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SEPARATION OF SOME METABOLICALLY IMPORTANT AROMATIC N-ACYLAMINO ACIDS OF THE BENZOYL AND CINNAMOYL SERIES BY THIN-LAYER, GAS–LIQUID AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separations of 30 different N-acylamino acids and peptides from the benzoyl and cinnamoyl series have been studied by thin-layer (TLC), gas–liquid (GLC) and high-performance liquid chromatography (HPLC). TLC separations on three different layers and with four different solvent systems are described. GLC separations of the trimethylsilyl derivatives of some of the compounds were carried out on a glass column packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 + 1.5% SE-52 and using a temperature program. HPLC separations on a reversed-phase column (LiChrosorb RP-18, 10 μm, Knauer) employed a combination of isocratic and a linear gradient elution [solvent A, water–formic acid (95:5 v/v); solvent B, methanol; 35°C]. The few compounds which could not be separated by the latter system were separated on a second column (LiChrosorb Si 60, 7 μm, Knauer) by means of a combination of isocratic and linear gradient elution [solvent A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v); solvent B, methanol; 30°C]. These methods can easily be combined, and allow the separation of N-acylamino acids and N-acylpeptides from biological fluids, extracts and partial hydrolysates from phenolic acid-containing plant proteins.

INTRODUCTION

In addition to N-benzoylglycine (hippuric acid), which is normally present in urine as a metabolite of dietary components, several other N-acylglycines of the substituted benzoyl series (*viz.*, *o*-, *p*- and *m*-hydroxybenzoylglycine, vanilloylglycine and isovanilloylglycine, etc.[1–10]), the related phenylacetyl series (*viz.*, N-phenylacetyl-

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glutamine[11,12] and N-phenylacetylglutamic acid[13]) and the cinnamoyl series (*viz.*, N-cinnamoylglycine[13], N-*p*-hydroxycinnamoylglycine and N-feruloylglycine[1,2,7]) have been reported to occur in urine. Exposure of toluene or *p*- or *m*-xylene to man resulted in the production of *p*- and *m*-methylhippuric acid[14–16], while ingestion of L-DOPA gave rise to homovanilloylglycine[17].

Aliphatic N-acylamino acids may also occur in urine. Recently, N^{δ}-acetylornithine, a substance that has frequently been observed in plants[18], was identified as a minor component of both urine and deproteinized human or bovine blood[19]. Abnormal excretions of certain aliphatic N-acylglycine conjugates have been found in a number of organic acidurias[10,20,21], in a patient with D-glyceric acidimia[22] as well as in several other diseases[10], and according to the latter authors the discovery of new organic acidurias may well be promoted by the identification of "new" acylglycines.

Besides urine and blood plasma, other biological fluids and materials may also contain N-acylamino acids. In this context it should be mentioned that N-phenylacetylglutamine has been detected in bovine milk[23], while N-benzoylglutamic acid has been identified as a metabolite of benzoic acid in Indian fruit bats[24].

Conjugation of aromatic carboxylic acids can also occur in plants, because N-feruloylglycine has been identified as a building stone of barley and Medicago sativa proteins[25–27]. An analogous N-acylamino acid of the cinnamoyl series, *viz.*, N-*p*-coumaroylglutamic acid, has been reported to occur in black tea[28], and two aromatic amides of aspartic acid, namely N-benzoylaspartate and its homologue N-phenylacetylaspartate have been isolated from pea seeds[29].

It is therefore possible that certain aromatic N-acylamino acids may be of importance in the metabolism of phenolic acids and related compounds. N-acylamino acids such as N-benzoyl-L-leucine and N-phenylacetyl-L-leucine inhibit the growth of several plant pathogens[30], while N-benzoylaspartic acid reduces the relative germination rate of rice seeds[31]. It seems thus that N-acylamino acids (possibly also N-acylpeptides), especially those of the benzoyl, phenylacetyl and cinnamoyl series, are of great biological interest.

Unfortunately, with the exception of hippuric acid and its derivatives, little information on the chromatography of these compounds (especially those of the cinnamoyl series) is available. Usually, N-acylglycines and related substances (*e.g.*, hippuric acid and its derivatives) have been analysed by means of paper chromatography (PC) or thin-layer chromatography (TLC)[21,32–37]. There are few reports of the separation of these substances (with the exception of hippuric acid) by gas–liquid chromatography (GLC)[8–13,38,39] or by high-performance liquid chromatography (HPLC)[40–44].

For the above reasons, several N-acylamino acids and peptides of the benzoyl and cinnamoyl series have been synthesized[45–50] and studied. In this paper the TLC, GLC and HPLC separation and detection of some of these compounds will be described.

MATERIALS AND METHODS

With the exception of N-benzoylglycine (hippuric acid) which was purchased from Aldrich, (Beerse, Belgium), all other compounds were synthesized. N-*o*-, *m*- and *p*-hydroxybenzoyl-, N-vanilloyl-, N-syringoyl-, N-cinnamoyl-, N-*p*-coumaroyl-, N-

feruloyl- and N-sinapoylglycine and N-feruloyl-L-alanine were prepared from the corresponding N-hydroxysuccinimide esters[49]. N-Protocatechuoyl- and N-caffeoylglycine as well as N-caffeoylglycyl-L-phenylalanine were obtained from the corresponding N-(O-ethoxycarbonyl) derivatives using the same active ester method, the protecting O-ethoxycarbonyl groups being removed with hydrazine[50]. N-*p*-Hydroxybenzoyl-L-alanine, N-cinnamoyl- and N-*p*-coumaroyl-DL-alanine, N-*o*-coumaroyl-, N-*p*-coumaroyl- and N-feruloyl-DL-aspartic acid, as well as N-feruloyl-L-phenylalanine, N-cinnamoylglycyl-L-phenylalanine, N-*p*-coumaroylglycyl-L-phenylalanine and N-feruloylglycyl-L-phenylalanine were prepared by means of the acid chloride method[47]. N-*p*-Coumaroyl- and N-feruloylsarcosine were obtained analogously. N-Cinnamoyl-, N-*p*-coumaroyl- and N-feruloyl-L-methionine as well as N-feruloyl-L-phenylalanyl-L-phenylalanine were synthesized via the mixed anhydrides as described by De Pooter *et al.*[46].

MN cellulose powder 300 (average particle size *ca.* 10 μm) and N-methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) were purchased from Macherey, Nagel & Co. (Düren, G.F.R.). Silica gel G and pre-coated TLC plates (silica gel 60F-254) were obtained from E. Merck (Darmstadt, G.F.R.), and Chromosorb W AW DMCS (80–100 mesh), SE-30 and SE-52 from Varian Aerograph. The analytical HPLC column (250 × 4.6 mm I.D.), containing LiChrosorb RP-18 (10 μm) and the semi-preparative LiChrosorb Si 60 (7 μm, 250 × 8 mm I.D.) were obtained from Dr. H. Knauer (Wissenschaftliche Geräte, Oberursel/Taunus, G.F.R.). The solvents used for HPLC were formic acid (suprapur) and methanol (LiChrosolv for chromatography) (E. Merck). "Baker analysed HPLC reagent water" was obtained from Baker Chemicals (Deventer, The Netherlands).

TLC

Preparation of the thin layers. For the preparation of cellulose plates, MN cellulose powder 300 was used. The silica gel–cellulose layers were prepared as described previously[51]. In certain experiments the internal water phase of the plates was increased by keeping the plates, after spotting, but before irrigation, in an atmosphere saturated with water for 20 min[52] or by steaming[51]. However, this procedure, which gives excellent results with phenols, phenolic acids, coumarins, etc., did not improve the chromatography of the N-acylamino acids and peptides.

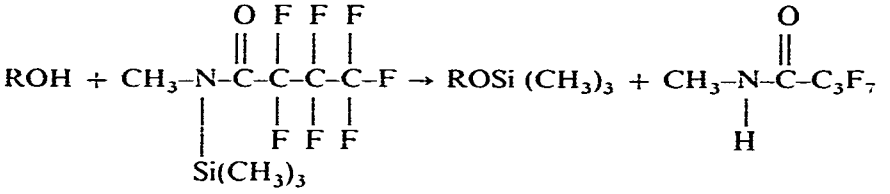
Chromatography. The compounds were dissolved in methanol–water (1:1 v/v) and 10–20 μg were applied on the thin layers. The following solvents were employed at room temperature: toluene–ethyl formate–formic acid (5:4:1) (TEF); *sec.*-butanol–water (4:1); 2% acetic acid and ethyl methyl ketone–pyridine–water–acetic acid (70:15:15:2) (EPWA).

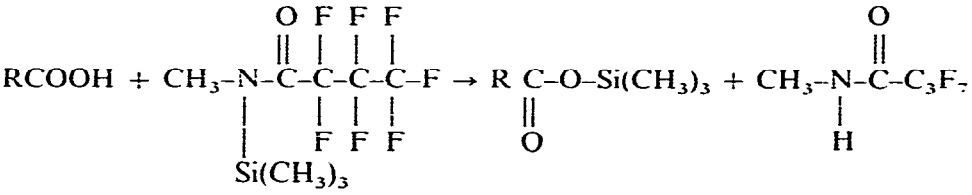
Detection. The compounds were detected by examination of the dried chromatograms under UV light (360 nm) before and after spraying the thin layers with 2 *M* sodium hydroxide. Thereafter the compounds were revealed by spraying with diazotized *p*-nitroaniline[51], treatment with Gaffney's reagent[32,34] or with 1% $KMnO_4$ in 0.1 *M* H_2SO_4.

GLC

Trimethylsilylation. Each component (± 0.4 mg) of a mixture was weighed. Subsequently, 400 μl MSHFBA were added and the reaction mixture was kept for 10

min at 125°C in a sealed vial. The possible reactions of the phenolic and acidic functions with MSHFBA are as follows:





Aliquots (20 μl) of the reaction mixture were injected directly in the gas–liquid chromatograph.

Chromatography. Analyses were performed on a Hewlett-Packard gas chromatograph 5730A equipped with glass columns and a thermal conductivity detector (temperature 350 C, filament current 150 mA). The glass column (3.0 m × 2 mm I.D.) was packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 plus 1.5% SE-52[53]. The carrier gas (helium) flow-rate was 30 ml/min. The injector port temperature was 300°C, and the column temperature was programmed at 8 C/min from 200 C to 310 C. The recorder (0–1.0 mV) chart speed was 1 cm/min. Attenuation:1.

HPLC

A Hewlett-Packard liquid chromatograph 1084B equipped with a Pye Unicam variable wavelength LC 3 UV-detector set at 280 nm was used throughout this work. Two different types of columns were employed. (a) An analytical LiChrosorb RP-18 (10 μm) prepacked column (250 × 4.6 mm I.D.). The eluent (gradient elution) and chromatographic conditions were: solvent A, formic acid–water (5:95, v/v); solvent B, methanol; gradient profile, linear; gradient range, 0–2 min, isocratic 7% B (% B in A), 2–8 min, 7–15% B, 8–25 min, 15–75% B, 25–27 min, 75–80% B, 27–29 min, isocratic 80% B; flow-rate 2.5 ml/min; oven and eluent temperatures 35°C; column pressure 80–100 bar; attenuation $6.4 \cdot 10^{-3}$ absorbance units per cm and recorder (0–1.0 mV) chart speed 0.5 cm/min. (b) A semi-preparative LiChrosorb Si 60 (7 μm) prepacked column (250 × 8 mm I.D.). Conditions: solvent A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v); solvent B, methanol; gradient profile, linear; gradient range, 0–5 min, isocratic 2% B (% B in A), 5–20 min, 2–17% B, 20–28 min, 17–35% B, 28–33 min, 35–60% B; flow-rate 4 ml/min; oven and eluent temperatures 30°C; column pressure and attenuation as above.

The N-acylamino acids and peptides were dissolved in methanol–water (1:1, v/v) and on both columns the sample size was 20 μl (loop-valve), 1–5 μg of each of the substances being injected.

RESULTS AND DISCUSSION

Table I shows the R_F values of 30 aromatic N-acylamino acids of the benzoyl and cinnamoyl series on cellulose (A), silica gel–cellulose (B) and precoated silica gel plates (C) (E. Merck), developed with TEF, *sec.*-butanol–water, 2% acetic acid or EPWA. Table II shows the fluorescence (UV light 360 nm) of the compounds before and after NaOH treatment as well as the colour reaction with diazotized *p*-nitroaniline. Also shown is the colour reaction of the compounds with either Gaffney's reagent[32] or 1% potassium permanganate in 0.1 *M* sulphuric acid. The colours produced were further standardized and matched against the "Derwent Colour pencils, series No. 19 (Cumberland Pencil Ltd., Keswick, Great Britain).

Table I shows that the R_F values of the compounds are generally higher on cellulose plates than on silica gel–cellulose and pre-coated silica gel layers, the values on these last two types of layers being relatively small. *sec.*-Butanol–water produces higher R_F values on cellulose layers than TEF, especially for compounds such as N-protocatechuoylglycine, N-caffeoylglycine and N-*p*-coumaroyl-DL-aspartic acid. The differences in R_F values obtained with the former solvent on silica gel–cellulose layers and precoated silica gel layers were less clear-cut. In the case of 2% acetic acid, only the R_F values on cellulose and silica gel–cellulose layers are given because separation of the compounds on pre-coated silica gel layers proved to be unsatisfactory. Finally, with EPWA the R_F values of the N-acylamino acids and peptides were again high, especially on cellulose layers. On the latter with TEF as solvent, the glycine derivatives separated according to the polarity and type of substitution of the phenolic moiety. The same holds true when compounds with the same acyl groups but different amino acid or peptide moieties are considered (*e.g.*, N-*p*-coumaroylglycine, N-*p*-coumaroylalanine, etc.).

Two-dimensional TLC of the compounds can be performed on cellulose or silica gel–cellulose layers with TEF and EPWA as solvents. Furthermore it should be noted that irrigation with 2% acetic acid gives rise to a separation of the pre-existing *cis* and *trans* isomers. This last effect can be prevented when the synthesis, spotting and chromatography are performed under an orange ICI Perspex filter No. 300.

As can be seen from Table II most of the substances were fluorescent at 360 nm, and with the exception of the cinnamoyl derivatives all compounds also showed a more or less pronounced colour reaction with diazotized *p*-nitroaniline. Some of the N-acylamino acids showed a further reaction with Gaffney's reagent, and all the compounds could be revealed with 1% $KMnO_4$ in 0.1 *M* H_2SO_4. GLC separations, especially GLC–mass spectrometry (MS) combinations, could also play a key rôle in the analysis of aromatic N-acylamino acids from the benzoyl or cinnamoyl series (see refs. 9–12, 14 and 39). Indeed, after trimethylsilylation and preparative GLC, N-feruloylglycine could be collected as a TMS derivative by means of a technique described by Vande Casteele *et al.*[53]. Overnight hydrolysis of the TMS compound with a drop of water and subsequent TLC showed that the GLC–TLC combination could be used for the purification and (or) identification of ferulic acid derivatives from complex mixtures.

Unfortunately, as shown in Fig. 1 and Table III, not all of the compounds studied could easily be derivatized and chromatographed. Moreover, the TMS com-

TABLE I

R_F VALUES OF THE COMPOUNDS STUDIED ON CELLULOSE (A), SILICA GEL CELLULOSE (B) AND PRECOATED SILICA GEL PLATES (C) (E. MERCK)

Compound	*Toluene–ethyl formate–formic acid (5:4:1)*			*sec.-Butanol–water (4:1)*			*2% Acetic acid*		*Ethyl methyl ketone–pyridine–water–acetic acid (70:15:15:2)*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*A*	*B*	*C*
N-Benzoylglycine (hippuric acid)	0.54	0.37	0.25	0.86	0.43	0.31	0.93	0.92	0.77	0.57	0.66
N-*o*-Hydroxybenzoylglycine	0.51	0.38	0.33	0.69	0.35	0.36	0.76	0.84	0.90	0.76	0.64
N-*p*-Hydroxybenzoylglycine	0.25	0.17	0.14	0.73	0.18	0.33	0.76	0.91	0.69	0.58	0.61
N-*m*-Hydroxybenzoylglycine	0.23	0.18	0.15	0.68	0.29	0.33	0.86	0.91	0.73	0.64	0.63
N-Protocatechuoylglycine	0.05	0.06	0.09	0.53	0.15	0.29	0.67	0.83	1.00	0.64	0.42
N-Vanilloylglycine	0.30	0.22	0.16	0.60	0.22	0.28	0.75	0.84	0.66	0.58	0.60
N-Syringoylglycine	0.20	0.17	0.11	0.62	0.17	0.25	0.70	0.80	–	–	0.53
N-Cinnamoylglycine	0.60	0.43	0.28	1.00	0.36	0.33	0.66	0.72	0.83	0.62	0.67
N-*p*-Coumaroylglycine	0.27	0.21	0.17	0.70	0.28	0.37	0.49	0.67	0.77	0.64	0.63
N-Caffeoylglycine	0.08	0.09	0.10	0.58	0.18	0.32	0.38	0.46	0.59	0.53	0.57
N-Feruloylglycine	0.28	0.27	0.18	0.59	0.21	0.34	0.37*/0.64**	0.39*/0.64**	0.65	0.55	0.60
N-Sinapoylglycine	0.24	0.20	0.12	0.50	0.13	0.33	0.40*/0.79**	0.47*/0.77**	0.61	0.47	0.54

N-*p*-Coumaroylsarcosine	0.60	0.31	0.19	0.93	0.33	0.29	0.23*/0.98**	0.16*/0.90**	0.96	0.72	0.65
N-Feruloylsarcosine	0.66	0.34	0.20	0.84	0.24	0.25	0.58*/0.90**	0.77*/0.90**	0.93	0.68	0.60
N-*p*-Hydroxybenzoyl-L-alanine	0.44	0.57	0.43	--	0.93	0.77	0.86	0.75	1.00	0.78	1.00
N-Cinnamoyl-DL-alanine	0.71	0.42	0.33	0.92	0.34	0.38	0.72	0.57	1.00	0.84	0.77
N-*p*-Coumaroyl-DL-alanine	0.59	0.36	0.22	0.97	0.40	0.39	0.59	0.76	0.97	0.82	0.78
N-Feruloyl-L-alanine	0.64	0.39	0.23	0.94	0.34	0.33	0.53	0.76	0.96	0.79	0.74
N-Cinnamoyl-L-methionine	0.78	0.52	0.38	1.00	0.42	0.42	0.65	0.63	1.00	0.82	0.84
N-*p*-Coumaroyl-L-methionine	0.53	0.39	0.28	0.93	0.47	0.45	0.64*/0.94**	0.79*/0.98**	0.79	0.86	0.80
N-Feruloyl-L-methionine	0.54	0.44	0.31	0.88	0.37	0.40	0.87	0.59	1.00	0.79	0.80
N-*o*-Coumaroyl-DL-aspartic acid	0.25	0.19	0.51	0.97	0.76	0.66	0.67*/0.97**	0.37*/0.82**	0.86	0.67	0.58
N-*p*-Coumaroyl-DL-aspartic acid	0.12	0.14	0.12	0.83	0.16	0.22	0.65	0.91	0.91	0.67	0.55
N-Feruloyl-DL-aspartic acid	0.08	0.17	0.12	0.77	0.12	0.23	0.56	0.77	0.83	0.60	0.51
N-Feruloyl-L-phenylalanine	0.83	0.50	0.33	0.96	0.43	0.43	0.26	0.37	0.98	0.89	0.77
N-Cinnamoylglycyl-L-phenylalanine	0.72	0.42	0.28	1.00	0.39	0.38	0.50	0.57	1.00	0.68	0.77
N-*p*-Coumaroylglycyl-L-phenylalanine	0.48	0.31	0.19	0.93	0.45	0.36	0.55*/0.79**	0.72*/0.79**	0.94	0.74	0.72
N-Caffeoylglycyl-L-phenylalanine	0.28	0.23	0.13	0.79	0.35	0.38	0.46	0.61	0.88	0.64	0.70
N-Feruloylglycyl-L-phenylalanine	0.51	0.36	0.20	0.85	0.36	0.34	0.43	0.53	0.93	0.66	0.66
N-Feruloyl-L-phenylalanyl-L-phenylalanine	0.89	0.53	0.35	0.95	0.54	0.48	0.07	0.62	1.00	0.93	0.88

* *trans* compound.
** *cis* compound.

TABLE II

APPEARANCE OF THE COMPOUNDS STUDIED IN UV LIGHT AND AFTER SPRAYING WITH ONE OF THE CHROMOGENIC REAGENTS

With the exception of N-benzoylglycine (hippuric acid), all the compounds tested showed on cellulose or silica gel–cellulose plates a primrose yellow colour reaction with 1 % $KMnO_4$ in 0.1 *M* H_2SO_4. On the pre-coated silica gel 60F-254 (Merck) plates, however, a zinc yellow colour was obtained with the same spray.

Compound	*Fluorescence (360 nm) before NaOH*		*Fluorescence (360 nm) after NaOH*		*Colour reaction with diazotized p-nitroaniline*		*Colour reaction with Gaffney's reagent*	
	Cellulose or silica gel cellulose plates	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*
N-Benzoylglycine (hippuric acid)	*	*	*	Blue-violet lake	Chinese white	Chinese white	Orange chrome	Deep chrome
N-*o*-Hydroxybenzoylglycine	Spectrum blue	Cobalt blue	Spectrum blue	Turquoise blue	Deep vermilion	Venetian red	Orange chrome	Spectrum orange
N-*p*-Hydroxybenzoylglycine	*	Blue-violet lake	*	Blue-violet lake	**	Deep vermilion	Orange chrome	Deep chrome
N-*m*-Hydroxybenzoylglycine	*	Blue-violet lake	Spectrum blue	Raw sienna	Crimson lake	Terra cotta	Orange chrome	Deep chrome
N-Protocatechuoylglycine	Dark violet	Raw sienna	Sky blue	Dark violet	French grey	Golden brown	French grey	**
N-Vanilloylglycine	Delft blue	Burnt carmine	Sky blue	Dark violet	Imperial purple-light violet	Burnt Carmine	Deep chrome	Spectrum orange
N-Syringoylglycine	Smalt blue	Middle chrome	Oriental blue	Raw sienna	Raw sienna	Straw yellow	**	**
N-Cinnamoylglycine	*	*	*	Blue-violet lake	**	Chinese white	Spectrum orange	Scarlet lake
N-*p*-Coumaroylglycine	Delft blue	Blue-violet lake	Blue-violet lake	Jade green	Dark violet	Raw umber	Orange chrome	Spectrum orange
N-Caffeoylglycine	Turquoise blue	Lemon cadmium	Lemon cadmium	Venetian red	Raw sienna	Raw umber	Raw umber	Spectrum orange
N-Feruloylglycine	Blue	Water green	Turquoise blue	Grass green	Prussian blue	Sepia	Orange chrome	Spectrum orange
N-Sinapoylglycine	Turquoise blue	May green	Water green	Grass green	Water green	Burnt yellow ochre	Orange chrome	Spectrum orange

N-*p*-Coumaroylsarcosine	Blue-violet lake	Blue-violet lake	Light blue	Jade green	Dark violet	Burnt carmine	Light violet	Indigo
N-Feruloylsarcosine	Smalt blue	Gold	Water green	May green	Bottle green	Red-violet lake	Light violet	Indigo
N-*p*-Hydroxybenzoyl-L-alanine	*	Blue-violet lake	*	Blue-violet lake	Madder carmine	Rose pink	**	**
N-Cinnamoyl-DL-alanine	*	*	*	*Blue-violet lake*	*Chinese white*	Chinese white	Orange chrome	**
N-*p*-Coumaroyl-DL-alanine	Blue-violet lake	Blue-violet lake	Light blue	Light blue	Gunmetal	Raw umber	**	**
N-Feruloyl-L-alanine	Spectrum blue	Water green	Turquoise green	Cobalt blue	Dark violet	French grey	**	**
NCinnamoyl-L-methionine	*	*	*	Blue-violet lake	**	Chinese white	**	**
N-*p*-Coumaroyl-L-methionine	Delft blue	Blue violet lake	Light blue	Venetian red	Dark violet	Raw umber	**	**
N-Feruloyl-L-methionine	*Light blue*	Water green	Turquoise green	Jade green	Delft blue	Gunmetal	**	**
N-*o*-Coumaroyl-DL-aspartic acid	Primrose yellow	Flesh pink	Lemon cadmium	Lemon cadmium	Red-violet blue	Madder carmine	**	**
N-*p*-Coumaroyl-DL-aspartic acid	Blue-violet lake	Blue-violet lake	Light blue	Kingfisher blue	Gunmetal	Raw umber	**	**
N-Feruloyl-DL-aspartic acid	Light blue	Water green	Turquoise green	Grass green	Bottle green	Sepia	**	**
N-Feruloyl-L-phenylalanine	Spectrum blue	Water green	Turquoise green	May green	Blue-grey	Blue-grey	**	**
N-Cinnamoylglycyl-L-phenyl-alanine	*	*	*	Blue-violet lake	**	Chinese white	**	**
N-*p*-Coumaroylglycyl-L-phenyl-alanine	Delft blue	Blue-violet lake	Light blue	May green	Dark violet	Raw umber	**	**
N-Caffeoylglycyl-L-phenyl-alanine	Light blue	Lemon cadmium	Raw sienna	Grass green	Raw umber	Burnt yellow ochre	Middle chrome	Middle chrome
N-Feruloylglycyl-L-phenyl-alanine	Light blue	Water green	Turquoise green	May green	Delft blue	Gunmetal	**	**
N-Feruloyl-L-phenylalanyl-L-phenylalanine	Smalt blue	Water green	Grass green	May green	Blue-grey	Blue-grey	**	**

* No fluorescence.
** No colour reaction.

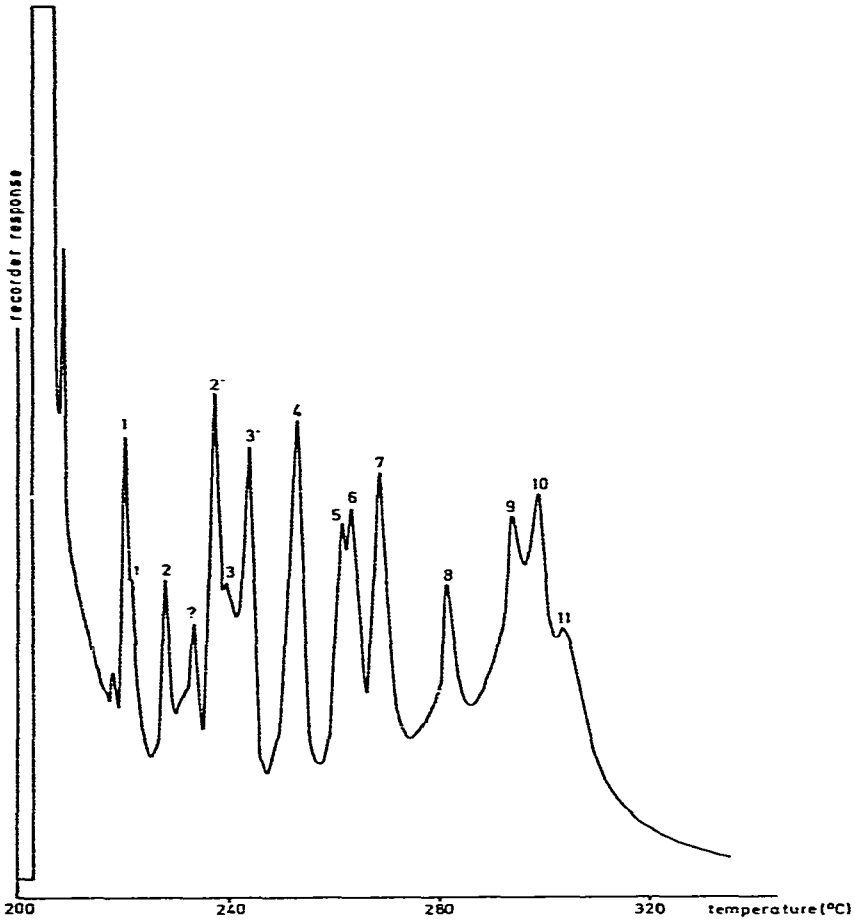


Fig. 1. Gas chromatogram of the TMS derivatives of some benzoyl and cinnamoyl amino acids. For peak identities see Table III.

pounds were very unstable, and could only be kept in the dark at room temperature for 24 h. In addition, most of these derivatives were extremely labile during GLC (stability: maximum of 15 min at higher temperatures) and subsequently the duration of the chromatography proved to be a limiting factor. The best results were obtained with an oven temperature of 200–310°C and a temperature program of 8°C/min. With a lower oven temperature (150°C) and (or) a program of 4°C/min the compounds could not be eluted, while an increase of the oven temperature at 16°C/min gave very poor separations. Thus, the GLC of these compounds is as yet not completely satisfactory. It should also be mentioned that certain of the substances gave rise to multiple peaks. This is a well known problem with N-acylglycines[10] and as indicated previously[5,4], single peaks (di- or tri-TMS) could not always be obtained by using longer reaction times.

For the above reasons, the complexity of the chromatograms containing N-acylglycines could not be reduced and although several silylating reagents and dif-

TABLE III

RELATIVE RETENTION TIMES AND COLUMN TEMPERATURES OF THE TMS DERIVATIVES OF SOME N-ACYLAMINO ACIDS

Retention time of N-cinnamoylglycine (5 min 27 sec) = 1.00.

Compound	*Relative retention*	*Column temperature (°C)*
1 Hippuric acid (N-benzoylglycine)	0.47	220
1′ Hippuric acid	0.50	222
2 *o*-Hydroxyhippuric acid	0.64	228
2′ *o*-Hydroxyhippuric acid	0.85	238
3 *m*-Hydroxyhippuric acid	0.91	240
3′ N-Cinnamoylglycine + *m*-hydroxyhippuric acid	1.00	244
4 *p*-Hydroxyhippuric acid	1.21	253
5 4-Hydroxy-3-methoxyhippuric acid	1.41	262
6 4-Hydroxy-3,5-dimethoxyhippuric acid	1.45	264
7 N-*p*-Coumaroylglycine, N-cinnamoyl-L-methionine	1.58	269
8 N-Feruloylglycine, N-caffeoylglycine	1.87	282
9 N-Sinapoylglycine	2.16	294
10 N-*p*-Coumaroyl-L-methionine	2.27	299
11 N-Feruloyl-L-methionine	2.38	304

ferent reaction conditions have been investigated, more research on the GLC separation of these compounds is required.

Since HPLC is more rapid and also much simpler than GLC, it has also been used for the separation of N-acylamino acids and peptides. HPLC techniques for hippuric acid and its derivatives are well known[15,16,42,44,55], but the separation of the present compounds has so far not been described. HPLC has the advantages that it does not require derivatization of the compounds, and when good separations can be obtained, a rapid quantitation of the substances is also possible.

For the HPLC separation of the N-acylamino acids of the benzoyl and cinnamoyl series, two different columns and eluting systems have been used (see Materials and Methods). The results obtained with the LiChrosorb RP-18 column, developed with a combination of isocratic and linear gradient elution [solvent A, water–formic acid (95:5, v/v) solvent B, methanol], were excellent because most of the 30 compounds investigated (with the exception of N-feruloylglycine and N-*o*-coumaroylaspartic acid; N-cinnamoyl-DL-alanine, N-*p*-coumaroyl-L-methionine and N-caffeoylglycyl-L-phenylalanine) could be separated (see Fig. 2 and Table IV).

The following conclusions can be drawn:

(a) Compounds with identical acyl groups but different amino acid or peptide moieties are eluted according to the polarity of the amino acid or peptide moiety. The retention times are in the order: aspartic acid derivatives < glycine derivatives < sarcosine derivatives ≈ alanine derivatives < methionine derivatives < glycyl-

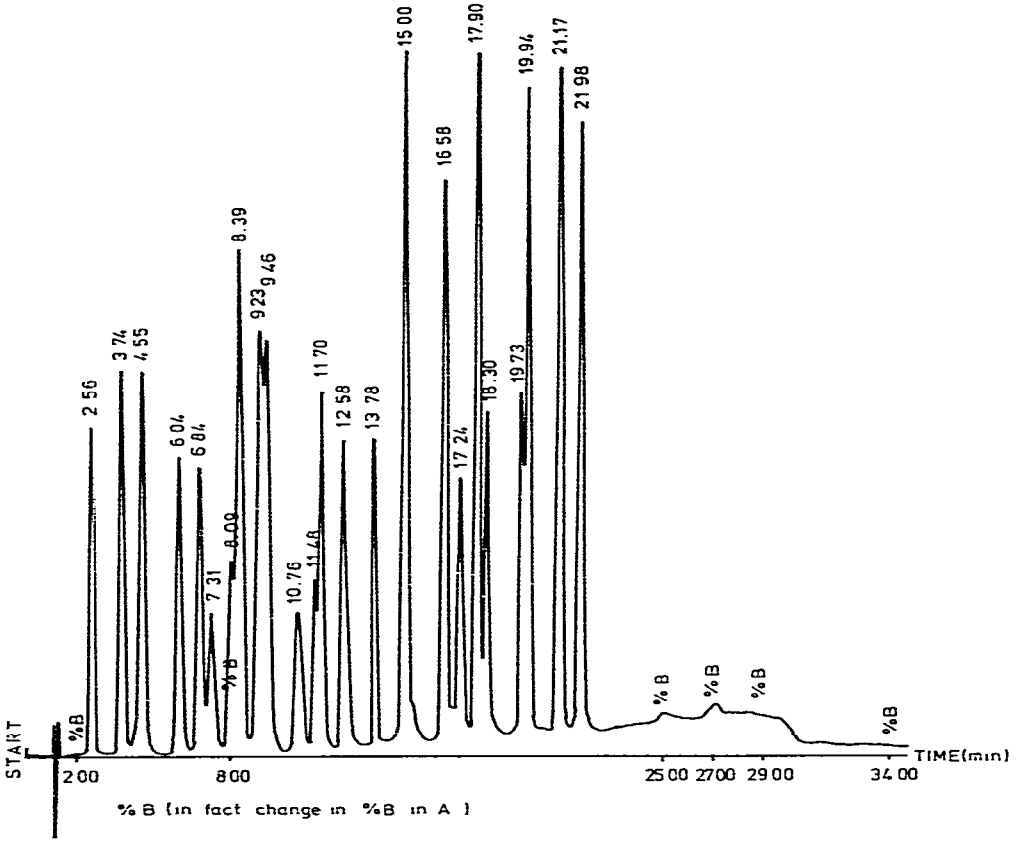


Fig. 2. Separation of N-acylamino acids and peptides of the benzoyl and cinnamoyl series on an analytical RP-18 column. For the eluting system see Materials and methods.

phenylalanine derivatives < phenylalanine derivatives < phenylalanylphenylalanine derivatives

(b) For compounds with the same amino acid or peptide moiety and the same substitution pattern of the acyl group, the order of retention is: N-benzoyl compounds < N-cinnamoyl compounds

(c) For compounds with acyl groups of the benzoyl type but different substitution patterns, the order of retention is: di-OH < *p*-OH < *m*-OH < -OH + $-OCH_3$ < *o*-OH < unsubstituted ring < -OH + di-OCH_3

(d) For compounds with acyl groups of the cinnamoyl type but different substitution patterns, the order of retention is: di-OH < *p*-OH < -OH + $-OCH_3$ < *o*-OH < -OH + di-OCH_3 < unsubstituted ring

Since certain of the above compounds could not be separated on the RP-18 column, a second system [LiChrosorb Si 60 (7 μm) (semi-preparative column); combination of isocratic and gradient elution with solvents A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v) and B, methanol] has been investigated. With the latter system the separation of N-feruloylglycine and *o*-coumaroylaspartic acid, and of N-cinnamoyl-DL-alanine, N-*p*-coumaroyl-L-methionine and N-caffeoylglycyl-L-phenylalanine could be achieved (Fig. 3, Table V).

TABLE IV

RETENTION TIMES OF N-ACYLAMINO ACIDS AND PEPTIDES ON AN ANALYTICAL RP-18 COLUMN

For the eluting system see Materials and methods.

Compound	*Retention time (min)*
N-Protocatechuoylglycine	2.56
N-*p*-Hydroxybenzoylglycine	3.74
N-*m*-Hydroxybenzoylglycine	4.55
N-Vanilloylglycine	6.04
N-Caffeoylglycine	6.84
N-*p*-Hydroxybenzoyl-L-alanine	7.31
N-Benzoylglycine	8.09
N-Syringoylglycine	8.39
N-*p*-Coumaroyl-DL-aspartic acid	9.23
N-*p*-Coumaroylglycine	9.46
N-*o*-Hydroxybenzoylglycine	10.76
N-Feruloyl-DL-aspartic acid	11.48
N-Feruloylglycine	11.70
N-*o*-Coumaroyl-DL-aspartic acid	11.70
N-*p*-Coumaroylsarcosine + N-*p*-coumaroyl-DL-alanine + N-sinapoylglycine	12.58
N-Feruloylsarcosine + N-feruloyl-L-alanine	13.78
N-Cinnamoylglycine	15.00
N-Cinnamoyl-DL-alanine + N-*p*-coumaroyl-L-methionine + N-caffeoylglycyl-L-phenylalanine	16.58
N-Feruloyl-L-methionine	17.24
N-*p*-Coumaroylglycyl-L-phenylalanine	17.90
N-Feruloylglycyl-L-phenylalanine	18.30
N-Feruloyl-L-phenylalanine	19.73
N-Cinnamoyl-L-methionine	19.94
N-Cinnamoylglycyl-L-phenylalanine	21.17
N-Feruloyl-L-phenylalanyl-L-phenylalanine	21.98

TABLE V

RETENTION TIMES OF N-ACYLAMINO ACIDS AND PEPTIDES ON A SEMI-PREPARATIVE LICHROSORB SI 60 (7 μm) COLUMN

For the eluting system see Materials and methods.

Compound	*Retention time (min)*
N-Cinnamoyl-L-methionine	12.78
N-Feruloyl-L-phenylalanyl-L-phenylalanine	13.44
N-Feruloyl-L-phenylalanine	13.97
N-Feruloyl-L-methionine	14.78
N-*p*-Coumaroyl-L-methionine	16.61
N-Feruloylglycyl-L-phenylalanine + N-feruloylglycine	17.22
N-Vanilloylglycine	18.18
N-*p*-Coumaroylglycyl-L-phenylalanine	18.54
N-Feruloyl-DL-aspartic acid	19.09
N-Caffeoylglycyl-L-phenylalanine	19.67
N-Caffeoylglycine	20.02

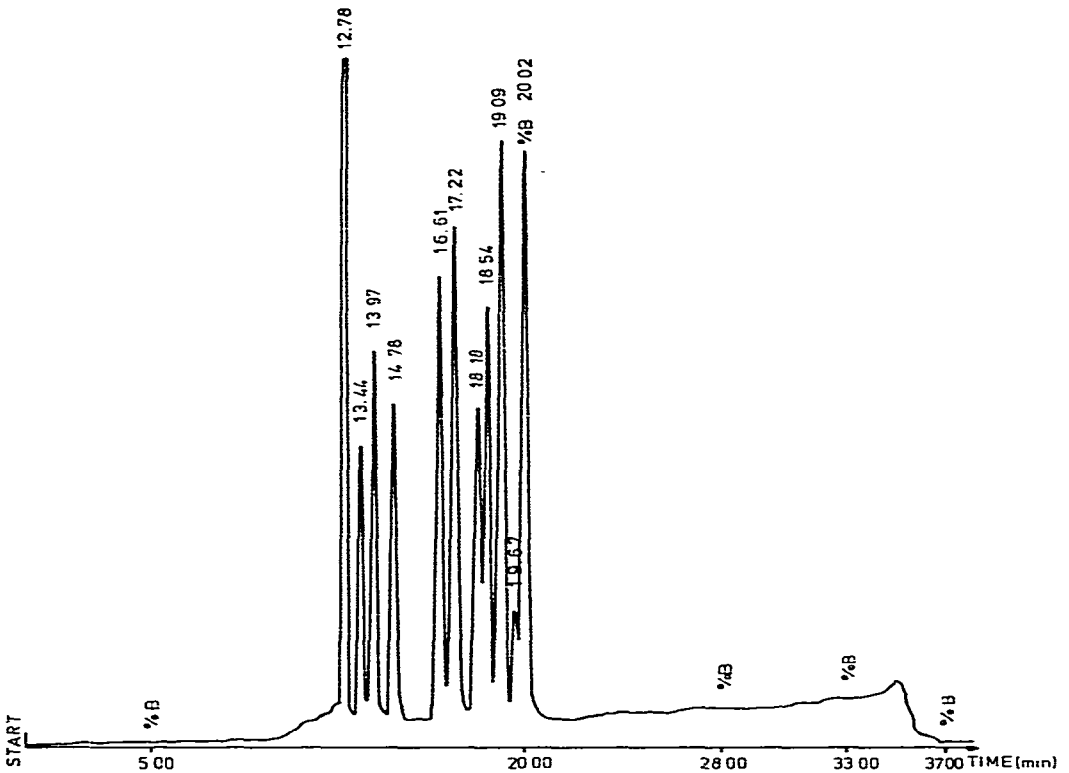


Fig. 3. Separation of N-acylamino acids and peptides of the benzoyl and cinnamoyl series on a semi-preparative LiChrosorb Si 60 (7 μm) column. For the eluting system see Materials and methods.

It can thus be concluded that with HPLC (possibly in combination with TLC or even, and where possible, TLC + GLC) all the N-acylamino acids and peptides studied can be separated. The methods described allow the separation and quantitation of such compounds in biological fluids and extracts, as well as in partial hydrolysates from plant proteins[27].

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