcholine
10	H	CH_3O	CH_3O	H	H	3,4-Dimethoxybenzoylcholine
11	H	HO	HO	HO	H	3,4,5-Trihydroxybenzoylcholine
12	H	CH_3O	HO	CH_3O	H	3,5-Dimethoxy-4-hydroxybenzoylcholine

TABLE II

CINNAMOYLCHOLINE DERIVATIVES INVESTIGATED



No.	R_2	R_3	R_4	R_5	R_6	*Name*
13	H	H	H	H	H	Cinnamoylcholine
14	H	H	HO	H	H	4-Hydroxycinnamoylcholine
15	H	CH_3O	HO	H	H	4-Hydroxy-3-methoxycinnamoylcholine
16	H	HO	CH_3O	H	H	3-Hydroxy-4-methoxycinnamoylcholine
17	H	CH_3O	CH_3O	H	H	3,4-Dimethoxycinnamoylcholine
18	CH_3O	CH_3O	H	H	H	2,3-Dimethoxycinnamoylcholine
19	CH_3O	H	CH_3O	H	H	2,4-Dimethoxycinnamoylcholine
20	CH_3O	H	H	CH_3O	H	2,5-Dimethoxycinnamoylcholine
21	H	CH_3O	HO	CH_3O	H	3,5-Dimethoxy-4-hydroxycinnamoylcholine
22	H	CH_3O	β-D-Gluco-pyranosyloxy	CH_3O	H	3,5-Dimethoxy-4-β-D-glucopyranosyloxy-cinnamoylcholine

natural sources[1,2]. Choline esters 1–20 were prepared by synthesis; these included the natural products[2] 4-hydroxybenzoylcholine (1), hesperaline (10), feruloylcholine (15) and isoferuloylcholine (16).

The method of synthesis was based on previously described principles[1,11] but was changed slightly to avoid oxidation of the phenols. Sodium carboxylates were prepared by careful titration with sodium hydroxide to pH 7. Equimolar amounts of sodium carboxylates and bromocholine bromide[1] were dissolved in water and, after evaporation to dryness, the residues were heated at 125–130°C for 2 h[11]. In most instances the residues melted and later recrystallized during the heating. The resulting mixture of choline ester bromide and sodium bromide were purified by column chromatography on Sephadex CM-25 (H^+) (30 × 1.5 cm I.D.) using 1 *M* acetic acid as the eluent according to a recently described method[3]. The identities of the compounds were established by paper and thin-layer chromatography, high-voltage electrophoresis and ultraviolet and nuclear magnetic resonance spectroscopy[1,2].

Chromatography

The liquid chromatograph used consisted of two Waters M-6000 A pumps, a Waters M-450 variable-wavelength absorbance detector, a Waters M-720 system controller and a Rheodyne Model 7125 injection valve with a 20-μl loop. The chromatograms were recorded on a Kipp and Zonen BD-41 recorder. All experiments were performed on 120 × 4.6 mm I.D. columns (Knauer, Berlin, G.F.R.) packed by the dilute slurry technique with either Nucleosil 5 C_8 (5 μm), Nucleosil 5 C_{18} (5 μm), Nucleosil 5 CN (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.) or Spherisorb Phenyl (5 μm) (E. Merck).

The gradient system consisted of solvent A [0.02 *M* phosphate buffer, 0.02 *M* dibutylamine and 0.02 *M* sodium heptanesulphonic acid (pH 2.0) modified with 50% acetonitrile] and solvent B [0.01 *M* phosphate buffer, 0.01 *M* dibutylamine and 0.01 *M* sodium heptanesulphonic acid (pH 2.0)].

The mobile phase was a linear gradient of solvent A–solvent B (2:8 to 7:3) for 30 min. The column was equilibrated under the initial conditions for 5 min prior to each injection. The flow-rate was 1.0 ml/min.

RESULTS AND DISCUSSION

UV spectra of solutions of the acetate salts of the aromatic choline esters were recorded prior to use in the HPLC analysis. The results are shown in Table III. The absorbance maxima for the different choline esters are at different wavelengths. All of the investigated compounds have an appreciable absorbance at 254 nm, most of the benzoic acid derivatives have their absorbance maxima below 300 nm and most of the cinnamic acid derivatives have an absorption peak above 300 nm, although exceptions exist. Knowledge of the UV data is of value in connection with the identification of the compounds, a reasonable choice of detection wavelength and the use of peak heights and/or areas in quantitative determinations.

Elaboration of the HPLC method was performed by use of solutions containing the choline esters alone, and later mixtures of these compounds were used. Investigations with different stationary phases revealed that chromatography on Nucleosil 5 CN and Spherisorb Phenyl gave unsatisfactory peak shapes and poor reso-

TABLE III

UV DATA AND k' VALUES FROM HPLC OF AROMATIC CHOLINE ESTERS

*Compound No.**	*k' values from HPLC***		*λ_{max} (nm) and corresponding (relative) E values**** *from UV spectra*					
			In CH_3OH			*In CH_3OH + CH_3ONa*		
	On C_8	*On C_{18}*						
1	5.0	5.5	211 (0.62)	262 (0.37)		224 (0.46)		302 (0.58
2	5.9	6.9			305 (0.50)			335 (0.52)
3	11.2	12.8		241 (1.20)	308 (0.49)		247 (0.78)	339 (0.60
4	6.5	8.2		255 (0.66)	338 (0.33)		Unstable	
5	6.6	7.9		243 (0.80)	345 (0.47)		Unstable	
6	5.5	5.9	223 (>1.5)	265 (1.00)	299 (0.58)		Unstable	
7	3.6	4.1		254 (1.00)	315 (0.36)		Unstable	
8	5.5	5.8	232 (1.00)	266 (0.55)	297 (0.38)	232 (>1.5)		320 (0.97)
9	5.9	6.2	218 (1.20)	269 (0.61)	300 (0.68)	234 (1.5)	276 (0.35)	333 (0.30)
10	8.6	8.7	230 (>1.5)	265 (0.68)	295 (0.46)	230 (>1.5)	264 (0.68)	295 (0.46)
11	1.9	2.3	220 (>1.5)	278 (0.71)			Unstable	
12	6.1	6.2	221 (1.5)	282 (0.67)		221 (>1.5)	239 (1.20)	328 (1.5)
13	13.7	16.0	218 (0.86)	278 (1.10)		222 (0.97)	282 (1.20)	
14	9.3	9.6	229 (1.35)	287 (1.32)	320 (sh. 0.9)	239 (1.10)	307 (0.87)	360 (0.75)
15	10.1	10.3	238 (0.87)		328 (1.29)		250 (0.74)	372 (1.49)
16	10.5	10.9	248 (0.27)	305 (0.31)	333 (0.35)	269 (0.41)	311 (0.32)	374 (0.19)
17	13.5	14.3	242 (0.51)	300 (0.64)	325 (0.78)		290 (0.63)	314 (0.63)
18	15.5	17.0		290 (0.93)			278 (0.89)	
19	17.9	19.3	243 (0.63)	297 (0.80)	332 (0.10)	235 (0.71)	289 (0.83)	322 (0.86)
20	17.1	18.2		286 (0.91)	360 (0.35)		274 (0.82)	337 (0.32)
21	9.9	9.7	230 (1.5)	245 (0.40)	335 (0.43)	230 (>1.5)	260 (0.70)	395 (0.71)
22	4.7	4.5	215 (>1.5)		310 (0.8)		Unstable	

* For structures and names of the compounds, see Tables I and II.

** For HPLC conditions and column types, see Experimental. $k' = (t_R - t_0)/t_0$; $t_0 = 6.5$ mm.

*** The absorbance values (relative E) indicate the relative intensities of the different peaks in the spectra (see ref. 1).

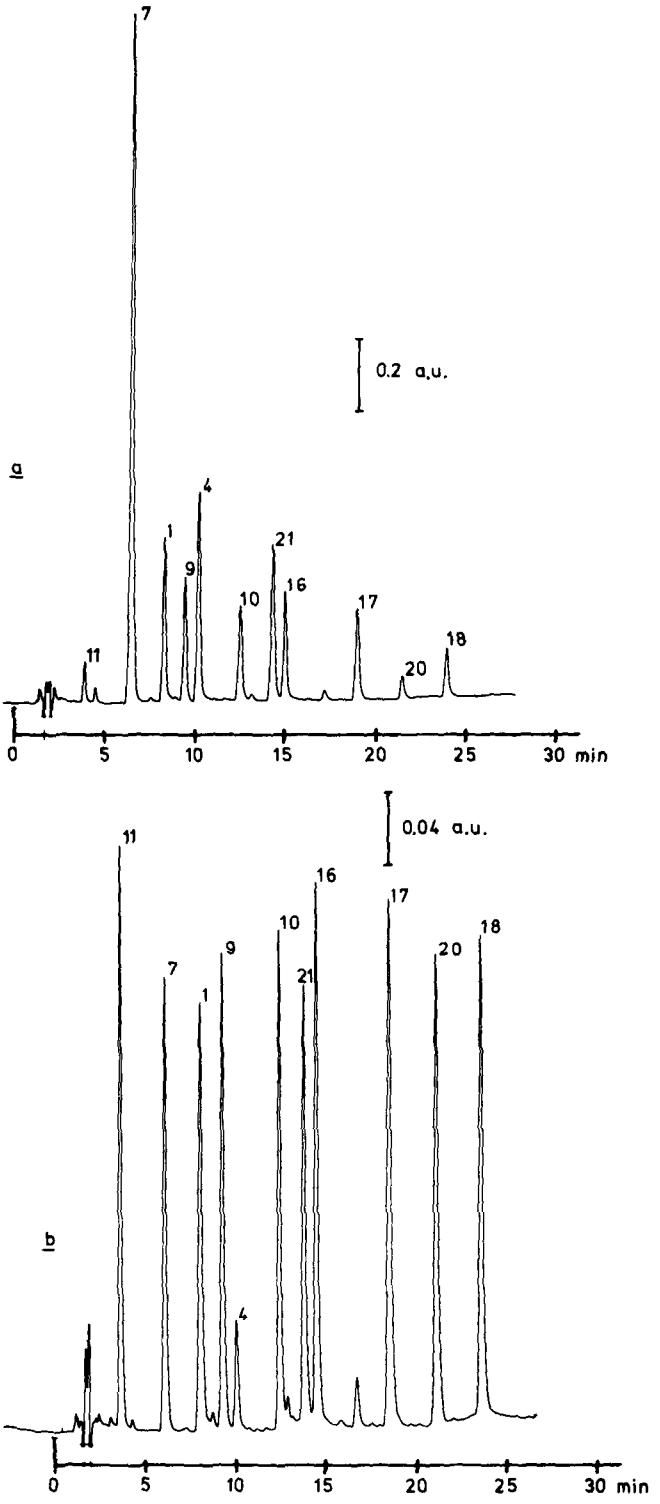


Fig. 1.

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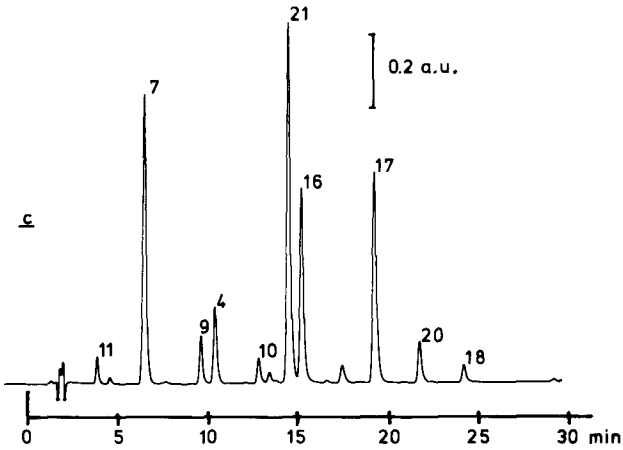

Fig. 1. Chromatogram of aromatic choline esters. Peak numbers correspond to those used for the compounds in Tables I and II. Support, Nucleosil 5 C_8; column, 120 × 4.6 mm I.D. Mobile phase, gradient system and flow as described under Experimental. Detection wavelength: (a) 254 nm; (b) 280 nm; (c) 313 nm.

lution of the choline esters. Reasonable results were obtained by use of the Nucleosil 5 C_{18} column, but better peak symmetry and resolution were obtained by use of Nucleosil 5 C_8 as the stationary phase. The values of k' obtained are given in Table III.

Fig. 1 shows the chromatograms of an artificial mixture of aromatic choline esters separated on the Nucleosil 5 C_8 column. Different wavelengths of detection were used, which resulted in differences between the relative intensities of the peaks corresponding to the differences in the UV spectra of the individual compounds (see above). The trend in the separation was similar to that obtained by use of the C_{18} column. However, minor differences were found for some of the compounds, as revealed from comparison of the k' values shown in Table III.

Optimal conditions for separation depend on which of the structurally closely related compounds the mixture consists of. Several different aromatic choline esters are to be expected in the total mixture of choline esters isolated from natural sources[1–3]. Detection at different wavelengths is helpful, as are the possibilities of choice between different columns. Further, as discussed previously for reversed-phase ion-pair HPLC, counter-ion and modifier concentrations affect the separations obtainable[12].

CONCLUSION

The HPLC method described here permits a rapid separation and determination of individual aromatic choline esters. Group separation of biological samples prior to the HPLC determination results in a more reliable analysis as it eliminates co-extractable compounds which otherwise are able to interfere[3]. Combination of these methods provides a gentle, highly sensitive, rapid and reproducible method for the determination of individual aromatic choline esters.

ACKNOWLEDGEMENTS

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