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Liquid chromatographic determination of biogenic amines in fermented foods after derivatization with 3,5-dinitrobenzoyl chloride

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Abstract

The reagent 3,5-dinitrobenzoyl chloride (DNBZ-Cl) was tested for pre-column derivatization of biogenic amines (BAs). Samples were derivatized within 3 min in 1 *M* NaOH at ambient temperature by adding 2-propanole and 50 m*M* DNBZ-Cl in acetonitrile. The reaction was terminated by addition of 2 *M* HCl. For high-performance liquid chromatography an encapsulated stationary reversed-phase and gradient elution using a ternary gradient system were used. The DNBZ derivatives were quantified by their UV-absorption at 260 nm. The structures of the derivatives were elucidated using coupling of HPLC with electrospray ionization mass spectrometry. Detection limits of BAs were approximately 124–864 $\mu g\,l^{-1}$ (injected amounts 203–1410 pg) at a signal-to-noise ratio of 3:1. The coefficients of determination were 0.989–0.996, with the exceptions of cadaverine (0.976) and serotonin (0.965). The method was applied to the quantitative determination of agmatine, cadaverine, histamine, octopamine, 2-phenylethylamine, putrescine, serotonin, spermidine, spermine, tryptamine and tyramine, in fermented cabbage juices, soy sauces, Misos (soy pastes), fermented fish sauces, and anchovy paste.

Keywords: Food analysis; Derivatization, LC; Biogenic amines; Dinitrobenzoyl chloride

1. Introduction

Biogenic amines (BAs) are low-molecular-mass amines having a recognized biological activity and which might occur naturally in food and beverages [1]. Their amounts usually increase during controlled or spontaneous microbial fermentation of food or in the course of food spoilage [2,3]. The major pathway of BA formation is the decarboxylation of free amino acids (AAs) mainly by microbial enzymatic activity. Amounts and ratios of some selected BAs might serve as indicators for the quality or hygiene of food [4]. Several characteristic BA indices (BAIs) are calculated from the amounts of the most abundant BAs in fish, i.e. histamine (Him), putrescine (1,4-diaminobutane, Put), cadaverine (1,5-diaminopentane, Cad), spermidine (Spd) and spermine (Spm) [5]. A further BAI is calculated from quantities of tyramine (Tym), Put, Him and Cad in particular for assessing the quality of tuna [6].

The uptake of milligram amounts of Him by humans, depending on individuals, might result in hypo- or hypertension, headache, or anaphylactic shock syndromes [7–9]. Furthermore, uptake of diamines like Put and Cad can synergistically increase the toxic effects of Him [10]. Because the aromatic BAs Tym, 2-phenylethylamine (Pea) and tryptamine (Trm) have vasoactive properties [11], their presence in food should also be limited.

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On the other hand, fermented foods like cheese, sauerkraut, dry raw sausages and so-called 'Oriental' fermented foods, such as soy sauce, Tempe or Miso (soy paste), obtain their final texture, taste, appearance and palatability as a result of microbial fermentation [12]. Depending on the starter cultures employed, and fermentation conditions used, inevitably BAs are formed [3,7,11,13–18]. Alcoholic fermented beverages such as beer and wine were also analyzed for BAs [19–21].

For BA analysis, beneath radioimmunological [22] or enzymatic [23] methods, chromatographic techniques like thin-layer chromatography [24] or gas-chromatography [25] are used. Another method for analyzing BAs is their separation by ion-exchange chromatography and pulsed amperometric detection [26]. Because of the high selectivity and sensitivity, most analysts prefer reversed-phase high-performance liquid chromatography (HPLC) for separation and quantification of BAs. Since many BAs occurring in food show neither satisfactory absorption in the visible (Vis) or ultraviolet (UV) range, nor have fluorescence properties, chemical derivatization is usually performed for their detection. Beneath classical post-column derivatization procedures [27,28], various pre-column derivatization methods are used.

For the fluorescence labeling of BAs, suitable pre-column derivatization reagents are *ortho*-phthaldialdehyde (OPA) together with thiols such as 3-mercaptopropionic acid (3-MPA) [29] or 2-mercaptoethanol [30], 9-fluorenylmethyl chloroformate (FMOC-Cl) [31], 2-naphthyloxycarbonyl chloride (NOC-Cl) [32], or 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [33]. Pre-column derivatization reagents for BAs used for UV–Vis detection are, for example, 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) [34,35] and 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) [36]. We have recently shown that *para*-nitrobenzyloxycarbonyl chloride (PNZ-Cl) is also a suitable reagent for the determination of BAs in fermented beverages and vinegars [19].

We now show that the reagent 3,5-dinitrobenzoyl chloride (DNBZ-Cl), which was previously used for the analysis of Put, Cad, Spd, Spm in biological fluids [37], is a very suitable reagent for quantitative determination of twelve BAs (including the internal standard 1,6-diaminohexane) being of relevance for fermented foods.

The aim of the work was: (i) to test the suitability of the reagent DNBZ-Cl for the pre-column derivatization of BAs; (ii) to optimize chromatographic conditions; (iii) to characterize derivatives formed by on-line HPLC–electrospray ionization mass spectrometry (ESI-MS); and (iv) to demonstrate the applicability of the method on fermented foods known to have interfering matrix effects.

2. Experimental

2.1. Instruments

For HPLC a HP 1100 Series comprising a Model G1311A pump with low-pressure gradient-former, G1313A autosampler, G1322A degasser, G1316A column thermostat, G1314A diode-array detector with 13-μl flow cell, and HP ChemStation software for LC (Rev. A.04.02) were used (all from Hewlett-Packard, Waldbronn, Germany).

For ESI-MS an LCQ instrument (Finnigan-MAT, San Diego, CA), equipped with an electrospray interface (spray voltage 4.27 kV), was used. The capillary temperature was set at 230°C, the capillary voltage to −9.37 V. Nitrogen served as the sheath gas and auxiliary gas and as trap gas helium (purity>99.9990%) was used. ESI-MS was performed in negative ionization mode. For generating abundant negative ions aqueous 4 *M* NH_3 was added to the HPLC eluate by a syringe pump at a flow-rate of 3 $\mu l\ min^{-1}$.

2.2. Solvents, chemicals and amino acid standard

From Merck were purchased: ethanol (EtOH), sodium acetate (NaOAc) trihydrate and tetrahydrofuran (THF). Sodium hydroxide (NaOH), potassium hydroxide (KOH), *n*-butanol, *n*-hexane, 2-propanol (2-PrOH), and acetic acid (HOAc) were from Carl Roth (Karlsruhe, Germany). Histamine dihydrochloride, 1,6-diaminohexane dihydrochloride, and L-citrulline (L-Cit) were from Aldrich (Steinheim, Germany). Agmatine sulfate, boric acid (H_3BO_3), DNBZ-Cl, DL-octopamine hydrochloride, 2-phenylethylamine hydrochloride, 1,4-diaminobutane dihydrochloride, serotonin hydrogenoxalate, tyramine hydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, hydrochloric acid (HCl, 32%),

tryptamine, L-cysteine (L-Cys), L-tryptophan (L-Trp), L-ornithine (L-Orn), and γ-aminobutyric acid (GABA) were from Fluka (Neu-Ulm, Germany). For preparing aqueous solutions doubly distilled water from a quartz distill was used.

2.3. Preparation of sodium acetate and borate buffers and reagents

Sodium acetate (NaOAc) buffer (100 m*M*, pH 7.0) was prepared in a volumetric flask from NaOAc·$3H_2O$ (13.6 g) in water (950 ml) by titration with 1 *M* HCl and final dilution to 1 l by addition of water. NaOAc buffer (100 m*M*, pH 4.3) was prepared from HOAc (100 mmol) in water (950 ml) by titration with 2 *M* NaOH and final dilution with water to 1 l.

Potassium borate buffers were prepared from boric acid (33.43 g, 0.5 mol) in water (950 ml) by titration with KOH (20%, w/v) to pH 8.0 and final adjustment with water to 1 l. Borate buffers with other pH values were prepared similarly.

As derivatizing reagent 50 m*M* DNBZ-Cl in MeCN was used and 2 *M* HCl served for termination of the reaction.

2.4. Preparation of standards of biogenic amines and amino acids

The corresponding hydrochlorides of BAs were dissolved in 0.1 *M* HCl to provide final concentrations of 50 m*M*. Other concentrations used for calibrations were prepared by appropriate dilution with 0.1 *M* HCl. As internal standard 10 m*M* 1,6-diaminohexane (Dhx) in doubly distilled water was used. For a standard containing BAs and amino acids, equal volumes of 2.5 m*M* BA standard mixture, 2.5 m*M* protein hydrolysate standard of amino acids (standard H, Pierce, Rockford, IL), and 2.5 m*M* solutions of the frequently occurring amino acids in fermented food, L-Cit, L-Cys, GABA, L-Orn, and L-Trp were combined. The mixture was evaporated to dryness in vacuo and 0.1 *M* HCl was added to provide a final concentration of 125 μ*M*.

2.5. Sources and characterization of samples

The lactic fermented cabbage juices and the anchovy paste were purchased at local retail outlets in Germany. Fermented products made from soy beans (soy sauces, Miso) and fermented fish sauces were purchased at retail outlets in Seoul, South Korea, or laboratory-made in South Korea following traditional recipes. Samples 1–19 are specified in the following.

Lactic fermented cabbage juices (sauerkraut juices): Samples 1–5 were bottled and pasteurized juices made from white cabbage by different manufacturers (Germany). According to the manufacturers' declaration, all samples contained table salt, and Samples 1, 2, 4 and 5 additionally contained ascorbic acid.

Soy sauces: Samples 6 [dry matter (DM) 25.9%] and 7 (DM 28.1%) were commercial products. According to the manufacturers' declaration, Sample 6 was a mixture of fermented soy sauce and acidic hydrolyzed soy beans (50:50, v/v), Sample 7 was a mixture of fermented soy sauce and acidic hydrolyzed soy beans (20:80, v/v). Both samples were prepared from Koji made from soy bean and wheat. Samples 8 (DM 29.2%, 3 months fermented) and 9 (DM 22.2%, 5 months fermented) were 100% fermented soy sauces, laboratory-made following traditional recipes using soy bean Koji. Koji is moulded cereal, e.g. cooked or steamed soy bean, wheat or rice. It serves as an inoculum for raw materials providing finally fermented foods [38].

Misos: Miso is a paste of soy beans, usually mixed with rice, wheat or barley, and table salt. It is used as a flavouring ingredient in, for example, soup [38]. Samples 10 and 11 were commercial products made from soy bean Koji (Sample 10, 28.3%; Sample 11, 23.0%) and wheat (Sample 10, 2.2%; Sample 11, 14.4%) with the addition of table salt. The laboratory-made Samples 12–14 were produced from soy beans, salt, and soy bean Koji and were fermented for 2–3 months.

Fermented fish sauces: fermented fish sauces are produced by breakdown of small fish, e.g. anchovies (*Engraulidea* spp.), by fish enzymes. They contain table salt and were used as flavouring in many dishes [38]. Sample 15 (78.0% fish extract, 22.0% table salt, w/w) and Sample 16 (80.0% fish extract, 20.0% table salt, w/w) were both industrial products.

Anchovy paste: Sample 17 is a non-fermented product made from anchovies (75%), fat, and salt. This (industrial) sample was investigated for comparison and since fish is a potential source of BAs.

2.6. Treatment of samples for analysis and derivatization procedure

For analyses of Samples 1–16, to aliquots (1 ml or 1 g), ethanol (0.5 ml) and a solution of the internal standard Dhx (50 μl) were added. A final volume of 10 ml (adjusted with 0.1 *M* HCl) was centrifuged for 20 min at 3500 *g* and aliquots of the supernatant (40 μl) were analyzed. In the case of the anchovy paste (Sample 17), after the centrifugation step the supernatant was extracted with *n*-hexane (3×2 ml). The organic phase was discarded. The aqueous phase was evaporated to dryness in vacuo and the remaining residue was dissolved in 0.1 *M* HCl (10 ml).

Aliquots of samples or BA standards (40 μl), 1 *M* NaOH (120 μl), 2-PrOH (70 μl), and DNBZ-Cl (210 μl) were mixed in a reaction vial. After 3 min of manual shaking at ambient temperature, 2 *M* HCl (50 μl) were added to stop the reaction. After 1 min of shaking, aliquots of 20 μl were analyzed.

2.7. Chromatography

Derivatized BAs were analyzed on a Grom-Sil ODS-3 CP 120 RP-18 encapsulated polymer coated column (250×4 mm I.D., particle size 5 μm, GROM Analytik, Herrenberg, Germany).

A ternary gradient consisting of 97% 0.1 *M* NaOAc (pH 7.0) and 3% THF (v/v) (eluent A), 0.1 *M* NaOAc (pH 4.3) (eluent B), and MeCN (eluent C) was used. For the gradient program see Table 1. The column was kept at 30°C in a heated column compartment. The DNBZ amines were detected by their absorption at 260 nm.

For on-line HPLC–ESI-MS the HP 1100 series (see above) was used (for chromatographic parameters see Table 2).

Table 1
Gradient program for the separation of DNBZ amines (for other chromatographic conditions see Section 2)[a]

Time (min)	Eluent A (%)	Eluent B (%)	Eluent C (%)	Flow-rate (ml min^{-1})
0.0	85.0	0	15.0	0.65
8.0	85.0	0	15.0	0.65
17.0	80.0	0	20.0	0.65
20.0	75.5	0	24.5	0.65
22.0	75.5	0	24.5	0.65
25.0	71.0	0	29.0	0.65
28.0	69.0	0	31.0	0.65
29.0	72.0	0	28.0	0.65
35.0	67.0	0	33.0	0.65
36.0	67.0	0	33.0	0.65
40.0	65.5	0	34.5	0.65
42.0	65.5	0	34.5	0.65
47.0	64.0	0	36.0	0.65
48.0	0	64.0	36.0	0.65
53.0	0	50.0	50.0	0.65
58.0	0	48.0	52.0	0.65
64.0	0	40.0	60.0	0.65
66.0	0	30.0	70.0	0.65
67.0	0	0	100.0	1.3
71.0	0	0	100.0	1.3
72.0	85.0	0	15.0	1.3
76.0	85.0	0	15.0	1.3

[a] Eluent A, 100 m*M* NaOAc pH 7.0; eluent B, 100 m*M* NaOAc pH 4.3; eluent C, acetonitrile.

3. Results and discussion

3.1. Optimization of the derivatization conditions

For the evaluation of optimal derivatization conditions for BAs, 0.5 *M* borate buffers with pH values 8.0 – 10.0, as well as 1 *M* NaOH, were tested. The results were compared to those obtained by derivatizing BA standard mixtures (c=250 μ*M*). In borate buffers with pH values between 8.0 and 9.0 precipitation of a white solid was observed after addition of the derivatization reagent. No precipitate was formed in borate buffers with pH 9.5 or 10.0 and in 1 *M* NaOH. Further, maximum absorption resulting in largest peak areas was obtained by derivatization in 1 *M* NaOH. By adding different volumes of 1 *M* NaOH (80 μl – 200 μl) for derivatization of 40 μl of BA standard mixtures, the minimum volume used for derivatization should be 120 μl. Use of larger volumes did not increase peak areas of the DNBZ amines.

Derivatization of 40 μl of a BA standard mixture (c=250 μ*M*) with various amounts and concentrations of DNBZ-Cl showed that largest areas were obtained using 210 μl of 50 m*M* DNBZ-Cl in MeCN. This corresponds to a 1050-fold molar excess of DNBZ-Cl for each BA in the standard. In order to avoid the separation of the reaction mixture in an organic and aqueous phase, 2-PrOH (70 μl) was added to the derivatization mixture.

Various derivatization times (1–20 min) and reaction temperatures (ambient temperature, 30°C,

Table 2
Gradient profiles (30°C column temperature) (a)–(c) for characterization of the DNBZ BAs via LC–MS[a]

(a)				(b)				(c)			
Time (min)	A (%)	B (%)	Flow-rate (ml min^{-1})	Time (min)	A (%)	B (%)	Flow-rate (ml min^{-1})	Time (min)	A (%)	B (%)	Flow-rate (ml min^{-1})
0.0	85	15	0.75	0.0	55	45	0.75	0.0	70	30	0.75
8.0	85	15	0.75	8.0	55	45	0.75	8.0	70	30	0.75
17.0	80	20	0.75	17.0	30	70	0.75	17.0	60	40	0.75
20.0	75	25	0.75	20.0	30	70	0.75	20.0	60	40	0.75
22.0	75	25	0.75	21.0	0	100	0.75	25.0	50	50	0.75
25.0	70	30	0.75	26.0	0	100	0.75	30.0	50	50	0.75
30.0	60	40	0.75	27.0	55	45	0.75	40.0	30	70	0.75
40.0	50	50	0.75	32.0	55	45	0.75	45.0	0	100	1.0
45.0	0	100	1.0					50.0	0	100	1.0
50.0	0	100	1.0					51.0	70	30	1.3
51.0	85	15	1.3					57.0	70	30	1.3
57.0	85	15	1.3								

[a] Elucidation of: (a) Him, Seo, Ocp and Tym; (b) Spd and Spm; (c) Trm, Put, Pea, Agm, Cad and Dhx. Eluent A, doubly distilled water; eluent B, acetonitrile.

40°C, 50°C and 60°C) were tested for assessing optimal reaction conditions. After derivatization of BA standard mixtures (c=250 μM), 2 M HCl was used to stop the reaction. The BA derivatives showed maximum peak areas with manual shaking for 3–5 min at ambient temperature. Use of higher reaction temperatures and change of the reaction times resulted in decreasing peak areas. In the case of Him, the largest peak area was observed after 3 min of derivatization. Since the differences of peak areas between a derivatization time of 3 and 5 min for all other BAs was very small, and since Him is among the most important BA, derivatization for 3 min at ambient temperature with shaking was selected. The autosampler used allowed an accurate injection of samples but was not reliable for automatic pre-column derivatization.

The last step of the derivatization procedure is the termination of the reaction by addition of 2 M HCl. This is also necessary because the strong basic reaction conditions would extremely shorten the shelf life of the separation column. Amounts of 50 μl of 2 M HCl were sufficient for neutralizing the reaction mixture.

3.2. Chromatographic conditions and calibration of standards

The reagent DNBZ-Cl forms derivatives with BAs as well as with amino acids. The aim of the work, however, was to determine food-relevant BAs exclusively.

Among several stationary phases tested the best resolution of derivatives was achieved on a polymer encapsulated octadecylsilyl column using a ternary gradient (see Section 2 and Table 1). At the beginning of the gradient program a pH 7.0 of the eluent was chosen. This pH, together with the addition of the modifier THF, allows the separation of the first eluting DNBZ BAs, i.e. the derivatives from Him, octopamine (Ocp), Tym and serotonin (Seo) (see Fig. 1). After 47 min elution time the pH was changed to 4.3. This led to a faster elution of the remaining DNBZ BAs. Testing different flow-rates and column temperatures established the use of a flow-rate of 0.65 ml min^{-1} at 30°C. At the end of the analyses the flow-rate was increased to 1.3 ml min^{-1} in order to purge the column with MeCN and for reconditioning the stationary phase with a mixture of 85% eluent A and 15% MeCN (v/v). Derivatives of BAs of interest are well separated in approximately 68 min, with the exception of Cad, Pea and agmatine (Agm) (Fig. 1). At the beginning of the chromatogram the reagent DNBZ-Cl (designated ‘A’) used in excess, as well as compounds (designated ‘X’) resulting from the reagent, are eluting. Separation of a standard of BAs and amino acids is shown in Fig. 2. For their complete, or almost complete, separation a run-time of 68 min is required. Therefore, a special sample work-up for the separation of BAs from

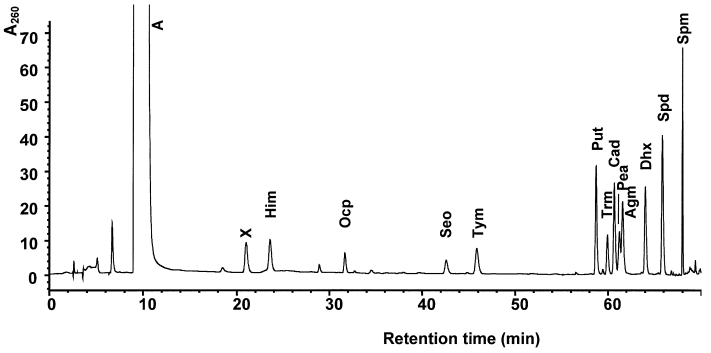

Fig. 1. HPLC of a standard of biogenic amines ($c=250$ μM) after derivatization with DNBZ-Cl. For chromatographic conditions see Section 2. A, DNBZ-Cl; X, derived from reagent. Peaks not assigned are unknown compounds. A_{260}, absorbance at 260 nm.

amino acids as described in the literature [21,30,35] is not required.

For calibration, standard mixtures of BAs (10, 25, 50, 75, 100 and 250 μM) were prepared and analyzed. The data of the linearity test are shown in Table 3. The relative standard deviations (RSDs) for peak areas of a standard mixture ($c=250$ μM) ranged from 2.21% (Put) to 9.54% (Pea). The coefficients of determination were 0.989–0.996 with the exception of Cad (0.976) and Seo (0.965).

For testing relative standard deviations (RSDs) of retention times, BA standard mixtures ($c=250$ μM and $c=100$ μM) were derivatized and injected ($n=10$). The RSDs of retention times ranged from 0.08 to 0.86% (see Table 3). Detection limits of DNBZ BAs at a signal-to-noise ratio of 3:1 were 124–864 $\mu g\,l^{-1}$ (injected amounts: 203–1410 pg). The lowest BAs standard concentration (10 μM) was chosen for the determination of quantification limits with a signal-to-noise ratio>5:1.

3.3. Characterization of the DNBZ derivatives by HPLC–ESI-MS

For structure elucidation of the DNBZ derivatives, on-line HPLC–ESI-MS were performed (see Section 2 and Table 2). Molecular ions and diagnostic fragments of derivatives were determined in the negative ionization mode. To increase the yield of negative ions, 4 M NH_3 was added to the effluent. MS proved that the BAs formed single derivatives (see Table 4). Most BAs provide fragments of the type $[(DNBZ)_nBA]^-$ with $n=1$–4 (n, degree of derivatization). Further, clusters of the type $[(DNBZ)_nBA]_2^-$, or, in the cases of Ocp and Tym, $[(DNBZ)_nBA]_3^-$, were detected. These clusters allow

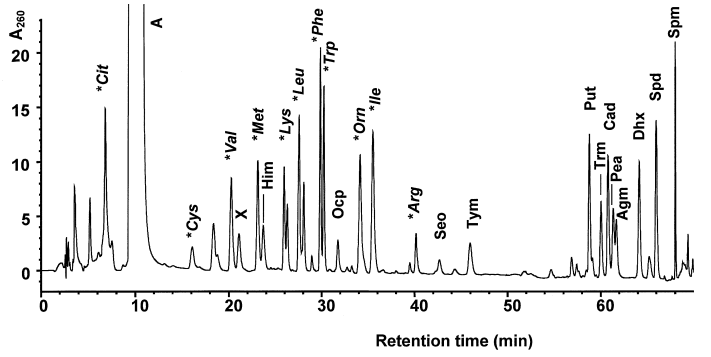

Fig. 2. HPLC of a mixed standard of biogenic amines and amino acids ($c=125$ μM) after derivatization with DNBZ-Cl. For chromatographic conditions see Section 2. A, DNBZ-Cl; X, from the reagent. Peaks not assigned are unknown compounds. Amino acids are designated with asterisks. A_{260}, absorbance at 260 nm.

Table 3
Results of repeatability test for retention times ($n=10$), peak areas ($c=250$ μM, $n=5$) and linearity test (six concentrations, $n=3$) for DNBZ derivatives of biogenic amines (BAs)

BA	Retention time		Peak area		Equation of linearity curve[a]	B
	(min)	RSD (%)	(mAU s)	RSD (%)		
Him	23.61	0.86	145.88	5.57	$y=0.457x-2.350$	0.996
Ocp	31.64	0.67	65.66	5.42	$y=0.292x+3.276$	0.990
Seo	43.45	0.41	62.88	6.22	$y=0.401x+3.133$	0.965
Tym	45.83	0.45	105.57	3.18	$y=0.453x-0.776$	0.996
Put	58.57	0.21	251.57	2.21	$y=1.074x-3.456$	0.990
Trm	59.80	0.24	94.55	3.06	$y=0.275x-2.237$	0.993
Cad	60.51	0.25	232.44	3.50	$y=0.944x+3.094$	0.976
Pea	61.08	0.25	218.83	9.54	$y=0.563x+4.470$	0.993
Agm	61.38	0.28	238.31	6.13	$y=0.420x+7.003$	0.993
Dhx	63.82	0.27	225.47	2.69	$y=0.930x+2.717$	0.994
Spd	65.65	0.27	289.57	5.77	$y=1.200x-3.598$	0.989
Spm	67.92	0.08	155.04	3.92	$y=0.660x-1.319$	0.989

[a] Equation of the linearity curve with y = peak area (mAU s) and x = concentration (μM). B, coefficient of determination. RSD, relative standard deviation (%).

an easier identification of the molecular masses and the order of derivatization [see Fig. 3(a) and (b)]. Monosubstituted DNBZ derivatives were formed from Ocp, Pea, Seo, Trm and Tym, bis-substituted from Agm, Him, Put, Cad and Dhx, tris-substituted from Spd, and tetrakis-substituted from Spm. From the data it is concluded that each primary and secondary amino groups of the di- and polyamines are singly derivatized. Derivatization of Seo lead to a number of peaks under HPLC–ESI-MS conditions among which only the monosubstituted DNBZ-Seo at $m/z=369.7$ could be assigned. As is characteristic for imidazolyl derivatives, fragment ions of DNBZ-Him are of low abundance under conditions of negative ESI-MS. Only the monosubstituted derivative could be detected, but no corresponding ions for bis- or tris-substituted derivatives were observed.

3.4. Amounts of BAs in food

The BAs determined in fermented cabbage juices, soy sauces, Misos, and fish products are listed in Table 5, and selected chromatograms are shown in Fig. 4(a)–(d). Addition of Dhx as internal standard

Table 4
Detected DNBZ derivatives and calculated molecular mass ($M_{r,\,cal}$) of BAs [$(DNBZ)_n$BA][a]

$(DNBZ)_n$BA	$M_{r,\,cal}$ (g mol^{-1})	Molecular ions (m/z)		
		$[(DNBZ)_n BA]^-$	$[(DNBZ)_n BA]_2^-$	$[(DNBZ)_n BA]_3^-$
$(DNBZ)_1$Ocp	347.0	346.8	693.2	1039.6
$(DNBZ)_1$Pea	315.0	314.9	629.2	–
$(DNBZ)_1$Seo	370.0	369.7	739.3	–
$(DNBZ)_1$Trm	354.0	353.8	707.6	–
$(DNBZ)_1$Tym	331.3	330.7	661.5	991.7
$(DNBZ)_2$Agm	498.2	499.8	999.2	–
$(DNBZ)_2$Cad	490.0	489.9	979.4	–
$(DNBZ)_2$Dhx	504.0	503.8	1007.4	–
$(DNBZ)_2$Him	499.0	499.4[b]	–	–
$(DNBZ)_2$Put	476.0	476.0	951.4	–
$(DNBZ)_3$Spd	727.0	727.1	1454.1	–
$(DNBZ)_4$Spm	978.0	978.0	–	–

[a] n, order of derivatization. $(DNBZ)_n$BA ($n=1$, 2, 3, 4), mono-, bis-, tris- or tetrakis-substituted derivative.
[b] Signal of low abundance.

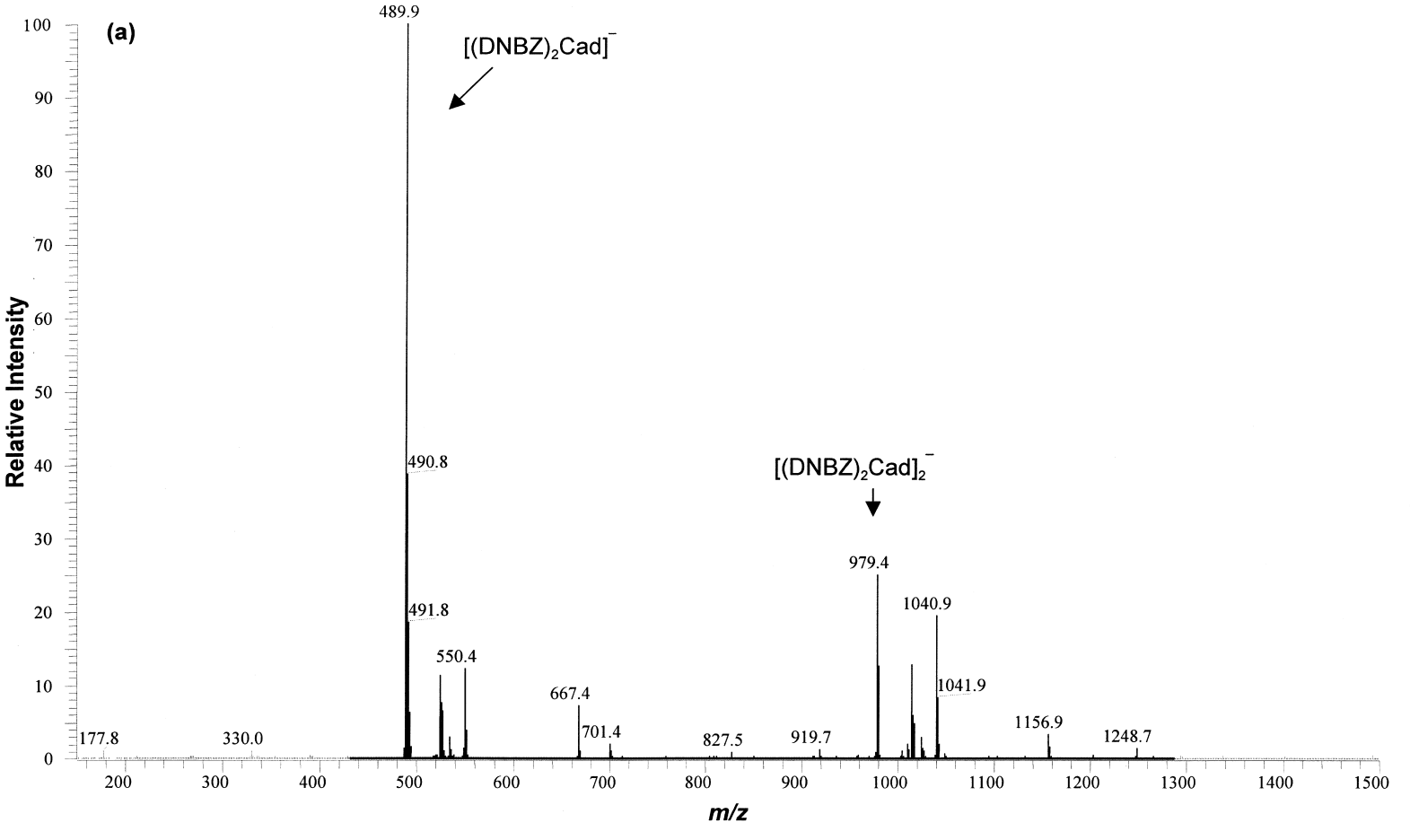

Fig. 3. (a) Electrospray ionization mass spectrum of the derivative $(DNBZ)_2Cad$. (b)–(d) Chromatograms of selected masses of the derivatives formed from the reaction of DNBZ-Cl and Cad: (b) selected ion range of the $[(DNBZ)_2Cad]^-$ fragment at $m/z=485–495$; (c) selected ion range of the $[(DNBZ)_2Cad]_2^-$ cluster at $m/z=975–985$; and (d) selected ion range of the $[(DNBZ)_1Cad]^-$ fragment at $m/z=290–300$ (not detected). For experimental conditions see Section 2 and Table 2(c).

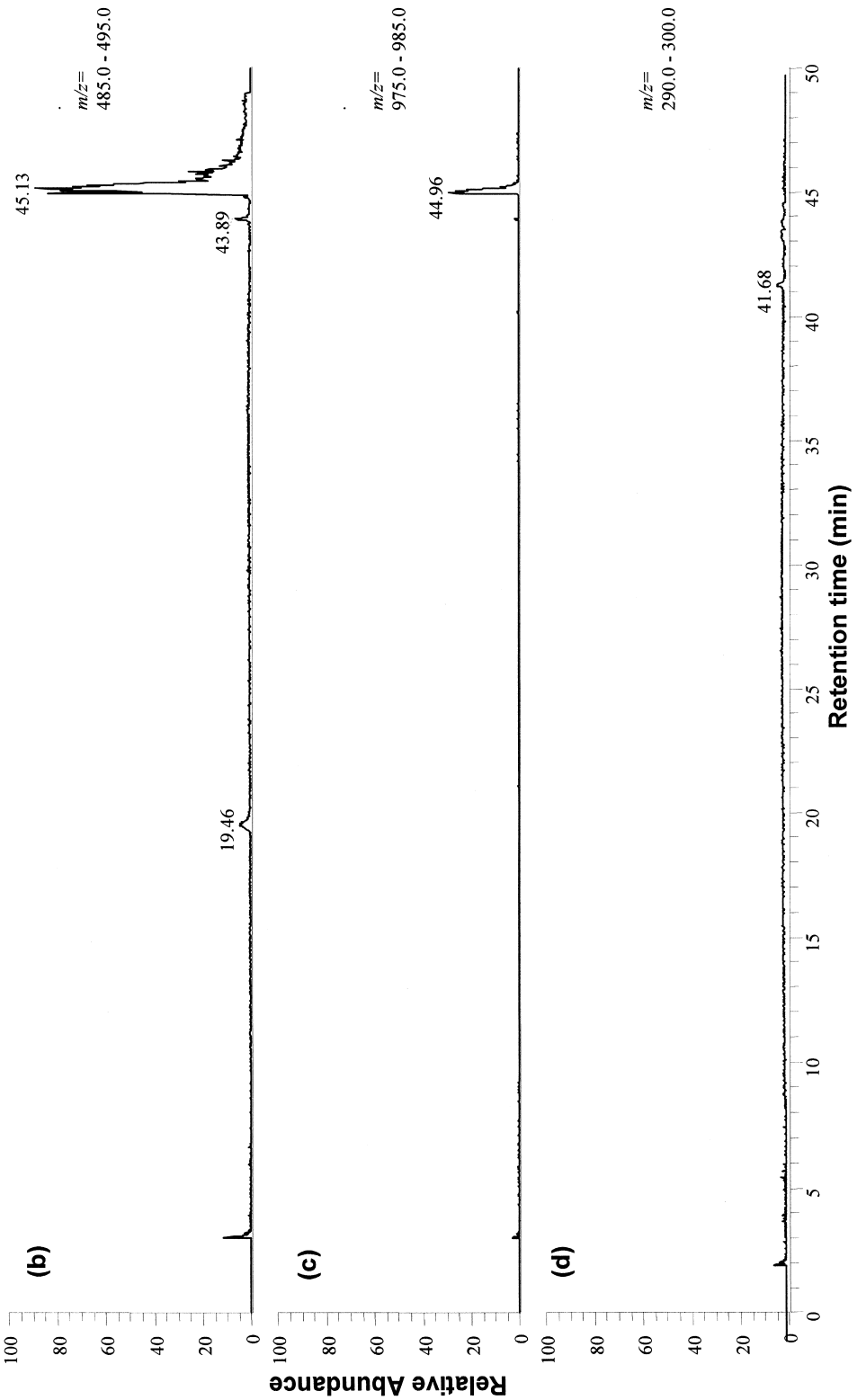

Fig. 3. (*continued*).

Table 5
Amounts of biogenic amines (BAs) in mg l^{-1} (Samples 1–9 and 15–16) or mg kg^{-1} (Samples 10–14 and 17)[a]

BA	Fermented cabbage juices					Soy sauces				Miso					Fish products		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Him	41.5	50.8	38.6	83.7	62.9	45.7	157	34.9	43.2	31.1	389	19.4	N.D.	136	757	721	31.3
Ocp	69.9	38.6	23.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	78.1	N.D.	N.D.
Seo	N.D.	N.D.	N.D.	N.D.	N.D.	168	9.1	N.D.	N.D.	N.D.	8.6	N.D.	N.D.	40.2	N.D.	N.D.	N.D.
Tym	73.0	37.1	50.4	64.3	37.9	89.2	172	28.1	17.7	40.4	215	81.6	24.6	349	357	276	20.5
Put	366	142	83.5	242	247	46.7	52.3	15.8	17.3	22.0	93.1	58.8	15.0	316	108	197	6.1
Trm	99.6	N.D.	49.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	739	337	N.D.
Cad	28.0	N.D.	N.D.	59.4	18.9	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	29.0	285	108	N.D.
Pea	50.8	N.D.	N.D.	N.D.	N.D.	7.6	68.5	N.D.	N.D.	N.D.	6.1	N.D.	N.D.	155	33.4	N.D.	N.D.
Agm	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	30.4	N.D.	N.D.	N.D.	N.D.
Spd	96.9	59.7	35.3	10.9	9.3	25.0	23.4	25.6	8.3	6.2	13.7	N.D.	N.D.	6.5	17.7	20.7	13.6
Spm	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Σ	826	328	280	460	376	382	482	105	86	100	726	160	70	1032	2374	1660	72

[a] $n=2$; N.D., not detected; Σ, total of BAs determined.

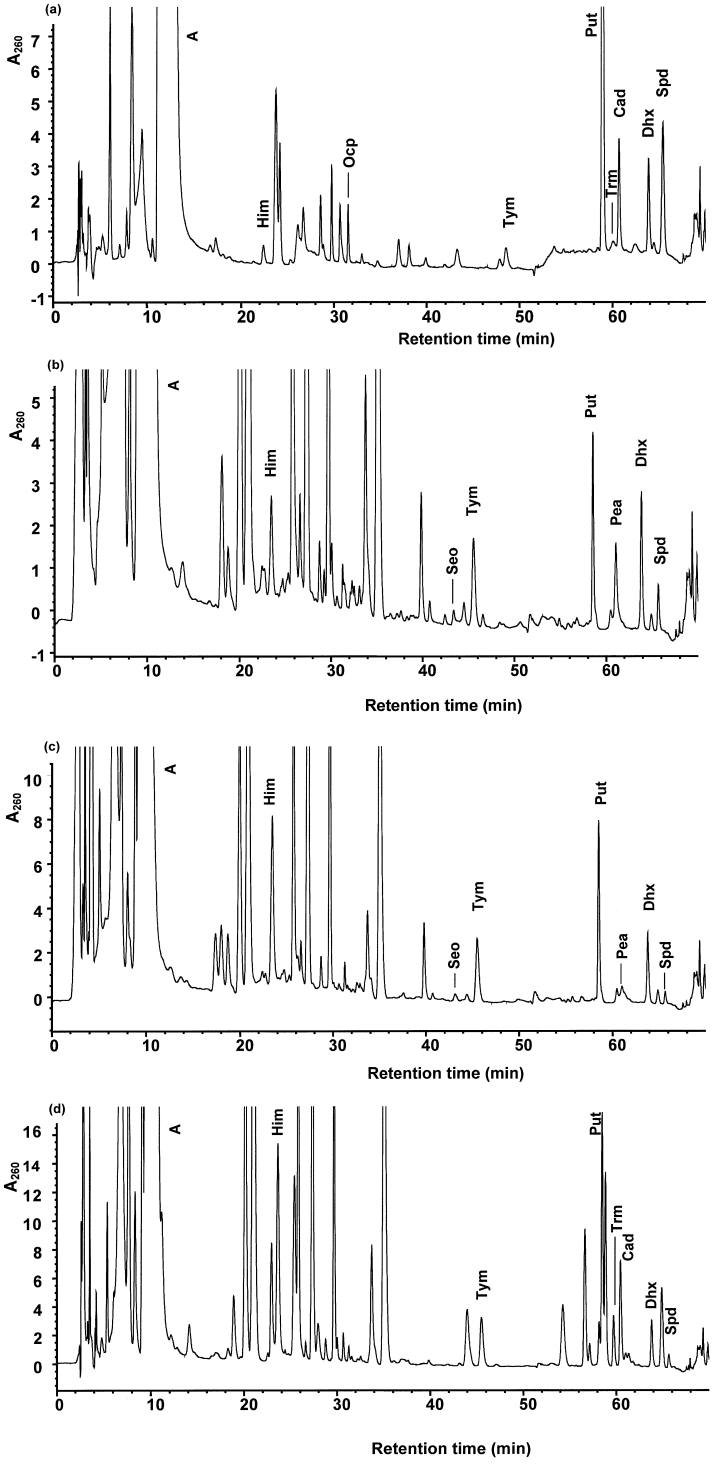

Fig. 4. HPLC of foodstuffs after derivatization with DNBZ-Cl: (a) fermented cabbage juice (Sample 1); (b) soy sauce (Sample 7); (c) Miso (Sample 11); and (d) fermented fish sauce (Sample 16). For characterization of samples and chromatographic conditions see Section 2. A_{260}, absorbance at 260 nm.

made possible the determination of recovery rates, ranging from 65 to 97%, depending on the sample investigated.

A good example for the demonstration of the increase of amounts of BAs in the course of bacterial fermentation is the lactic fermentation of cabbage juice. For this process, mixed cultures of *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp. and *Pediococcus* spp. are used. Large quantities of Put (amounting to 366 mg l^{-1}) as well as Him (39 – 63 mg l^{-1}), Tym (37–73 mg l^{-1}), and Spd (9 – 97 mg l^{-1}) were detected in all samples [for a chromatogram of sauerkraut juice Sample 1 see Fig. 4(a)]. Amounts of Him and Tym result from decarboxylation of histidine and tyrosine by the microorganisms used for the lactic fermentation [14,39]. The data agree favorably with previous investigations of sauerkraut juices [19], the contents of Tym (37–73 mg l^{-1} vs. 30–69 mg l^{-1} in Ref. [19]) were very similar. Samples 3–5 contain almost identical amounts of Him. In the cases of the Samples 1 and 2, the amounts from analyses using the DNBZ-Cl method are different compared to the analyses carried out with PNZ-Cl [19]. Quantitative data resulting from the DNBZ-Cl method are assumed to be more reliable considering the fact that DNBZ-Cl forms one derivative with Him, whereas PNZ-Cl yields two derivatives. Deviating amounts of Put with both methods were probably the result of dilutions of the samples before derivatization, required prior to the analyses.

Soy sauce and Miso are the most important among many fermented foods consumed for millenia in Asian countries. Traditional soy sauce is made by fermentation of soy beans, with or without the addition of rice or wheat. As starters the Koji moulds *Aspergillus oryzae* or *Aspergillus soyae*, the yeast *Saccharomyces rouxii*, and lactic acid bacteria such as *Pediococcus halophilus* and *Streptococcus faecalis* are used [40–44]. Studies have shown that BAs in fermented soy products are most likely formed by the lactic microflora active during fermentation [14,45]. The BAs Him, Tym, Put and Spd were detected in all soy sauces. Sample 7 showed the highest amounts of Tym (172 mg l^{-1}) and Him (158 mg l^{-1}). Total amounts of BAs in the samples investigated were lower than those reported in the literature [14,45,46]. In Samples 6 and 7, representing mixtures of fermented and hydrolyzed soy sauces, amounts of BAs were approximately four to five times higher in comparison to fermented soy sauces Samples 8 and 9. This can be explained by the different manufacturing processes for industrial and laboratory-made soy sauces. Notably, sensations and symptoms such as headaches, flushing and palpitations, known as 'Chinese Restaurant Syndrome', formerly attributed to excessive use of monosodium glutamate, are now attributed to the occurrence of large quantities of BAs, as well as other components, in particular allergic proteins in soy sauces [47].

Miso is a paste made of soybeans, rice or barley using Koji with the moulds *Aspergillus oryzae* or *Aspergillus soyae*, the yeasts *Saccharomyces rouxii* and *Torulopsis* spp., and lactic acid bacteria such as *Pediococcus halophilus*, *Pediococcus cerevisiae*, and *Streptococcus faecalis* [44]. In contrast to soy sauce fermentation, proteolysis is limited. Strongly varying amounts of Tym and Put were detected in all Miso samples. Samples 11 and 14 had remarkably high total amounts of BAs (726 mg kg^{-1} and 1032 mg kg^{-1}, respectively). The amount of Him in Sample 11 (389 mg kg^{-1}) was very high, whereas in Sample 13 no Him was detected. In Miso Sample 14 exceptionally large quantities of Tym (349 mg kg^{-1}) and Put (316 mg kg^{-1}) could be detected. Such concentrations of BAs might be harmful to people taking monoamine oxidase (MAO) inhibitors [7]. It was reported that in most Miso samples Put, but no Him, was analyzed. In contrast, in all Misos investigated by us Put was detected, and in four out of five samples Him was detected [48].

Fermented fish sauces are popular in Asian countries as liquid spices. Various fish pastes are also made from different species of fish [49]. The fermentation process is mainly carried out by halophilic microorganisms, which partially belong to the native microflora of fish. The fish sauces investigated show very high amounts of Tym, Cad, Put, Trm and Him (BAs all exceeding 100 mg l^{-1}). The high amounts of Put, Cad and Tym, which usually are indicators for very long storage or spoilage, are indicators for proteolysis [5,7,50]. Microbial fermentation of Samples 15 and 16 is indicated by high quantities of BAs. In contrast, an anchovy paste (Sample 17), which was not fermented, showed much lower

amounts of BAs (see Table 5). The calculated BAI values according to Karmas [5] (61.5 for Sample 15, and 47.4 for Sample 16) for both samples are indicators of low quality. Furthermore, the very high values of these BAIs, and the high absolute amounts of Him and Tym in both samples, might possibly cause intoxication through consumption of these products.

4. Conclusions

The reagent DNBZ-Cl was found to be highly suitable for the quantitative determination of dietary BAs using Dhx as the internal standard. The derivatives formed are well resolved on an octadecylsilyl encapsulated stationary phase using a ternary gradient system. The method shows good repeatability of retention times and excellent linearity of derivatization. UV detection at 260 nm also allows the determination of Trm and Seo, which is not possible by derivatization with the fluorescent reagents FMOC-Cl or NOC-Cl as a result of fluorescence quenching.

Further advantages of this method are the commercial availability of DNBZ-Cl and fast reaction with BAs at ambient temperature leading to well-defined stable derivatives in most cases in comparison to derivatization with oxycarbonyl chlorides, which might result in the formation of two or more derivatives in the cases of Tym or Him. The standard of BAs and amino acids is completely, or almost completely, separated. Therefore, a special sample work-up for the separation of BAs from amino acids is not required. The excellent resolution, however, is at the expense of a relatively long run-time. Further, the stability of the reagent for several months in the freezer, its reasonable price and the high purity are worth noting. A disadvantage is the appearance of an intensive signal in the chromatogram resulting from the reagent.

The quantitative data also demonstrate that fermented foods in general should be analyzed for amounts of BAs. The so-called Asian or Oriental fermented foods, which attract increasing acceptance also in Western cuisine, have to be taken into account as rich sources of BAs. In order to prevent formation of high amounts of BAs in fermented foods, selection of suitable starter microorganisms with low amino acid decarboxylase activity is necessary.

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