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Pre-column derivatization of biogenic amines and amino acids with 9-fluorenylmethyl chloroformate and heptylamine

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ABSTRACT

A rapid and fully automated pre-column derivatization method for the determination of primary and secondary biogenic amines and amino acids is described. The derivatization reagent 9-fluorenylmethyl chloroformate is used, together with heptylamine for removing the excess of reagent. The derivatization procedure is described in detail. Chromatographic separation and detection of biogenic amines only or of biogenic amines and amino acids is possible. Repeatability data and applications of the technique are presented.

INTRODUCTION

Biogenic amines and amino acids are natural compounds of different food products, such as fish, seafood, cheese and wine. During fermentation or spoilage biogenic amines are produced by decarboxylation of the corresponding amino acids. The latter are decomposed to different products [1–3]. Consequently, biogenic amines are indicators of food quality. Therefore, it is important to determine certain biogenic amines in the presence of amino acids in different food matrices. In 1981 Karmas [4] introduced the "biogenic amine index" (BAI) for quality control of fish and fish products.

$$\text{BAI} = \frac{(\text{mg/kg histamine} + \text{mg/kg putrescine} + \text{mg/kg cadaverine})}{(1 + \text{mg/kg spermidine} + \text{mg/kg spermine})}$$

BAI values > 10 indicate as a rule a clear reduction of quality. During ripening of cheese, biogenic amines are produced by decarboxylation of amino acids. The main products are tyramine, putrescine, cadaverine, tryptamine and β-phenylethylamine.

Most of the amines show neither natural UV absorption nor fluorescence. Therefore, chemical derivatization is necessary to obtain detectable derivatives of the amines after HPLC separation. Different derivatization reagents were tested for the analysis of amines, *e.g.* ninhydrin in amino acid analysers with post-column derivatization

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[5] and 5-dimethylaminonaphthalene-1-sulfonylchloride as well as *o*-phthaldialdehyde as pre-column derivatization reagents [6–12]. Recently, naphthalene-2,3-dicarboxaldehyde and 9-fluorenylmethyl chloroformate (FMOC-Cl) are introduced for analysis of amines with pre-column derivatization [13–17]. Primary and secondary amines react with FMOC-Cl to give the corresponding 9-fluorenylmethyl carbamates [18]. The fluorescent FMOC derivatives of biogenic amines and amino acids are reasonably stable. Therefore, the reaction is well suited for pre-column derivatization of both biogenic amines and amino acids.

EXPERIMENTAL

Apparatus

The following apparatus were used: D-6000 HPLC-manager version 2, revision 3 with Interface (Merck–Hitachi), degasser (Hitachi) with helium (5.0, Messer–Griesheim), L-6200A intelligent pump (Merck–Hitachi), T-6300 column thermostat (40°C column temperature) (Merck), AS-4000 intelligent autosampler (Merck–Hitachi), F-1050 fluorescence spectrophotometer (Merck–Hitachi) (excitation wavelength 265 nm, emission wavelength 315 nm), 250-4 Supersphere 60 RP-8 column (E. Merck)

Chemicals

Chemicals used were obtained from commercial sources, including: Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany) and Sigma (Deisenhofen, Germany). All chemicals were of the highest purity available and were used without further purification. All HPLC solvents were obtained from Merck.

Preparation of standards

For the preparation of standards of biogenic amines and amino acids, 10 mg of each amino compound (or of the corresponding hydrochloride) were dissolved in 1000 ml of water. The resulting concentration of each amine (or its hydrochloride) was 10 μg/ml in the standard solution. Other concentrations were obtained by dilution of this standard solution, which was stored at −30°C.

Sample preparation

Wine and fruit and vegetable juices were injected directly into the HPLC system after filtration through a membrane (regenerated cellulose, 0.45 μm pore size, Sartorius G, No. 11606-13) and dilution (*e.g.* 1:5 or 1:20).

Samples of fish were chopped up and homogenized in a relationship of fish–0.1 *M* HCl (2:1, w/w). To 30 g paste (equivalent to 20.0 g fish) 40 ml 0.1 *M* HCl were added. After centrifugation and decantation of the supernatant solution, the residue was extracted twice with 40 ml 0.1 *M* HCl and once with 20 ml 0.1 *M* HCl. After filtration the extracts were made up to 100 ml with 0.1 *M* HCl and stored at − 30°C until HPLC analysis.

Samples of cheese were chopped up and homogenized in a relationship of cheese–0.1 *M* HCl (4:3, w/w). A 43.75-g amount of the paste (equivalent to 25.0 g cheese) were suspended with 40 ml 0.1 *M* HCl. After centrifugation the supernatant solution was filtered. The residue was extracted again twice with 20 ml 0.1 *M* HCl. The resulting combined extracts were made up to 100 ml with 0.1 *M* HCl and stored at − 30° until HPLC analysis.

Chromatographic conditions

Separation of biogenic amines only. Eluent A: 0.1 *M* sodium acetate, adjusted with NaOH to pH 4.4–acetonitrile (50:50). Eluent B: 100% acetonitrile. Gradient:

Time (min)	%B	Flow (ml/min)	Time (min)	%B	Flow (ml/min)
0	0	0.05	40	90	1.20
0.1	0	1.20	43	100	1.20
7	0	1.20	52	100	1.20
12	10	1.20	53	0	1.20
27	30	1.20	60	0	1.20
33	30	1.20	60.1	0	0.05

Separation of biogenic amines and amino acids. Eluent A: 0.1 *M* sodium acetate, adjusted

with NaOH to pH 4.4–acetonitrile (78:22). Eluent B: 100% acetonitrile. Gradient:

Time (min)	%B	Flow (ml/min)	Time (min)	%B	Flow (ml/min)
0	0	0.05	57	55	1.25
1	0	1.25	63	55	1.25
17	20	1.25	70	93	1.25
19	29	1.25	73	100	1.25
20	23	1.25	82	100	1.25
27	23	1.25	83	0	1.25
30	36	1.25	93	0	1.25
37	36	1.25	94	0	0.05
42	42	1.25			

Derivatization procedure

For derivatization, 50 μl sample (neutralization of strong acidic samples is sometimes necessary) were added to 200 μl borate buffer (0.2 *M* boric acid solution adjusted to pH 8.5 with 30% potassium hydroxide solution). After addition of 200 μl FMOC reagent (3 m*M* in acetone), the solution was mixed 3 min at room temperature. Then 50 μl heptylamine reagent (3 ml heptylamine and 15 ml acetonitrile, adjusted with 175 ml 0.1 *M* HCl to pH 7–8) were added to remove the excess of FMOC reagent. After 3 min of mixing, 80 μl of the reaction solution were diluted with 320 μl eluent A. Then 20 μl were injected for HPLC analysis. The procedure was used either in the manual mode or by utilizing a modified autosampler.

RESULTS AND DISCUSSION

Discussion of the derivatization conditions

Dependence of the derivatization on buffer and pH. 0.2 *M* Boric acid, adjusted to pH 8.5 with potassium hydroxide, was used for the derivatization buffer. Higher buffer concentrations sometimes led to separation of phases. The range of pH values, which was reported in earlier literature, was between pH 7.7 (derivatization of amino acids) [19] and pH 8.5 (derivatization of biogenic amines) [20]. Values of pH outside this range led to worse yields. Derivatization was therefore carried out at a pH of 8.5.

Dependence of the derivatization on concentration and solvent of the FMOC-Cl solution. When acetonitrile was used as the solvent for FMOC-Cl, a separation of phases occurred at high buffer concentrations. This could be avoided by use of acetone instead of acetonitrile. When the concentration of FMOC-Cl was too low (< 2 m*M*), there was not enough FMOC-Cl for reaction, especially in samples in which many amino acids were present as well as biogenic amines. Histamine seemed to react particularly slowly, and in order to quantify, it was necessary either to separate the amino acids from the biogenic amines or to use higher FMOC-Cl concentrations (*e.g.* 5 m*M*).

If higher concentrations of FMOC-Cl were used, two problems appeared: (1) when the sample had low concentrations of amines and amino acids, the excess of FMOC-Cl was so high, that the FMOC derivative of heptylamine (which was used for reducing the excess of FMOC-Cl) was precipitated, and (2) an excess of FMOC-Cl caused interferences in the chromatographic separation at the retention time of spermidine and spermine.

Dependence of the derivatization on reaction time. Various reaction times (90 s–20 min) were tested for derivatization of the biogenic amines. The dependence of the peak heights of the amine derivatives on reaction time is shown in Fig. 1. Spermidine, spermine and β-phenylethylamine show optimum yields at a reaction time of 5 min whereas putrescine, histamine, cadaverine and tyramine have a maximum at 3 min. In the derivatization of the amino acids, a reaction time of more than 3 min led, particularly in the cases of glutamic acid and aspartic acid, to peaks which had a strong pronounced shoulder. Sometimes even double peaks were detected. Therefore, a reaction time of 3 min was chosen for derivatization of amino acids and biogenic amines.

Reactants to remove the excess of FMOC-Cl. Excessive FMOC-Cl had to be removed by extraction or reaction with an other amine, because disturbances appeared in the chromatog-

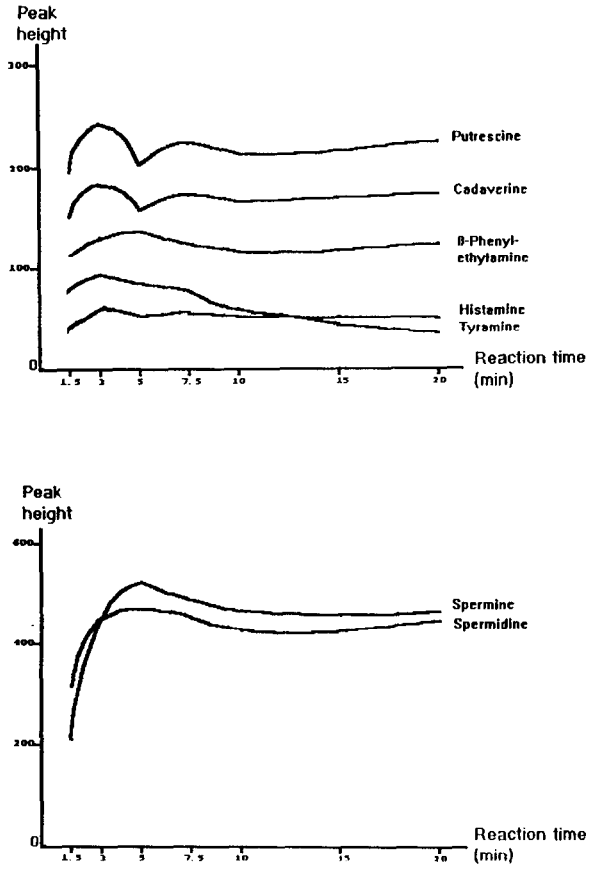

Fig. 1. Dependence of peak heights of FMOC derivatives on reaction time.

raphy, which made detection of tyramine and β-phenylethylamine impossible. Extraction with hexane removes FMOC-Cl from the water phase, but it also leads to the partial removal of most of the FMOC-amines. We also tested some amines, which react with FMOC-Cl after addition to the derivatization mixture. We obtained the best results with heptylamine, because no interferences were seen between its FMOC derivative and other FMOC-amines and -amino acids. When pure heptylamine was used, the pH would rise to such high values, that cleavage of the resulting FMOC derivatives took place. It was therefore necessary to adjust the pH of the heptylamine solution with 0.1 *M* HCl to a value in the range 7–8.

Influence of dilution after derivatization on chromatography. On injection into the HPLC system after derivatization and removal of the excess of FMOC-Cl it was found that the analysis was complicated by the presence of a greater number of interfering peaks from non-polar compounds. Most of these peaks could be suppressed by a fivefold dilution of the reaction mixture with starting eluent before injection. A loss of detection limit results from this dilution, but this factor is <5 because a better signal-to-noise ratio.

Discussion of eluents

Mixtures of aqueous buffers and acetonitrile were tested as eluents. We obtained best separations with 100 m*M* sodium acetate solution, which was adjusted with potassium hydroxide to pH 4.4, a column temperature of 45°C, and a flow-rate of 1.20–1.25 ml/min.

Chromatographic separation of biogenic amines

A mixture of seven amines (in the form of their hydrochlorides) was injected after derivatization for chromatographic separation of the biogenic amines. β-Phenylethylamine, putrescine, cadaverine, spermidine and spermine showed only one peak in their chromatograms (Fig. 2). Histamine and tyramine each gave two peaks, and in the case of histamine the peak with the retention time of 27.8 min clearly dominated. For tyramine the mono-substituted derivative (at the amino group) at a retention time of 9.0 min and the bis-substituted derivative (at the amino and at the hydroxy group) at 37.6 min were obtained. The yield of the bis-substituted derivative rose with higher concentrations, whereas the yield of the mono-substituted derivative decreased.

The presence of two derivatives showed no effect on the linearity for tyramine determination. We preferred to use the peak at 9.0 min in the chromatogram for quantitative analysis of tyramine, because of interferences with condensation products, which were eluted in the region of the second derivative.

For determination of calibration curves, standard solutions of 10 μg/ml, 5 μg/ml, 2.5 μg/ml and 1 μg/ml were derivatized and injected five times. The corresponding injection concentrations were 4 ng/20 μl, 2 ng/20 μl, 1 ng/20 μl

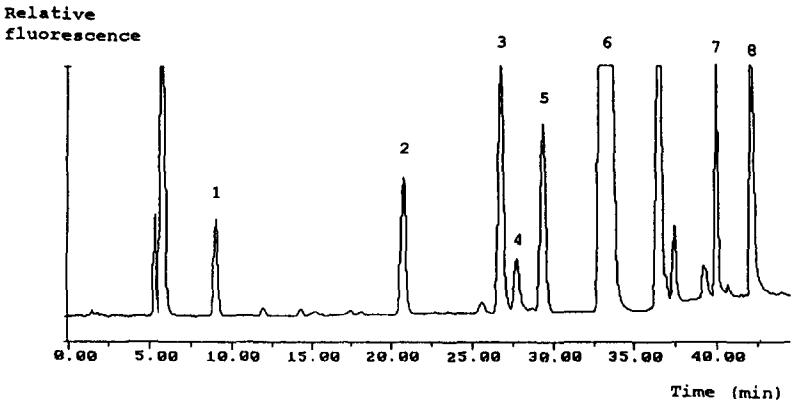

Fig. 2. HPLC chromatogram of a standard mixture (concentration 10 μg/ml) of biogenic amines after derivatization with FMOC-Cl and removing the excess of FMOC reagent with heptylamine. Peaks: 1 = tyramine; 2 = phenylethylamine; 3 = putrescine; 4 = histamine; 5 = cadaverine; 6 = heptylamine; 7 = spermidine; 8 = spermine.

and 0.4 ng/20 μl (calculated for the corresponding hydrochloride of the amine) (Table I).

In the last of these solutions the excess of FMOC-Cl became so high that condensation products were formed, which interfered with the chromatography of spermidine and spermine. Therefore, this concentration was neglected in the determination of correlation coefficients of spermidine and spermine.

The repeatability data determined in this analysis are shown in Table II.

A number of different food samples was successfully analysed for biogenic amine content using this procedure, *e.g.* wine, cheese, and fish (Fig. 3).

Chromatographic separation of amino acids and biogenic amines

A mixture of sixteen amino acids and seven biogenic amines (in the form of their hydrochlorides) was injected after derivatization with FMOC-Cl and removal of the excess of FMOC-Cl with heptylamine for chromatographic separation. The chromatogram (Fig. 4) showed a clear temporal separation of amino acids and biogenic

TABLE I

RESULTS OF THE LINEARITY TEST (ANALYSIS OF PEAK HEIGHT)

A = Correlation coefficient; B–E = relative standard deviations (%) of peak heights [injection concentrations: B 4.0 ng/20 μl ($n = 5$), C 2.0 ng/20 μl ($n = 5$), D 1.0 ng/20 μl ($n = 5$), E 0.4 ng/ 20μl ($n = 5$)].

Compound	A	B	C	D	E
Tyramine	0.9970	2.20	6.61	2.07	11.21
β-Phenylethylamine	0.9947	3.95	5.53	3.01	5.92
Putrescine	0.9971	2.91	4.88	5.64	15.51
Histamine	0.9954	2.98	1.40	4.94	5.17
Cadaverine	0.9965	3.20	5.52	6.65	10.97
Spermidine	0.9952	2.99	1.87	8.91	–
Spermine	0.9828	1.71	4.00	3.96	–

TABLE II

REPEATABILITY DATA (ANALYSIS OF RETENTION TIME AND OF PEAK HEIGHT)

A = Retention time (average) (min); B = standard deviation ($n = 20$); C = relative standard deviation (%) ($n = 20$); D = relative standard deviation (%) of peak heights ($n = 5$, injection concentration = 4 ng/20 μl).

Compound	A	B	C	D
Tyramine	9.00	0.016	0.18	2.20
β-Phenylethylamine	20.75	0.048	0.23	3.95
Putrescine	26.81	0.028	0.10	2.91
Histamine	27.79	0.036	0.13	2.98
Cadaverine	29.41	0.032	0.11	3.20
Spermidine	39.97	0.007	0.02	2.99
Spermine	42.09	0.019	0.05	1.71

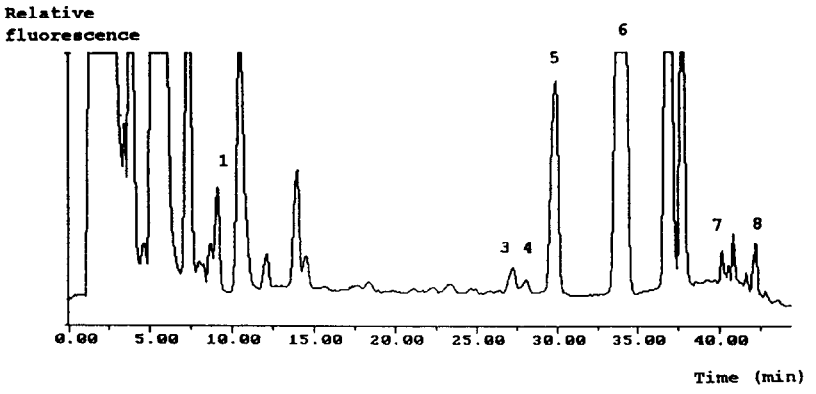

Fig. 3. HPLC chromatogram of salmon extract (with gradient for separation of biogenic amines). Peaks as in Fig. 2.

amines. Tyramine was the only representative of biogenic amines which eluted before the amino acid lysine.

Determination of calibration curves and repeatabilities were carried out analogous to the method described in the section *Chromatographic separation of biogenic amines*. The linearities and repeatabilities are as good as those for the chromatographic separation of biogenic amines [21].

Different food samples were successfully analysed for both biogenic amines and amino acids using this procedure, *e.g.* wine, fish and cheese (Fig. 5).

SUMMARY AND CONCLUSIONS

Primary and secondary amines react very fast with FMOC-Cl in slightly alkaline solution to give the corresponding fluorescent 9-fluorenylmethyl carbamates, which exhibit high stability. Therefore, selective determination of FMOC derivatives is possible by fluorescence detection after pre-column derivatization and reversed-phase HPLC separation. Disadvantages of this method are the fluorescence of FMOC-Cl and the fluorescence of hydrolysis products such as 9-fluorenylmethanol. Therefore, an additional step in the derivatization procedure is necessary

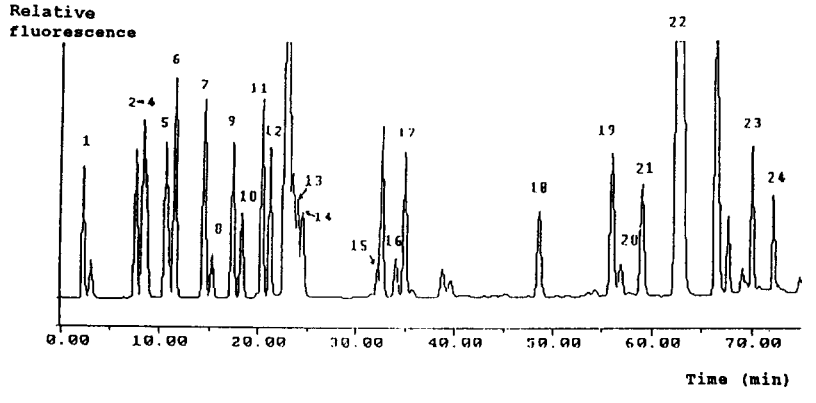

Fig. 4. HPLC chromatogram of a standard mixture (concentration 5 μg/ml) of biogenic amines and amino acids after derivatization with FMOC-Cl and removing the excess of FMOC reagent with heptylamine. Peaks: 1 = cysteic acid; 2 = aspartic acid; 3 = serine; 4 = glutamic acid; 5 = threonine; 6 = glycine; 7 = alanine; 8 = tyrosine; 9 = proline; 10 = methionine; 11 = valine; 12 = phenylalanine; 13 = isoleucine; 14 = leucine; 15 = histidine; 16 = tyramine; 17 = lysine; 18 = phenylethylamine; 19 = putrescine; 20 = histamine; 21 = cadaverine; 22 = heptylamine; 23 = spermidine; 24 = spermine.

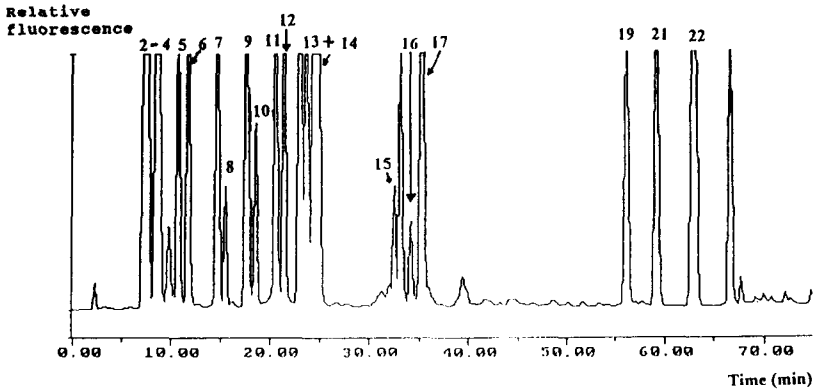

Fig. 5. HPLC chromatogram of cheese extract (with gradient for separation of biogenic amines and amino acids). Peaks as in Fig. 4.

for removing excess of reagent. There are two possibilities for this additional step: Extraction of excess reagent with *e.g.* hexane [18], or addition of an amine, *e.g.* adamantaneamine, which reacts with the excess of FMOC-Cl [19,20]. We prefer the second possibility using heptylamine as additional amine.

With this method we succeeded in detection of biogenic amines and amino acids. Additionally the determination of all biogenic amines (necessary for the calculation of the "biogenic amine index") is possible after HPLC separation of the FMOC derivatives and fluorescence detection. Yet, fluorescence detection is not sensitive enough for cystine, tryptophan, tryptamine and serotonine, because the fluorescence quantum yield is too low. However, UV detection at 265 nm is possible. The method allows full automatization with an autosampler such as the AS-4000 (Merck–Hitachi). Other advantages of the method are the good repeatabilities and good linearities for standards over a wide range of concentrations, and short run times.

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