



ELSEVIER

Journal of Chromatography A, 715 (1995) 67–79

JOURNAL OF
CHROMATOGRAPHY A

Simultaneous determination of amino acids and biogenic amines by reversed-phase high-performance liquid chromatography of the dabsyl derivatives

Ingolf Krause*, Annette Bockhardt, Herbert Neckermann, Thomas Henle,
Henning Klostermeyer

*Forschungszentrum für Milch und Lebensmittel, Institut für Chemie und Physik, Technische Universität
München-Weihenstephan, Voettinger Str. 45, D-85350 Freising, Germany*

First received 13 February 1995; revised manuscript received 4 May 1995; accepted 15 May 1995

Abstract

An efficient, sensitive and reliable method for the simultaneous determination of dabsyl derivatives of numerous proteinogenic and physiological amino acids and biogenic amines in complex matrices by reversed-phase high-performance liquid chromatography (RP-HPLC) is described. A linear relation between peak area and concentration was observed from 1.25 to 1250 pmol and the detection limit was between 0.12 and 0.52 pmol. The average repeatability ranged between 1.3 and 3.1%, and the recovery values between 98 and 104%. An automated derivatization/injection unit clearly improved the performance of the method. Triethylamine was found to be a very effective additive to optimize separation efficiency. The method was successfully applied to the analysis of amino acids from protein hydrolyzates and of amino acid and biogenic amines of biological samples and food. More than 40 compounds could be separated simultaneously.

1. Introduction

After the introduction of dabsyl chloride as a pre-column derivatization reagent for amino acids [1] as well as primary and secondary amines [2], numerous applications have been published, most of them related to the analysis of amino acids in biological fluids or protein hydrolyzates by high-performance liquid chromatography (HPLC) [3–12,17]. Several advan-

tages of the dabsyl method over pre- or post-column derivatization with OPA, FMOC, PITC or fluorescamine were reported [6,13,14], e.g. dabsyl derivatives of primary and secondary amino acids are stable at room temperature and detection can be carried out in the visible region ($\lambda = 436\text{--}460\text{ nm}$) with high specificity and sensitivity at the picomole level [3–4,6].

Several modifications of the basic method of Chang et al. [3] were directed to optimize dabsylation and chromatographic separation [4–8,10,12]. The stability of the dabsyl derivatives, which is commonly described as being “perfect” up to 4 weeks at room temperature

* Corresponding author.

[5,8,14,16] or over one year at -20°C [16–18], has been questioned by Vendrell and Avilés [12]. Observations of these authors clearly indicated that peak-area values of certain amino acids (bis-dabsylated lysine, tyrosine and histidine) are not reproducible from one experiment to another if the standard conditions of Chang et al. [3] for derivatization are applied. Furthermore, a non-quantitative derivatization yield of several proteinogenic amino acids (aspartic acid, glutamic acid, serine, threonine, arginine) was found [5]. Vendrell and Avilés [12] proposed to increase the final concentration of dabsyl chloride and to adjust properly the concentration of organic solvent in the reaction mixture to overcome these problems.

Chromatographic separations of the dabsyl derivatives of selected physiological amino acids, decarboxylated amino acids (taurine, β -alanine, γ -amino butyric acid) and selected polyamines (putrescine, spermine, spermidine) as well as of phosphorylated amino acids were published by several authors [2,4,10,11,15].

In addition, the influence of chromatographic parameters such as pH, buffer composition, ionic strength, organic modifier, temperature, column length and type on the separation efficiency of certain amino acids has been described [5–7,9,12,17], but most of these parameters were only tested on the separation of the 18 hydrolyzate amino acids or selected additional compounds. Although several methods for the determination of biogenic amines in food or biological material have been reported—for a review see Refs. [19–21]—a simultaneous determination of biogenic amines and amino acids, including the precursors of biogenic amines, has not yet been published.

Our attempt was to develop a method for the simultaneous determination of proteinogenic and physiological amino acids as well as biogenic amines with special focus on derivatization yield and derivative stability, even in complex matrices as food or biological material, thus providing a flexible method for several fields of applications.

2. Experimental

2.1. Materials

Doubly recrystallized dabsyl chloride, triethylamine (sequencing grade) and 6 M HCl were from Pierce (Rockford, IL, USA), dimethylformamide and ANOTOP filter were obtained from Merck (Darmstadt, Germany). Ultrafiltration inserts (UFC, M_r cut-off: 5000) were from Millipore (Eschborn, Germany). Acetone and bis-2-carboxyethyl-sulfide (3,3'-thiodipropionic acid, TDPA) were from Fluka (Neu-Ulm, Germany) and acetonitrile was from Riedel-de-Haen (Seelze, Germany). Amino acid standard kits were obtained from Pierce or Sigma (Deisenhofen, Germany). Additional amino acid and amine standards of highest purity available were obtained from Aldrich (Steinheim, Germany), Fluka, Merck, Sigma and Serva (Heidelberg, Germany). All glassware and polypropylene reaction vials were rinsed thoroughly with 70% ethanol and water and dried before use. Glass vials for protein/peptide hydrolysis were heated at 500°C for 3 h to remove any organic contaminants [5]. Highly purified water (Milli-Q, Millipore) was used throughout for preparation of all buffers and reagents.

Amino acid and amine standards

Composite amino acid and amine standards were prepared from stock solutions ($2.5\ \mu\text{mol/ml}$ 0.1 M HCl) to yield an overall concentration of 250 nmol/ml per component (except for physiological standards with varying concentrations). Norleucine or norvaline were used as internal standards at final concentration of 250 nmol/ml. TDPA (0.2%, w/v) was generally added to all standards as an antioxidant.

2.2. Protein/peptide hydrolysis [22]

An aliquot of protein or peptide solution in 0.1 mol/l HCl–0.2% TDPA containing 100 ng to 5 μg was transferred into micro glass-vials and dried in vacuo. Hydrolysis was carried out in the gas phase over 6 M HCl–0.2% TDPA at 110°C

for 24 h. Amino acids were redissolved in 50 to 200 μl of 0.1% HCl–0.2% TDPA and a 10- μl aliquot was submitted to derivatization.

2.3. Extraction of free amino acids and biogenic amines

Between 0.1 and 1.0 g of thoroughly homogenized solid samples, as food (cheese, meat, sausages, fish), tissue or plant material, or lyophilized samples (plasma, tissue) was dispersed with 10 ml of 0.1 mol/l hydrochloric acid solution containing 0.2% TDPA and the internal standard (250 nmol/ml) in a 25-ml centrifuge vial using an Ultra-Turrax homogenizer for 2 min at 20 000 rpm. The tube was covered with Parafilm and kept in an ultrasonic bath for maximal 30 min, centrifuged at 5000 g for 20 min, and any topping fat layer was removed. The supernatant was filtered through a 0.45- μm membrane and 3 ml of the filtrate was deproteinized by passing through ultrafiltration inserts (prewashed with 200 μl of the extraction medium prior to use) via centrifugation at maximal 3500 g for about 1 h. Membrane-filtrated liquid food samples were diluted with 0.1 mol/l HCl–0.2% TDPA–250 mmol/l internal standard and ultrafiltrated as described above.

Alternatively, 1 ml of supernatant or diluted liquid sample was deproteinized by mixing with 1 ml of 40% (w/v) trichloroacetic acid, and the mixture was kept for 10 min in an ice bath. After centrifugation (15 000 g, 15 min), a 100- μl aliquot of the supernatant was dried in a vacuum concentrator (Speed-Vac) and redissolved in 100 μl of reaction buffer immediately before derivatization.

2.4. Dabsylation procedure

Reagents

Reaction buffer (0.15 mol/l NaHCO_3 , pH 8.6) consisted of 630 mg sodium hydrogen carbonate, dissolved in 40 ml Milli-Q water, adjusted to pH 8.6 with diluted NaOH and made up to 50 ml with water.

Dilution buffer was a mixture of 50 ml acetoni-

trile, 25 ml ethanol and 25 ml elution buffer A (see HPLC section).

Dabsyl chloride reagent (12.4 mM in acetone) was prepared by dissolving 40 mg dabsyl chloride in 10 ml of acetone by ultrasonic treatment (10 min) and filtering through an ANOTOP filter into brown-glass vials, and was stored at -20°C .

Manual derivatization

Aliquots of 20 μl of amino acid and amine standards, containing 0.2–50 nmol per component, or deproteinized sample extracts—if necessary diluted with 0.1 mol/l HCl–0.2% TDPA according to the expected total free amino acid and amine content—were transferred into 1.8-ml screw-cap vials and diluted with 180 μl reaction buffer. After thorough mixing on a vortex-mixer, 200 μl of dabsyl chloride reagent was added and the vials were stoppered tightly and shortly mixed again. Samples were incubated at 70°C for 15 min with intermediate mixing at 1 min and 12 min. The reaction was stopped by placing the vials in an ice bath for 5 min. Subsequent to a short centrifugation step (10 s, 10 000 g), 400 μl of the dilution buffer was added, followed by thorough mixing and centrifugation (5 min, 15 000 g). The clear supernatants were directly set for injection or stored at -20°C until chromatography.

Automated derivatization

Using an automated pre-column dabsylation device for dabsylation (AS 3500, Thermo Separation Products, Darmstadt, Germany), 100 μl of dabsyl chloride reagent, 10 μl of amino acid–biogenic amine standards or appropriate diluted sample extracts and 90 μl of reaction buffer were subsequently loaded into a teflon loop. The content of the loop was then transferred to an empty vial, mixed and incubated at 70°C for 15 min. While heating, the vial was mixed during the first five minutes and during the last minute of the incubation period. After cooling to 12°C during 5 min, 200 μl of dilution buffer was added. The solution was mixed again for 0.5 min and kept at least 20 min at 12°C before submitting to injection.

2.5. High-performance liquid chromatography

HPLC equipment

The HPLC equipment (Waters, Eschborn, Germany) consisted of a WISP 712 autosampler, two Model 510 high-pressure pumps and an UV 486 variable-wavelength detector interfaced to a NEC APC computer via a SIM box. Maxima 820 software was used for system controlling and peak integration. For automatic derivatization, the WISP 712 autosampler was replaced by the AS 3500 pre-column derivatization unit/autosampler.

HPLC conditions

Dabsyl derivatives of amino acids and amines were separated either on a 150 × 4.6 mm I.D. stainless-steel column filled with 3 μm Spherisorb ODS-2 (Knauer, Berlin, Germany), including a 10 × 4 mm I.D. guard cartridge containing the same stationary phase, or on a 150 × 3.9 mm I.D. Novapak C₁₈ column, 4 μm (Waters, Eschborn, Germany), including a guard cartridge. The column was thermostated at 50°C. Usually 10 μl of the derivatized samples was injected.

Mobile phase A, consisting of 9 mM sodium dihydrogenphosphate, 4% dimethylformamide and 0.1–0.2% triethylamine (TEA), was titrated to pH 6.55 with phosphoric acid. Mobile phase B was 80% (v/v) aqueous acetonitrile.

Dabsylated amino acids and amines were eluted at a flow-rate of 1 ml/min using the gradient system listed in Table 1; detection was at 436 nm and the data acquisition rate was 5 Hz.

3. Results and discussion

3.1. Deproteination

Deproteination by ultrafiltration was found to be preferable compared to trichloroacetic acid (TCA) precipitation, as with a relative high concentration of residual TCA after drying in vacuo, the derivatization yield was sometimes observed to be decreased remarkably. In addition, the ultrafiltration procedure increased the shelf-life of the chromatography column (100–200 analyses). Recovery of amino acids from ultrafiltrated amino acid standards (each 250 nmol/ml) was between 98 and 102% from batch to batch when prewashed filters were used. Similar results were reported by Cohen and Strydom [23].

3.2. Dabsylation

Preliminary experiments were performed with combined standards or extracts of free amino acids and biogenic amines at greatly varying concentration. Initial results indicated that the concentration of dabsyl chloride in the reaction mixture had to be increased to 6.2 mM in order to achieve optimal derivatization and stability of the bis-dabsyl derivatives of lysine, tyrosine, histidine and carnosine and of the biogenic amines histamine, tyramine, putrescine and cadaverine. Similar observations were reported by Vendrell and Avilés [12]. Although the derivatization yield (judged by corresponding peak response factors) of glutamic and aspartic acid decreased with increasing concentration of

Table 1
Scheme of elution gradient

	Time (min)									
	0.0	2.0	7.0	35.0	45.0	66.0	71.0	77.0	77.5	90.0
Solvent B (%)	8.0	8.0	20.0	35.0	50.0	100.0	100.0	100.0	8.0	8.0
Curve type ^a		6	5	7	6	6	6	6	6	6

^a 5 = convex, 6 = linear, 7 = concave according to Maxima 820 (Waters) gradient-controlling software.

dabsyl chloride in the reaction mixture, derivatization was found to be linear over a wide concentration range (see section 3.3., linearity). It was also observed that methionine was readily oxidized to methionine sulfone and methionine sulfoxide under these conditions, but oxidation could be completely inhibited by adding 0.2% of the antioxidant TDPA to sample and standard solutions. For the reaction buffer, a molarity of 0.15 mol/l and pH 8.6 was encountered to be most suitable with respect to the derivatization yield of the bis-dabsyl derivatives and the repeatability of derivatization for all amino acids and biogenic amines tested. However, Jansen et al. [17] did not report a significant difference of peak heights when varying the pH of the reaction mixture within pH 7.5 to 9.0, but their data were restricted to a limited number of amino acids (glutamic acid, alanine, valine, leucine, lysine). Furthermore, their contrary findings may result from the low buffering capacity of the reaction buffer (0.05 mol/l) not guaranteeing to maintain the pH of the reaction mixture at the desired level. The volume ratio of sample, dabsyl reagent and dilution buffer, as well as the composition of the dilution buffer, significantly influenced the yield of the derivatives and the linearity range of the derivatization procedure. These findings were in congruence with the results of Vendrell and Avilés [12]. In derivatized samples, which were diluted according to Knecht and Chang [8], crystallization of amino acid derivatives or dabsonate (see Table 2) occasionally occurred upon prolonged standing, resulting in a concomitant clearance of the peak area of hydrophobic and basic amino acids and some biogenic amines (histamine, tyramine). After replacing Chang's dilution buffer by a modified solvent mixture (see Experimental section), all derivatives were observed to be stable within 24 h (decrease of peak area below 4%). With extended storage of derivatized samples at room temperature, a decrease of the peak area of asparagine and tyramine was observed in deproteinated samples of free amino acids in biological fluids or food extracts. Nevertheless, 24 h stability is a reasonable period for manual day-to-day preparation of the samples and consecu-

tive chromatographic analysis. A general improvement of the repeatability of the peak area was achieved by using an automated derivatization unit (AS 3500), serving to maintain a constant standby period of the derivatized samples prior to injection and a more precise fully automated liquid handling.

3.3. Chromatographic separation

Qualitative results

Using the chromatographic parameters described in the Experimental section, the separation of the dabsyl derivatives of numerous amino acids including phosphorylated or carboxymethylated compounds as well as biogenic amines was investigated, most of them being separated simultaneously in one chromatographic run. The elution order and separation efficiency of 68 dabsyl derivatives tested on a 4- μ m NovaPak C₁₈ column at a TEA concentration of 0.16% are summarized in Table 2; the separation of a combined amino acid and biogenic amine standard is shown in Fig. 1.

We observed that elution order and separation efficiency were strongly depending on the TEA concentration in the elution buffer A, not confirming the findings of Jansen et al. [17], who reported that additives like TEA or dimethylformamide (DMF) had no substantial effect upon chromatographic separation. From repeated experiments with increasing concentration of TEA in elution buffer A titrated to pH 6.55, an obvious non-linear relationship between TEA concentration and retention time was observed for several amino acid derivatives eluting between 13 and 55 min, as shown in Figs. 2A–2E. Whereas in the last part of the gradient, little influence on retention time for the hydrophobic biogenic amines was observed (Fig. 2F). Retention of dabsyl derivatives of almost any amino acid and amine reached a maximum at 0.18% TEA. Critical pairs of closely eluting amino acid and amine derivatives (phosphothreonine/aspartic acid, glycine/arginine, alanine/ β -alanine, α - γ -aminobutyric acid, lanthionine/agmatine and histidine/carnosine) were sufficiently separated at 0.16% TEA. Except for glycine/arginine, the

Table 2
Elution order and retention time of dabsyl derivatives (0.16% TEA)

Compound	Abbreviation ^a	Incomplete separation	Co-elution	Retention time (min)	Compound	Abbreviation ^a	Incomplete separation	Co-elution	Retention time (min)
O-Phosphoserine	PS			13.95	Norleucine	NLE			40.90
Aspartic acid	D	PT		14.94	Phenylalanine	F			41.57
O-Phosphothreonine	PT	D		15.15	Ammonia	NH ₃			42.05
Glutamic acid	E			15.91	Lanthionine	LAN			43.62
Carboxymethylcysteine	CMC			16.39	Agmatine	RN			44.00
S-Sulfo cysteine	SC			16.89	2-Aminoethanol	SN		CYT	44.76
β -Amino adipic acid	AAA			17.10	Cystathionine	CYT		SN	44.76
Hydroxyproline	OHP			22.51	Cysteine	C			45.65
Asparagine	N			23.13	Homocystine	HCT			46.83
Glutamine	Q			24.72	1-Amino-2-propanol	TN			48.02
Citrulline	CIT			25.35	Hydroxylysine	OHK			50.04
Serine	S			26.17	Ornithine	ORN			51.02
Phosphoethanolamine	PSN		MESO	27.43	Lysine	K			51.58
Methionine sulfoxide	MESO		PSN	27.69	Histidine	H			52.10
(two diastereomers)	MESO		PSN	28.15	Carnosine	CAR	AN		52.38
Threonine	T			28.45	Ethylamine	AN	CAR		52.59
Glycine	G		MH	28.94	Tyrosine	Y			54.30
1- and 3-Methyl-histidine	MH		G	28.94	Pyrrrolidine	PN		WN	57.22
Arginine	R			29.38	Tryptamine	WN		PN	57.22
Alanine	A	β A		30.65	Isobutylamine	VN			57.79
β -Alanine	β A	A		30.92	3,4-Dihydroxyphenylalanin	DOPA			58.34
Anserine	AS			31.52	Phenylethylamine	FN			59.20
Taurine	TAU			32.31	Methylbutylamine	IN			59.94
Sarcosine	SAR		AABA	32.58	Putrescine	PUT			62.16
α -Aminobutyric acid	AABA	GABA	SAR	32.75	Cadaverine	CAD			63.29
γ -Aminobutyric acid	GABA	AABA		33.27	Histamine	HN			63.87
Proline	P			33.99	Cystamine	CN			63.87
β -Amino isobutyric acid	BAIBA			34.40	Serotonine	OHWN			65.00
Norvaline	NVA			35.06	Tyramine	YN			67.31
Valine	V			35.55	Spermidine	MN			67.98
Methionine	M			37.60	Noradrenaline	NADN			68.49
Isoleucine	I			39.35	Dopamine	DOPN			69.05
Leucine	L			40.03	Adrenaline	ADN			70.61
Tryptophan	W			40.51	Spermine	SPN			71.94

^a One-letter code was used for the nomenclature of proteinogenic amino acid; for most of their decarboxylated products (biogenic amines) a "N" ("amine") was added as a second letter except for those indicated in the table. Hydrolyzed dabsyl chloride (dabsonate) elutes as a broad peak at $t_R = 20$ min.

elution order of most of the "critical" pairs of dabsylated amino acid and amines was inverted when increasing the TEA concentration from 0.15 to 0.22%. Separation of the derivatives of the remaining amino acids and amines of Table 2, not illustrated in Fig. 2, was not deteriorated with varying TEA concentration, although they showed the same tendency of retention behaviour.

Lowering the initial concentration of solvent B

led to an improved separation of O-phosphoserine, O-phosphothreonine, aspartic acid, glutamic acid and α -amino adipic acid, but also resulted in an increased total gradient time. A convex flattening of the gradient with an average slope of 0.5% B/min positively affected the separation of the dabsonate to proline group. Dimethylformamide, added to mobile phase A, was found to improve the separation efficiency remarkably [17].

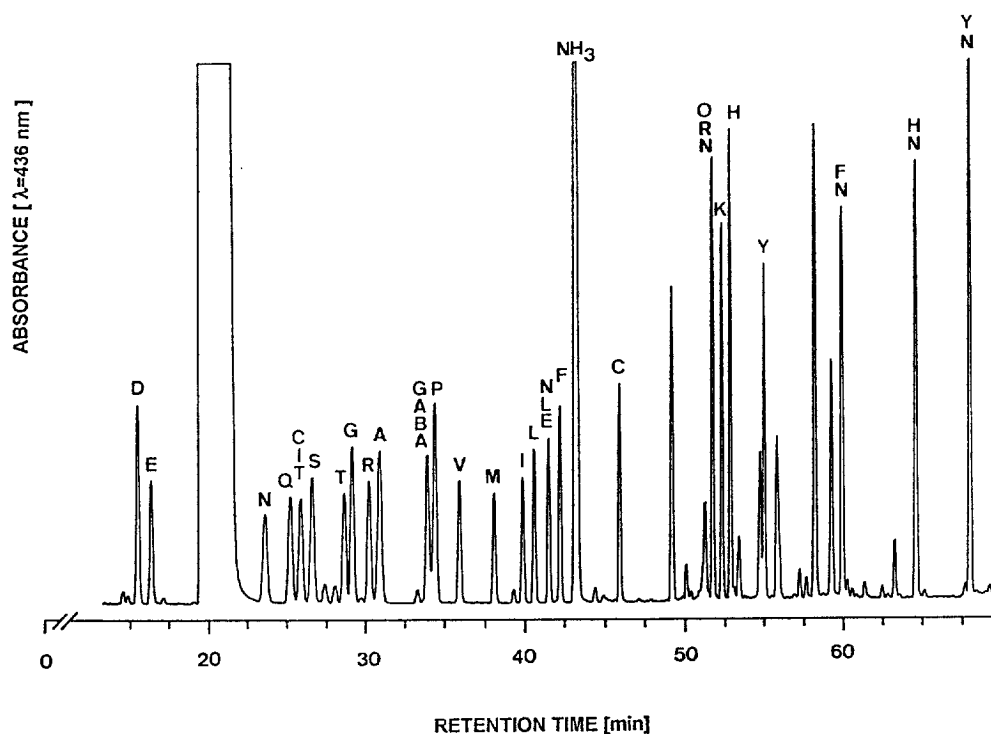


Fig. 1. RP-HPLC analysis of dabsyl derivatives from proteinogenic and physiological amino acids and selected biogenic amines, using elution buffer A containing 0.16% TEA, on a Novapak C₁₈ column. For abbreviations of amino acids and biogenic amines see Table 2.

The elution system indicated in the Experimental section using an optimized TEA concentration for a 150-mm column with 3- μ m Spherisorb ODS-2 or 4- μ m NovaPAK C₁₈ was most suitable for the simultaneous separation of a large number of components within a reasonable analysis time. Depending on the number and kind of amino acids and amines to be separated for an individual application, the gradient profile and, moreover, the appropriate TEA concentration, which may have to be adjusted according to type, batch and age of the chromatography column, has to be chosen carefully. Norleucine–eluting between leucine and phenylalanine–or norvaline–eluting between valine and methionine–proved to be suitable internal standards, which were included during the extraction of amino acids and biogenic amines or added to a protein/peptide prior to hydrolysis. By using a low molarity of phosphate and TEA as buffering additive in solvent A and aqueous

acetonitrile as solvent B the risk of salt precipitation during gradient formation was abandoned. The lifetime of the chromatography column usually was at least 200 runs.

Quantitative aspects

Linearity

A general, linear relationship between peak area and concentration, evaluated on a large number of samples ($n = 34$) of a combined standard of amino acids and biogenic amines, was observed over a range of 1.25 to 1250 pmol, provided that the samples were analyzed on the same day they had been derivatized. The correlation coefficients were greater than 0.999; data of regression analysis are compiled in Table 3.

Detection limit and determination limit [25]

According to Table 3, the detection limit of most of the dabsyl derivatives ranged between

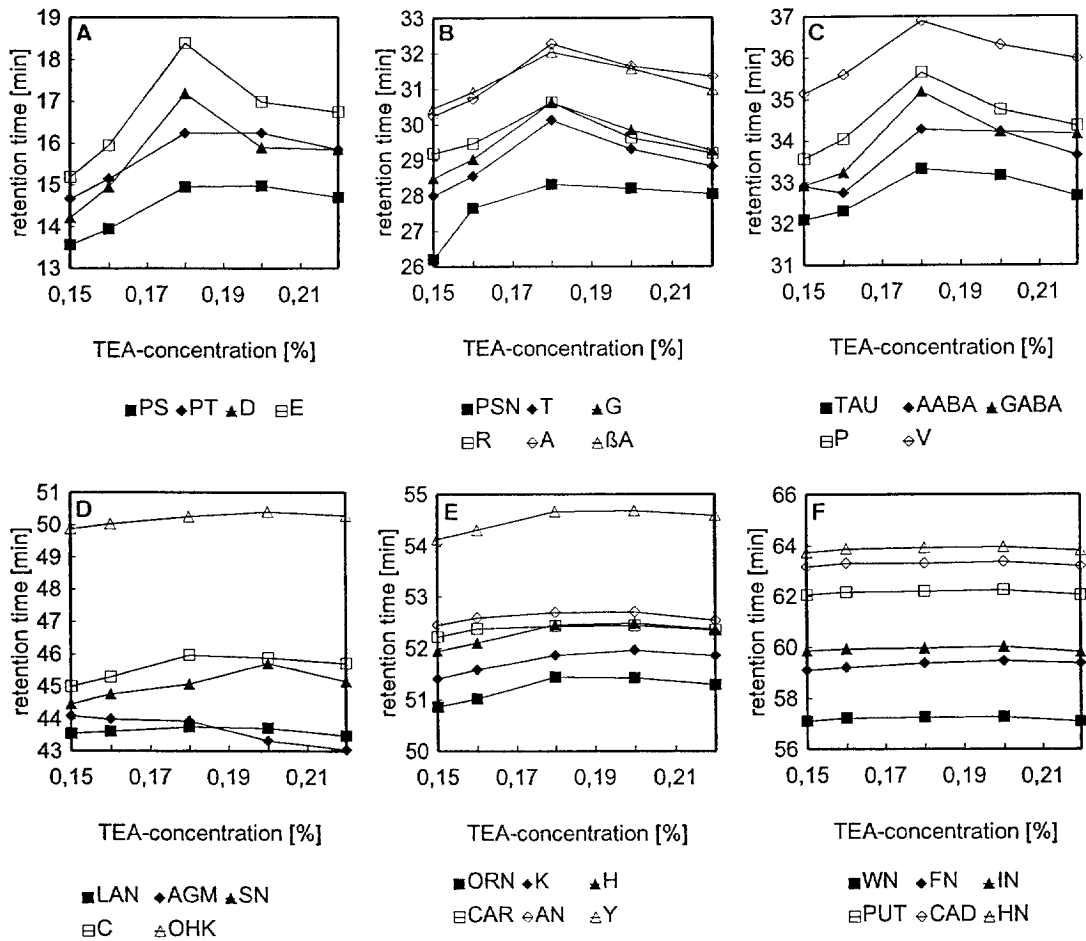


Fig. 2. Influence of the TEA concentration of elution buffer A on the elution behaviour of dabsylated amino acids and biogenic amines on a Spherisorb ODS-2 column. For abbreviations of amino acids and biogenic amines see Table 2.

0.12 and 0.52 pmol, with an average of approximately 0.3 pmol, a value being slightly lower than those reported by Chang and co-workers [6–8], Watanabe et al. [16] and Jansen et al. [17]. The determination limit ranged between 0.4 and 1.5 pmol. Nevertheless, when analyzing amino acids in the low picomole range, special attention has to be paid to avoid contamination of hydrochloric acid and glassware used for protein hydrolysis as well as of the derivatization buffer. Chang et al. [3,5,6] and Vendrell and Avilés [12] recommended that the starting amount of protein to be hydrolyzed and analyzed necessarily has to exceed 100 pmol in order to avoid extraneous contaminations of the sample and losses of

amino acids and derivatives. Consequently, the main clue for the amino acid analysis at the femtomole level is not the sensitivity of the method, but the sample handling.

Recovery

Recovery was evaluated for selected biogenic amines, which were spiked at two concentration levels onto a Parmesan cheese extract, in which these amines were either absent or present at a low level. Spiking was done prior to deproteination by ultrafiltration. Table 4 summarizes the recovery values for biogenic amines in Parmesan cheese, which were found to range between 98 and 104%. Recovery values usually exceeded

Table 3
Regression data, determination and detection limit of amino acid standards

Amino acid	Slope <i>m</i>	Intercept <i>y</i>	S.D. slope	S.D. intercept	Correlation coefficient	Detection limit (pmol)	Determination limit (pmol)
D	1.12	0.43	0.0023	1.47	0.999962	0.46	1.58
E	0.92	-2.01	0.0033	1.99	0.999875	0.51	1.76
N	0.80	1.21	0.021	1.52	0.999341	0.27	0.93
Q	1.04	-2.91	0.014	1.01	0.999828	0.28	0.97
CIT	1.12	-2.54	0.0076	0.56	0.999954	0.21	0.73
S	1.07	4.10	0.0075	5.56	0.999482	0.12	0.42
T	0.68	0.12	0.0020	1.30	0.999922	0.21	1.01
G	1.37	-0.055	0.000035	0.012	1.00	0.26	0.89
R	1.04	-0.43	0.0039	2.42	0.999858	0.33	1.15
A	1.38	0.62	0.0016	0.84	0.999992	0.17	0.61
GABA	1.12	2.92	0.0180	1.35	0.999735	0.26	0.93
P	1.41	0.011	0.000030	0.014	1.00	0.29	1.03
V	1.20	-9.29	0.0087	6.16	0.999274	0.52	1.80
M	1.17	-10.10	0.0090	6.63	0.999141	0.10	0.35
I	1.14	-13.59	0.0089	6.00	0.999275	0.41	1.40
L	1.25	-10.97	0.0086	6.02	0.999356	0.23	0.79
NLE	1.11	-1.91	0.029	2.12	0.99956	0.13	0.46
F	1.32	-9.21	0.0071	5.05	0.999592	0.31	1.09
C	0.99	-2.67	0.0041	2.56	0.999854	0.29	0.99
ORN	2.62	-8.04	0.027	1.97	0.999897	0.40	1.38
K	2.37	-13.17	0.020	14.69	0.998938	0.28	0.98
H	2.28	-2.38	0.0065	3.65	0.999919	0.30	1.05
Y	2.18	0.017	0.0021	1.27	0.999993	0.26	0.89
FN	1.75	1.07	0.027	1.99	0.999765	0.17	0.59
HN	2.26	-2.08	0.063	4.69	0.999219	0.31	1.07
YN	2.64	-4.33	0.025	1.88	0.999907	0.17	0.61

Relationship between peak area (*V_s*) and the amount of amino acids (pmol). Each 7 amino acid standards of 1250 and 1.25 pmol and each 5 samples of 1000, 50, 500 and 250 pmol, respectively, were analyzed. Claims of linearity are supported by regression data, which include slope, intercept, standard deviations of slope and intercept and correlation coefficients. The determination and detection limit was calculated in the range between 0 and 25 pmol according to DIN 32 645 [34].

Table 4
Recovery (%) of selected biogenic amines spiked onto an extract of a Parmesan cheese

Amino acid	Parmesan + 500 ppm amine (<i>n</i> = 5)	Parmesan + 1000 ppm amine (<i>n</i> = 5)
Tryptamine	103.15 ± 0.05	97.90 ± 0.48
Phenylethylamine	103.78 ± 0.18	97.57 ± 0.78
Putrescine	102.27 ± 0.57	100.37 ± 1.63
Cadaverine	102.32 ± 1.13	99.73 ± 1.43
Histamine	103.26 ± 0.30	99.39 ± 0.27
Tyramine	102.39 ± 0.85	102.31 ± 0.94

100% for spiking with 500 ppm biogenic amines as a consequence of single-point calibration.

Repeatability

The influence of the several steps of the method—extraction, automated derivatization and chromatographic separation—on its overall repeatability was investigated using Parmesan cheese sample (see Fig. 4), containing between 68 and 17 633 ppm of free amino acids and biogenic amines. When analyzing five subsamples of a single extract of free amino acids and biogenic amines after automated derivatization following a fixed time schedule, the repeatability was found to range between 0.2 and 3.3%, the average repeatability (1.3%) was merely influenced by chromatographic determination, including sample injection and automated peak integration. In a second series, five subsamples of the free amino acid extract were derivatized and analyzed separately. The average value of

repeatability was found to be slightly increased to 2.0%, due to an increased number of liquid-transferring steps during derivatization. As could be expected, a more pronounced increase of the average repeatability (3.1%) was observed when analyzing five separate samples of the Parmesan cheese submitted to extraction, deproteination and derivatization.

Furthermore, performance studies were extended using protein hydrolyzates, food samples as wine, meat, poultry meat conserves, ham, salami, Asian fish sauce, seasonings, and biological materials as plant and cortex extracts and lyophilized human plasma samples, giving similar results for repeatability and recovery values (data not shown). The repeatability values reported by several authors [12,14,16]—in some cases called “reproducibility” values—lie within the same range as described here, although some of the investigations were only performed on a limited number of amino acids, sometimes mere-

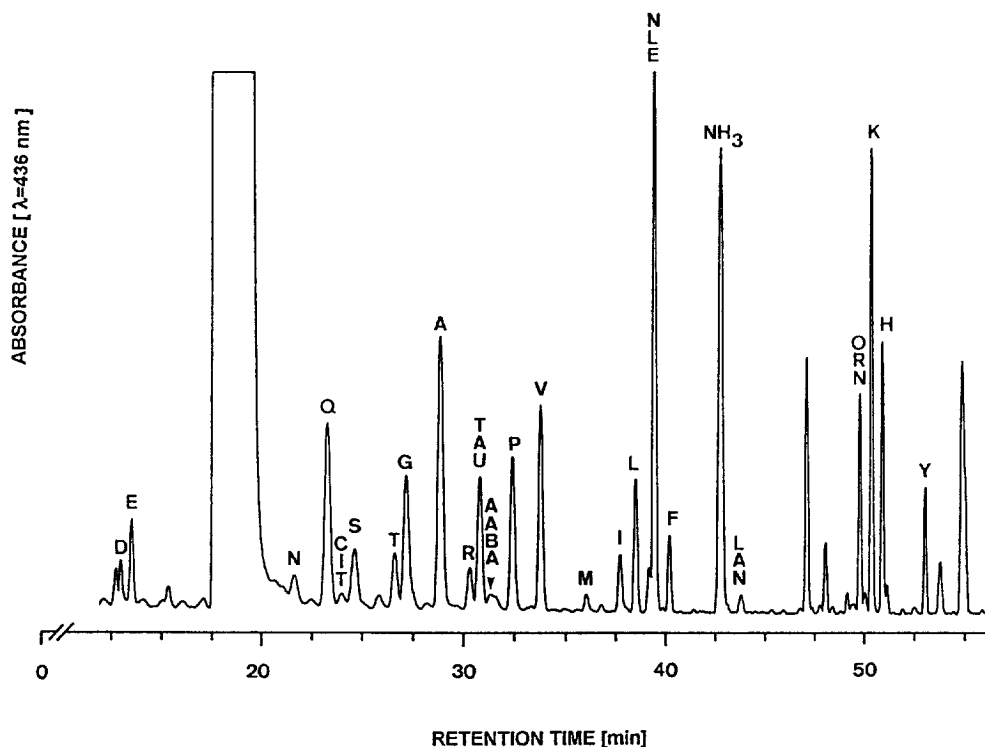


Fig. 3. RP-HPLC separation of dabsyl derivatives from human plasma, using elution buffer A containing 0.16% TEA, on a Spherisorb ODS-2 column. For abbreviations of amino acids and biogenic amines see Table 2.

ly using a single hydrolyzate standard sample or a protein hydrolyzate for controlling. In a collaborative study on the determination of free amino acids in cheese samples, the results of the dabsyl method were compared with those obtained by ion-exchange chromatography and RP-HPLC of FMOC/OPA derivatives and found to be in good accordance [26].

4. Applications

We have applied our method to the analysis of free amino acids and biogenic amines in human plasma (Fig. 3), cortex, Parmesan cheese (Fig. 4), ham (Fig. 5), seasonings and rice wine. As could be expected, amino acid and biogenic amine profiles are greatly varying within different types of individual samples, but no interference of the different matrices on the separation of the dabsyl derivatives was observed. With the possibility of obtaining data sets of usually more

than 30 components for a food sample like cheese, the method is valuable for quality ("identity") control [24]. Furthermore, the complex information that can be obtained for numerous amino acids and amines in various biological materials may be helpful in clinical diagnosis or protein analysis.

5. Conclusion

The sample preparation and derivatization procedure was optimized to achieve an efficient sensitive and reliable simultaneous determination of free amino acids and biogenic amines over a wide range of concentration. For the chromatographic separation of the dabsyl derivatives of amino acids and amines, TEA has proved to be a very effective additive to the aqueous mobile phase. The use of an automated derivatization device clearly improves the performance of the method, additionally avoiding

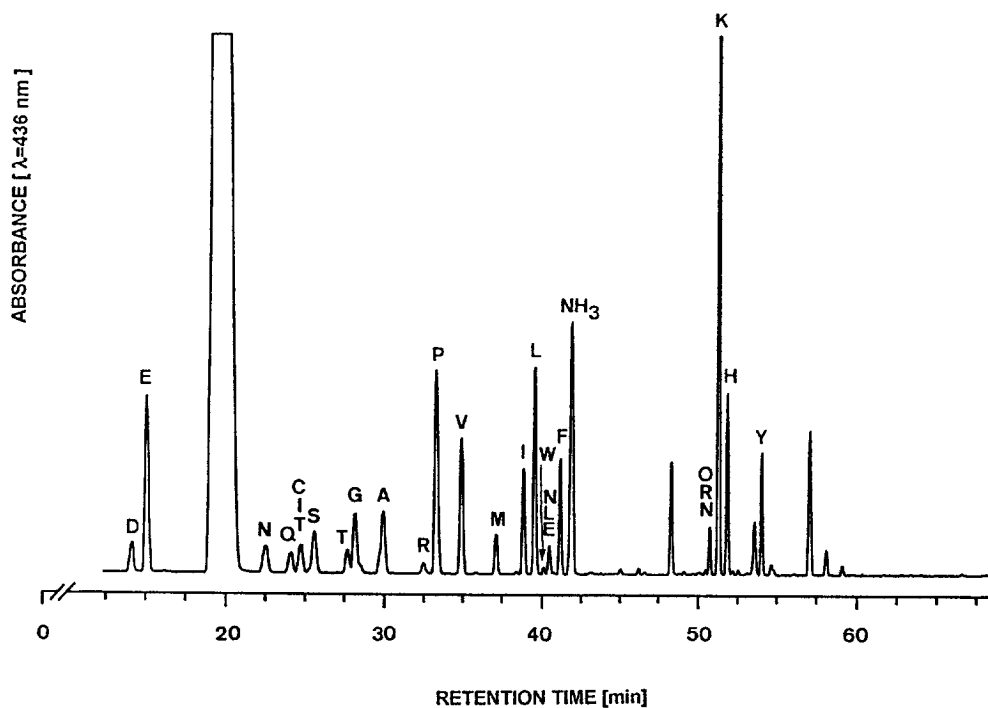


Fig. 4. RP-HPLC separation of amino acids extracted from Parmesan cheese. For chromatographic conditions and abbreviations see Fig. 3 and Table 2.

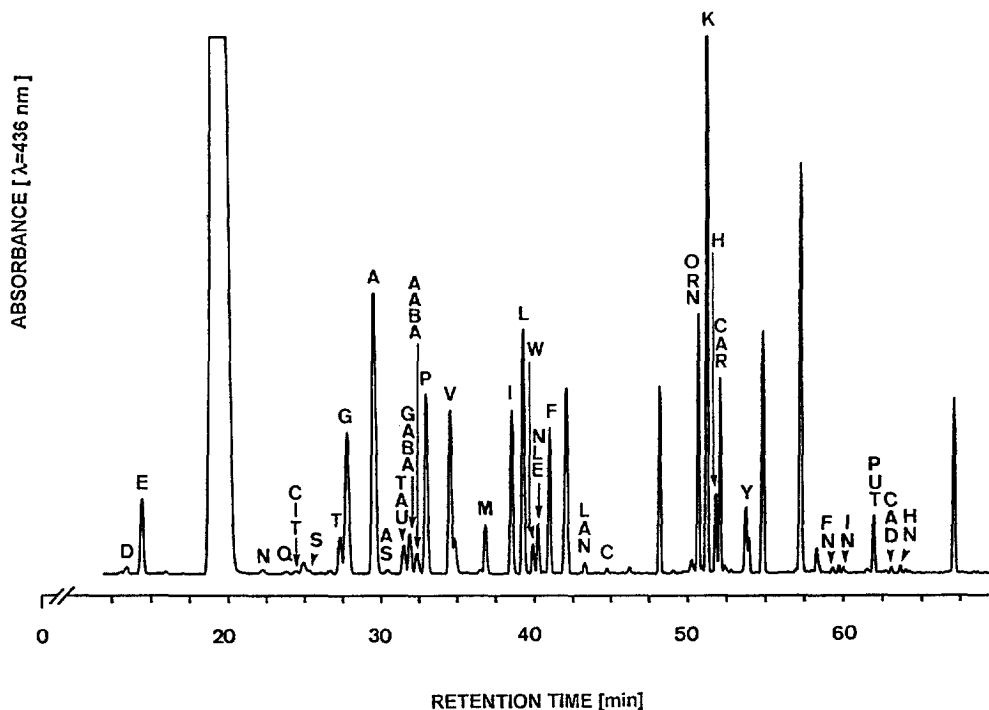


Fig. 5. Amino acid and biogenic amine profiles of ham. For chromatographic conditions and abbreviations see Fig. 3 and Table 2.

laboratory hazard from dabsyl chloride. Acceptable linearity, repeatability and recovery values in combination with feasible analysis time makes the method a serious alternative to conventional amino acid and amine analysis by the ion-exchange/ninhydrin or to RP-HPLC separation methods in combination with pre- or post-column derivatization procedures.

Acknowledgements

This work was kindly supported by the Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten – Sondervermögen der Milch- und Fettwirtschaft.

The authors thank Mrs. D. Luginger and Mrs. S. Ebert for excellent technical assistance.

References

- [1] J.K. Lin and J.Y. Chang, *Anal. Chem.*, 47 (1975) 1634.
- [2] J.K. Lin and C.C. Lai, *J. Chromatogr.*, 227 (1982) 369.
- [3] J.-Y. Chang, R. Knecht and D. Braun, *Biochem. J.*, 199 (1981) 547.
- [4] J.-Y. Chang, P. Martin, R. Bernasconi and D. Braun, *FEBS Lett.*, 132 (1981) 117.
- [5] J.-Y. Chang, R. Knecht and D. Braun, *Biochem. J.*, 203 (1982) 803.
- [6] J.-Y. Chang, R. Knecht and D. Braun, *Methods Enzymol.*, 91 (1983) 41.
- [7] J.-Y. Chang, *J. Chromatogr.*, 295 (1984) 193.
- [8] R. Knecht and J.Y. Chang, *Anal. Chem.*, 58 (1986) 2375.
- [9] J.Y. Chang, R. Knecht, P. Jenoe and S. Vekemans, in T.E. Hugli (Editor), *Techniques in Protein Chemistry*, Academic Press, San Diego, CA, 1989, p. 305.
- [10] V. Stocchi, L. Cucchiari, G. Piccoli and M. Magnani, *J. Chromatogr.*, 349 (1985) 77.
- [11] V. Stocchi, G. Piccoli, M. Magnani, F. Palma, B. Biagiarelli and L. Cucchiari, *Anal. Biochem.*, 178 (1989) 107.
- [12] J. Vendrell and F.X. Avilés, *J. Chromatogr.*, 358 (1986) 401.
- [13] M. Nieder, G.J. Hughes, S. Frutiger and E. Grom, *Labor Praxis*, 14 (1990) 26.
- [14] H.J. Schneider, *Chromatographia*, 28 (1989) 45.
- [15] D.A. Malencik, Z. Zhao and S.R. Anderson, *Anal. Chem.*, 184 (1990) 353.
- [16] A. Watanabe, J. Semba, A. Kurumaji, S. Kumashiro and M. Toru, *J. Chromatogr.*, 583 (1992) 241.

- [17] E.H.J.M. Jansen, R.H. van den Berg, R. Both-Miedema and L. Doorn, *J. Chromatogr.*, 553 (1991) 123.
- [18] D. Drnevich and T. Vary, *J. Chromatogr.*, 613 (1993) 137.
- [19] E. Mentasti, C. Sarzanini, O. Abollino and V. Porta, *Chromatographia*, 31 (1991) 41.
- [20] H. Treptow and A. Askar, *Ernährung*, 14 (1990) 9.
- [21] W.J. Hurst, *J. Liq. Chromatogr.*, 13 (1990) 1.
- [22] A. Otto, S. Kostka, A. Müller and G. Etzold, in B.J. Radola (Editor), *Elektrophorese Forum '91*, Bode, Freising, 1991, p. 351.
- [23] S.A. Cohen and D. Strydom, *Anal. Biochem.*, 174 (1988) 1.
- [24] I. Krause, A. Bockhardt, H. Neckermann and H. Klostermeyer, in preparation.
- [25] German Normative Institute, DIN 32 645, Beuth, Berlin, 1994.
- [26] U. Bütikofer, Evaluation of FLAIR/COST 902 collaborative test III, FAM Federal Dairy Research Institute Report, CH-Liebefeld, 25-8-1992.