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Journal of Chromatography B, 779 (2002) 229–239

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Comparison of a capillary electrophoresis method with high-performance liquid chromatography for the determination of biogenic amines in various food samples

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Received 14 February 2002; received in revised form 2 May 2002; accepted 23 May 2002

Abstract

A capillary electrophoresis (CE) and a high-performance liquid chromatography (HPLC) method to analyze biogenic amines in food were compared. An automated precolumn derivatization with *o*-phthaldialdehyde (OPA) allows for the determination of aliphatic amines and amino acids by HPLC. In contrast, for the measurement of histamine and tyramine by CE, no laborious sample pretreatment was necessary. The biogenic amines were separated by CE or HPLC in less than 9 or 20 min, respectively. The calibration curves were linear to at least 100 mg/kg ($r=0.999$) and 1000 mg/kg for HPLC and CE, respectively, with detection limits for histamine of 0.5 mg/kg (fluorescence detector) or 1 mg/kg (diode array detector) with HPLC and 2 mg/kg with CE. The detection limits for tyramine were 1.5 mg/kg with HPLC and 6 mg/kg with CE and for further amines (e.g., putrescine, spermidine, cadaverine, agmatine) ranging from 1.0 to 8.5 mg/kg with HPLC. There was a good correlation between CE and HPLC (correlation coefficient for histamine: 0.994).

Keywords: Biogenic amines; Tyramine

1. Introduction

Biogenic amines are organic bases of low-molecular mass comprising aliphatic, mono-, di- and polyamines, catecholamines, indolyl and imidazolyl amines. As their formation and metabolism occurs widely in living organisms they are also present in a variety of different foods, primarily as a consequence of microbial amino acid decarboxylation. Proteolytic processes take place, in general, during preparation, ripening and storage of food high in protein content (e.g., fish, meat, dairy products). Low levels of biogenic amines in food are not considered a serious risk. However, if the amount consumed is high enough or normal routes of amine catabolism are inhibited various physiological effects may be the consequence such as hypotension (in the case of

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00372-0

histamine, putrescine, cadaverine) or hypertension (as in the case of tyramine), nausea, headache, rash, dizziness, cardiac palpitation and emesis, and even intracerebral hemorrhage, anaphylactic shock syndrome and death, in very severe cases [1]. A considerable interest in the investigation of biogenic amines is focused on the neurotransmitter histamine. Therefore, the 'Fischhygieneverordnung' [2] in Germany prescribes a maximum permissible limit for histamine in fish and fish products of 200 mg/kg. If kept under temperatures of below 4 °C a histamine concentration in fish muscle flesh exceeding 50 mg/kg could be detected rather rarely. However, higher histamine levels might occur and are most often combined with the so-called scombroid fish poisoning derived from fish belonging to the family Scombridae and Scomberesocidae (such as tuna and mackerel) or nonscombroid fish (such as mahimahi, bluefish, sardines, pilchards, anchovies, herring and black marlin) as otherwise called histamine poisoning. In scombroid fish poisoning, histamine is taken as an indicator compound although the presence of further biogenic amines is known to prolong the symptoms due to a competitive inhibition of intestinal enzymes metabolizing histamine. Analysis of certain biogenic amines in food is a necessity to assess potential health hazards before consumption. Several methods to analyze biogenic amines in food have been described so far, including thin-layer chromatography, the use of amino acid analyzers, liquid chromatography [3–8], gas chromatography [9], and even several biochemical assays [10–12]. The method routinely used for histamine [13] involves an extraction with methanol, subsequent ion-exchange chromatography, and a chemical reaction with OPA under defined conditions to measure the resulting fluorescent reaction products. A major drawback of this official method for control of seafood for histamine contents is analysis speed and the requirement for defined conditions with regard to sample handling. HPLC methods use a pre- or post-column derivatization step to facilitate detection because the majority of the biogenic amines does not possess chromophoric or fluorophoric moieties, in general. Many of these derivatization procedures are time-consuming. Therefore, the main goal was to establish a rapid analysis system for the food processing industry taking into account the analysis of different food samples with only minor sample pretreatment required.

A modified HPLC method on the basis of an automated pre-column derivatization described by Petridis and Steinhart [14] was established. In addition, CE methods [15,16] were studied. Both HPLC and CE methods were optimized in terms of sensitivity, reproducibility, linear range, accuracy, precision and efficiency.

2. Experimental

2.1. Chemicals

The amine standards (as free bases or their hydrochlorides) histamine (His), tyramine (Tyr), spermidine (Spd), spermine (Spm), cadaverine (Cad), putrescine (Put), and agmatine (Agm), the OPA reagent (purified and concentrated), trichloroacetic acid (TCA), mercaptoethanol (2-ME) and triethylamine (TEA) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Boric acid, the HPLC-grade solvents methanol, isopropanol, *n*-butanol, *n*-heptane, tetrahydrofuran, and acetonitrile, were from Mallinckrodt Baker (Griesheim, Germany). All other chemicals were of reagent grade. The CE water was purchased from Hewlett-Packard (Waldbronn, Germany) and the water used for eluent preparation (HPLC) was purified with a SG Reinstwassersystem (SG, Barsbüttel, Germany).

The HPLC eluents were either used immediately after preparation or stored at −20 °C and filtered before use (RC-type, 0.2 μm, Sartorius, Göttingen, Germany).

2.2. Equipment

The HPLC system HP1100 series from Hewlett-Packard, equipped with the ChemStation software comprised the following modules: a high pressure gradient pump (binary or quaternary), an online vacuum degasser, an autosampler, a thermostated column compartment, a diode array detector (DAD) and a fluorescence detector (FLD).

For CE analyses a 3D-CE device from Hewlett-Packard, with a DAD including the ChemStation software for data analysis was applied.

An Ultra-Turrax T25 blender from IKA-Werk (Jahnke & Kunkel, Staufen, Germany), and a Hettich Universal 30 RF centrifuge from Hettich Zentrifugen (Tuttlingen, Germany), were used for sample preparation. In addition, BakerBond cartridges (Bakerbond spe RP-18 or CBA, both 3 ml) and a spe-12G device both from J.T. Baker (Mallinckrodt Baker, Griesheim, Germany), were used for sample cleanup.

2.3. Preparation of standard amine solution

Stock solutions containing 1000 mg/l biogenic amines (histamine, putrescine, cadaverine, tyramine, spermidine, spermine, agmatine) either in 0.1 *M* HCl or 5% TCA were prepared. Solutions containing each biogenic amine in concentrations of 0.5, 1, 5, 10, 50, 100, 200, and 500 mg/l were obtained by appropriate dilutions of the stock solution in 0.1 *M* HCl or 5% TCA, respectively. 1,7-diaminoheptane was used as internal standard (I.S.) for HPLC analysis.

2.4. Samples

Table 1 shows the food samples and the solvents which were used for extraction of the biogenic amines. For some of the samples, different extraction procedures were studied. All extracts were stored at 4 °C and filtered through a membrane filter (RC-type, 0.2 μm, Sartorius, Göttingen, Germany) prior to HPLC or CE analysis.

Table 1
Food samples and solvents used for extraction of the biogenic amines

Food sample	Extraction solution
Fish:	
Salmon	TCA, HCl, methanol
Herring	TCA
Cod	TCA
Cheese:	
Roquefort	HCl, TCA, methanol
Gorgonzola	HCl
Edamer	HCl
Meat products:	
Salami	TCA, HCl, methanol
Ham	TCA
Onion sausage	TCA
Vegetarian products:	
Olives in oil	Water, methanol, TCA, HCl
Tomatoes in oil	TCA
Canned sauerkraut	TCA

Storage experiments for 7 days comprised two different temperatures: 4 °C for salmon, ham, and Edamer cheese and room temperature for the onion sausage, salami, canned sauerkraut, and olives and tomatoes under oil.

2.5. Sample pretreatment

Following solvents were studied according to their suitability for amine extraction from food: 0.1 *M* HCl, two different concentrations (5%, 10%) of trichloroacetic acid (TCA), methanol in water (50 and 75%), and 100% methanol.

All food samples were homogenized prior to the extraction using a mill or a blender. Ten grams of each food sample (with exception of the cheese samples) were extracted twice with 25 ml of the respective solvent and treated with an Ultra-Turrax at 6433 *g* (8000 rpm) for 3 min. After centrifugation for 10 min at 1609 *g* (4000 rpm) the supernatants were filtered through a filter paper (595 1/2 type, Schleicher & Schuell, Dassel, Germany), combined and made up to 50 ml with the respective solvent.

The cheese samples were treated according to a method as described by Vale and Gloria [17]. Ten grams of grated cheese were suspended in 20 ml 0.1 *M* HCL and mixed in a Vortex for 5 min. Subsequent to a centrifugation step (3619 *g* for 30 min at room temperature) the supernatant was collected. The extraction was repeated with 20 ml of 0.1 *M* HCl for three times. The supernatants were combined and stored at 4 °C to crystallize most of the fat. The agglomerated fat layer was removed, and the supernatant was filtered.

For CE measurements the extracts prepared as described were injected directly after membrane filtration (RC-type, 0.2 μm, Sartorius). For HPLC analyses a neutralisation step (adjusting the pH to 6–7) was necessary. In addition, further purification prior to the analysis is required consisting of either liquid–liquid extraction (LLE) with butanol–*n*-heptane or solid-phase extraction (SPE).

2.5.1. Liquid–liquid extraction (LLE)

The LLE procedure as described by Mahendradatta and Schwedt [18] was modified. One ml sample extract was added to a mixture of 0.25 ml of 5 *M* NaOH, 0.75 g NaCl and 5 ml *n*-butanol. After shaking this mixture for 3 min and centrifugation at 1609 *g* (4000 rpm) for 10 min the *n*-butanol layer was separated. The aqueous layer (1.25 ml) was extracted again with 5 ml of *n*-butanol. The *n*-butanol of every extraction step was transferred in another tube containing 5 ml 0.1 *M* NaOH saturated with NaCl. To eliminate any free histidine this second centrifuge tube was shaken and centrifuged in the same way as before. An 8-ml aliquot of the butanol extract was transferred to a third tube containing 2.5 ml of 0.1 *M* HCl and 7 ml *n*-heptane, shaken for 1 min and subsequently centrifuged. After removing the organic layer the acidic aqueous layer (2.5 ml) containing the biogenic amines was analyzed.

2.5.2. Solid-phase extraction (SPE)

A RP-18 BakerBond cartridge (volume 3 ml) was used for SPE. After preconditioning of the column with 3 ml methanol followed by three column volumes of distilled water and 3 ml of 1 *M* HCl, 3 ml of the sample extract were applied onto the column. A washing step with 0.1 *M* potassium citrate buffer (with the volume required depending on the food matrix) was followed by evacuation of air in the column. Elution was performed with 3 ml of 0.1 *M* potassium citrate buffer–isopropanol.

2.6. Recovery studies

Recovery studies for CE and HPLC were performed with food samples (fish, cheese, sauerkraut) spiked with 20, 50, 100, 200 or 1000 mg/kg of histamine or tyramine.

2.7. HPLC conditions

An automated pre-column method using *o*-phthaldialdehyde (OPA) and mercaptoethanol (2-ME) was applied to reduce the time needed for HPLC analysis [19]. The conditions for HPLC measurement are given in Table 2.

2.8. CE conditions

For CE analysis a method modified from Mahendradatta and Schwedt [20] was applied. Two running buffers (sodium citrate and sodium phosphate buffer) at pH 2.5 and 6.5 were compared. In addition, two different separation temperatures, 25 and 35 °C, were tested. The conditions for CE separation are listed in Table 3.

3. Results

Several extraction methods and cleanup steps prior to HPLC and CE were compared in terms to shorten the overall analysis time.

3.1. Extraction and sample cleanup

Three solvents were studied according to their suitability for amine extraction from food: methanol (50, 75 and 100%), hydrochloric acid and trichloroacetic acid (5 and 10%). With 100% methanol the lowest signal/noise ratios and the highest peaks could be observed. An extraction with 10% TCA was less useful because of high signal/noise ratio and low peak resolution. On the other hand, the use of 5% TCA resulted in the best peak resolutions. Therefore, 5% TCA was used normally for extraction of the food samples (except cheese, see Table 1). Fig. 1 shows the HPLC chromatograms of a standard mixture of biogenic amines and of a cod extract containing trace amounts of histamine, tyramine, putrescine, and cadaverine. The presence of histamine in this sample could only be assumed. However, further sample cleanup subsequent to the extraction step appeared to be necessary. In general, this sample cleanup can be performed by column chromatography with ion-exchange resins, solid-phase extraction (SPE) or liquid–liquid partitioning (LLE). SPE required a number of single steps (activation or equilibration, washing, and elution) which are time consuming. Consequently, the recovery rates for histamine, tyramine, cadaverine, and putrescine using 1,7-diaminoheptane as internal standard after LLE were examined. After LLE the recovery rates were nearly 100% for histamine, 76% for tyramine, 94% for putrescine, and 100% for

Table 2
HPLC conditions

Column	LiChrospher RP 18, 250×4 mm, 5 μm	
Precolumn	LiChrospher RP 18, 4×4 mm, 5 μm	
Eluent	Phase A: 20 m*M* sodium acetate, 0.01% triethylamine (pH 7.2 adjusted with 1–2% acetic acid), 0.3% tetrahydrofuran Phase B: 20% 100 m*M* sodium acetate (adjusted to pH 7.2 with 1–2% acetic acid), 40% acetonitrile, 40% methanol	
Flow-rate	0.9 ml/min	
Gradient	Time (min)	Mobile phase B (%)
	0	30
	5	70
	16	100
	22	100
	23	30
Injector program	1 draw 5 μl from vial 1 2 draw 1 μl from vial 1 3 draw 0 μl from vial 1 4 draw 1 μl from vial 1 5 draw 0 μl from vial 1 6 mix 8 μl in seat, maximum speed, six times 7 injection of 1 μl With Vial 1 filled with 1 ml 0.4 N borate solution, adjusted to pH 10.4 with KOH Vial 2 filled with 1 ml water (HPLC grade) Vial 3 filled with the derivatization reagent prepared from 10 mg OPA, 1 ml 0.4 M borate solution (pH 10.4), 3 μl mercaptoethanol, 10 μl methanol, mixed and filtered through a membrane filter (RC-type, 0.2 μm, Sartorius, Göttingen, Germany	
Detection	(a) DAD at wavelengths 210 nm, 338 nm (b) FLD excitation wavelength 340 nm, emission wavelength 455 nm	
Total analysis time	35 min (post-time 5 min included)	

cadaverine (see Fig. 2). In sample extracts, the histamine assignment was only possible by application of the DAD.

For CE measurements sample purification by LLE could be omitted as shown by the electropherograms in Figs. 3 and 4. As no derivatization step was performed prior to CE histamine and tyramine were detected only by the DAD.

3.2. HPLC separation

Histamine, tyramine, putrescine, and cadaverine could be well resolved on the LiChrospher column. In the case of histamine separation amino acids which react also with OPA/2-ME might lead to interferences because they were eluted mainly during the first 8 min. Agmatine (t_R=10.2 min) and spermidine (t_R=14.9 min) were detectable with a small tailing. Only spermine was not detectable using the pre-column derivatization with OPA/2-ME.

3.3. CE separation

One important parameter for CE is the buffer system, especially the pH applied. To optimize the buffer system, a herring extract containing 0.5 mg/kg histamine was used. The extract was measured comparing a citrate and a phosphate buffer by pH

Table 3
CE conditions

Capillary	HP extended light path capillary (length 56 cm, inner diameter 50 μm)	
Cassette temperature	25 °C	
Working buffer	(a) 20 m*M* sodium citrate buffer, pH 2.5 and 6.5 (b) 25 m*M* sodium phosphate buffer, pH 2.5 and 6.5	
Preconditioning of the capillary	Flush 1 min with 0.1 N NaOH, 1 min with water, 2 min with working buffer	
Injection by pressure	25 mbar, 10 s	
Polarity	Positive (power 4 W, current 100 μmA)	
Gradient	Time (min)	Voltage (kV)
	0	0
	0.20	10
	1.20	25
Detection	DAD with wavelengths 210, 214 and 320 nm (spectra from 190 to 600 nm)	
Total analysis time	15 min (post-time 5 min included)	

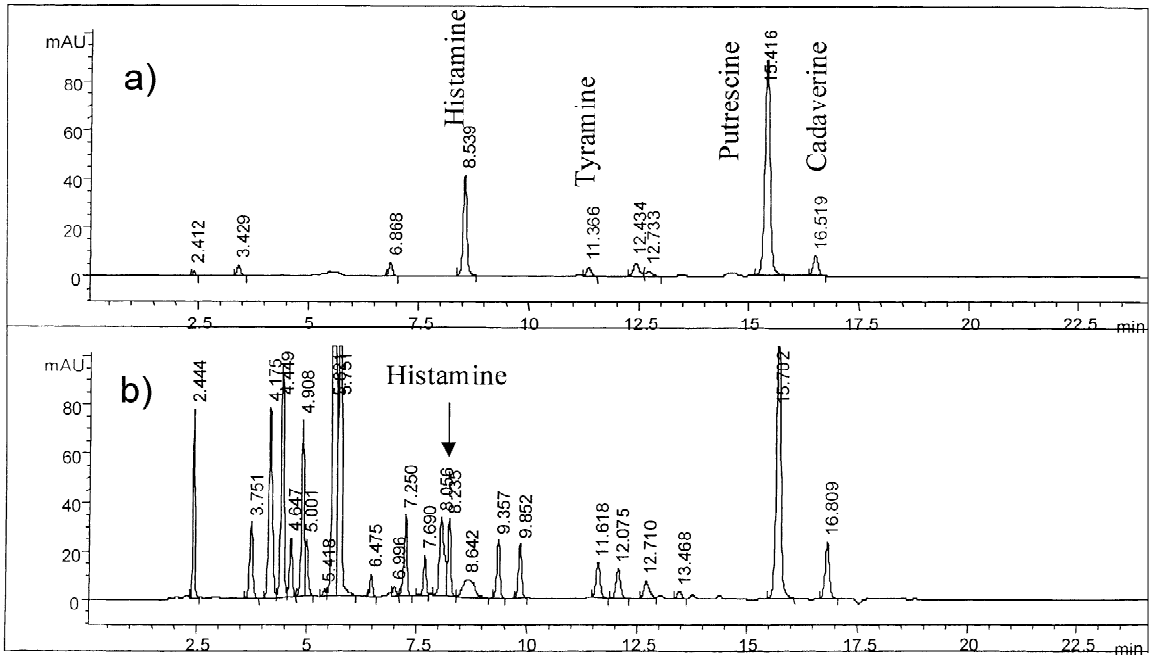

Fig. 1. HPLC separation of biogenic amines after extraction with 5% TCA, pre-column derivatization with OPA/2-ME, and UV detection (for chromatographic conditions see Table 2). (a) Standard solution, containing 1.0 μg/ml of histamine, tyramine, putrescine, and cadaverine. (b) TCA extract of a cod sample.

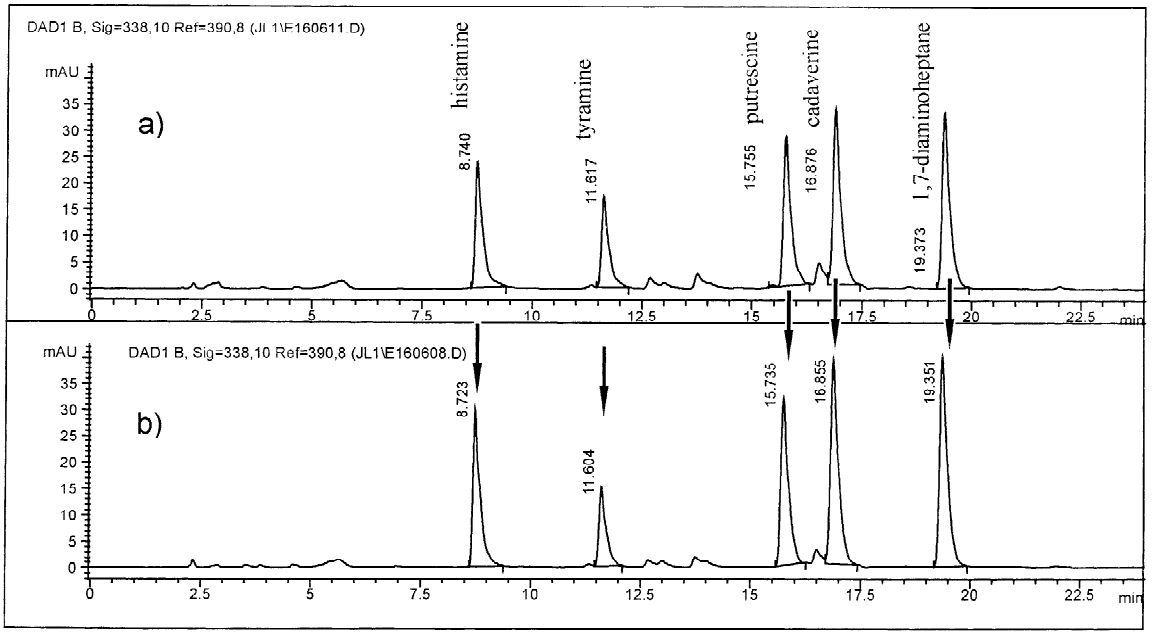

Fig. 2. HPLC separation of biogenic amines (standard solution, containing 5.0 μg amine/ml). (a) Before, and (b) after purification by LLE (detector: DAD) (for chromatographic conditions see Table 2).

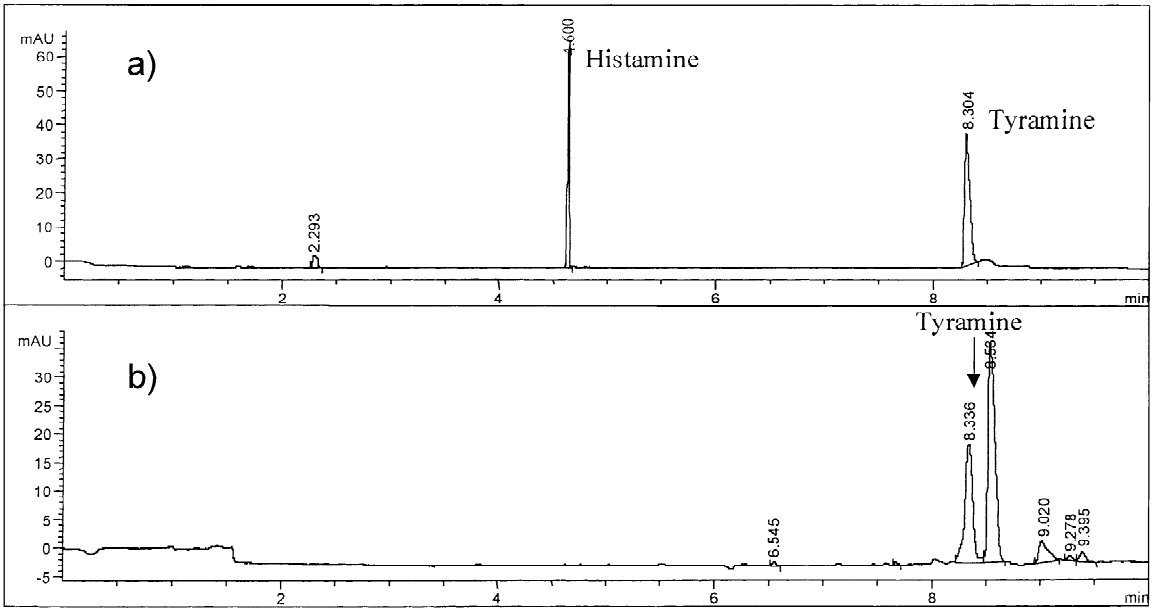

Fig. 3. CE separation of biogenic amines (for electrophoretic conditions see Table 3). (a) Standard solution, containing 10.0 μg/ml histamine and 10.0 μg/ml tyramine. (b) TCA extract of a Gorgonzola cheese sample.

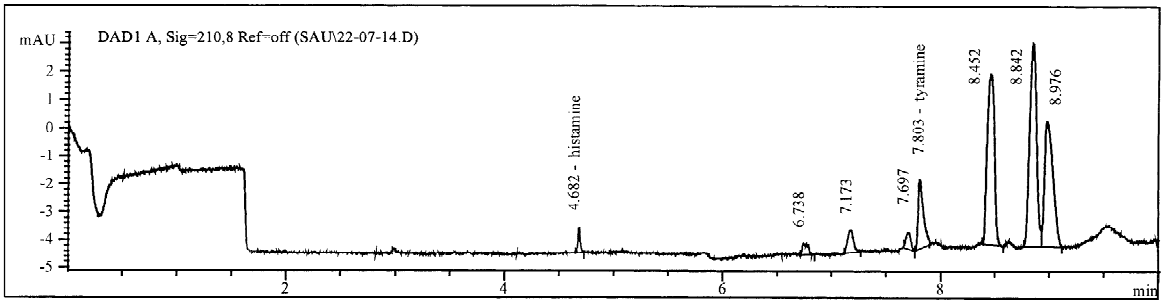

Fig. 4. CE separation of an TCA extract of canned sauerkraut (for CE conditions see Table 3).

values 2.5 and 6.5. The best result was obtained using a sodium phosphate buffer at pH 2.5. Histamine and tyramine dissolved in 5% TCA had migration times of 4.600 and 8.304 min, respectively, as shown in Fig. 3. For every sample analysis the capillary had to be fitted with two new buffer vials and a rinse of the capillary with 0.1 m*M* NaOH (1 min), water (1 min) and with the running buffer (2 min) had to be performed after every sample run. In addition, a re-calibration step had to be performed every five sample runs to overcome migration time differences. Only underivatized histamine and tyramine were detectable by CE with DAD (see Fig. 4).

3.4. Detection limit, linear range

To control the linear range of HPLC and CE method relations between the area responses of injected standard solutions to the corresponding amine concentrations were measured. The calibration curves for HPLC (carried out by 10 single measurements) and for CE (five measurements at the standard solutions in two different solvents) are depicted in Fig. 5. As is obvious the lower slope/sensitivity for CE results in a higher linear range. The detection limits by application of HPLC were 1 mg/kg for histamine and 1.5 mg/kg for tyramine, for other amines ranging from 1.0 to 8.5 mg/kg. Relative

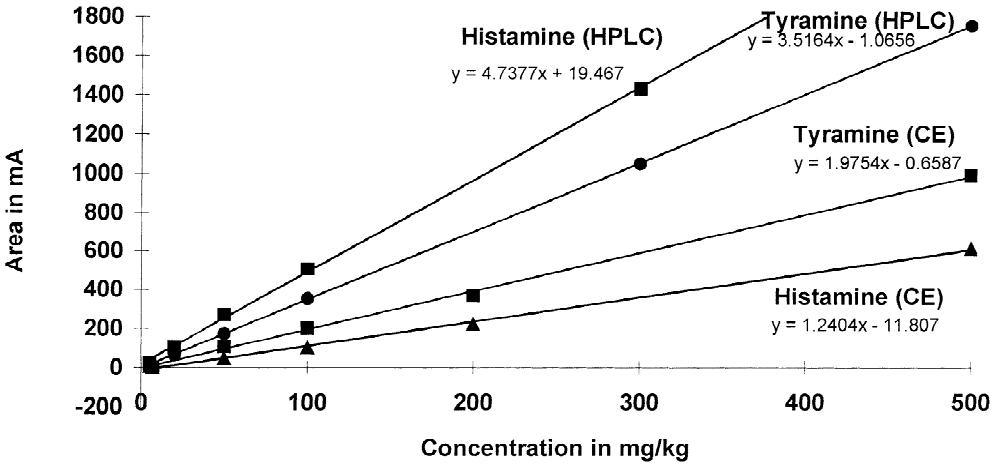

Fig. 5. Calibration curves (indicating the 2-fold RSD as error bars) for histamine and tyramine as measured by HPLC (for conditions cf. Table 2) and CE (for conditions cf. Table 3).

standard deviations (RSD) were 1.4% for range 5–100 mg/kg. CE detection limits were 2 mg/kg for histamine and 6 mg/kg for tyramine with a linear range up to 1000 mg/kg ($r=0.990$). Mean RSD values were 2% for CE within the measuring range from 5 to 100 mg/kg.

3.5. Biogenic amines in food samples

The samples were measured in duplicates or quadruplicates, respectively. The results are shown in Table 4. In this table were no samples exceeding the maximum amount of 200 mg histamine per kg in fish samples. In addition, no problems occurred for salami production during the ripening process and the control of canned sauerkraut also revealed no higher histamine levels. In the case of the Roquefort cheese it was evident that total amine content may exceed 200 mg/kg.

Some of the food samples were spiked with histamine and tyramine in concentrations ranging from 20 to 1000 mg/kg. The estimated recovery rates for the two amines in the different food were determined by HPLC and CE. From the results it could be assumed that there were no bigger differences between both methods (see Table 5).

4. Discussion

Two analytical techniques for the determination of biogenic amines in food were compared. HPLC and CE could be applied for the determination of histamine in food samples, e.g., regarding the regulation limit for histamine in fish products of 200 mg/kg. Both methods exhibited satisfying results concerning sensitivity, linear range, detection limit, reproducibility, accuracy and precision. In Table 6 the parameters of the HPLC and CE method are compared. The biogenic amines were separated in less than 9 min by CE or less than 20 min by HPLC. Detection limits of 1.0 mg/kg for histamine and further amines could be

Table 4
Histamine and tyramine contents of food samples determined by HPLC and CE

Sample	HPLC analysis		CE analysis	
	Histamine (mg/kg)	Tyramine (mg/kg)	Histamine (mg/kg)	Tyramine (mg/kg)
Fish:				
Herring	0.5[a] putrescine: 32	<d.l. cadaverine: 15	<d. l.	<d.l.
Salmon	<d.l. putrescine: 3	<d.l.	<d.l.	<d.l.
Cod	0.75[a] putrescine: 8	2 cadaverine: 10	<d.l.	<d.l.
Cheese:				
Roquefort	<d.l. putrescine: 80	152 cadaverine: 320	<d.l.	154
Gorgonzola	<d.l.	8	<d.l.	25
Edamer	<d.l.	13.5 cadaverine: 3	<d.l.	17
Meat products:				
Salami	<d.l. putrescine: 10	17	<d.l.	25
Onion sausage	<d.l.	32	<d.l.	35
Ham	<d.l.	7.5	<d.l.	10
Vegetarian products:				
Canned sauerkraut	<d.l. putrescine: 200 spermidine: 64	6 cadaverine: 25	<d.l.	<d.l.
Tomatoes	<d.l. putrescine: 7	4 cadaverine: 5	<d.l.	<d.l.
Olives	<d.l.	<d.l.	<d.l.	<d.l.

[a] Measured with FLD; d.l., detection limit. The detection limits using HPLC were: 1 mg/kg histamine (0.5 mg/kg using the FLD), 1.5 mg/kg tyramine and from 1.0 to 8.5 mg/kg for other amines as putrescine, cadaverine, agmatine, spermidine. The detection limits with CE were 2 mg/kg for histamine and 6 mg/kg for tyramine.

Table 5
Recovery rates for histamine and tyramine in several fortified food samples determined by CE and HPLC

Food	Spiked with histamine (in mg/kg)	Recovery rate in % with HPLC	Recovery rate in % with CE	Spiked with tyramine (in mg/kg)	Recovery rate in % with HPLC	Recovery rate in % with CE
Salmon	50	101	97	50	87	96
	100	97	102	100	92	97
Salami	20	100	198	50	83	100
	1000	85	90	200	94	95
Ham	100	98	95	20	78	102
	200	90	98	1000	76	97
Edamer	100	98	100	200	75	90
cheese	1000	87	96	1000	74	99
Canned	20	103	90	50	80	102
sauerkraut	50	101	101	1000	77	96

reached by HPLC compared with 2 mg/kg for histamine and 6 mg/kg for tyramine by the CE method. Higher sensitivity but reduced linear range could be reached with the HPLC method as the result of an increase in UV absorption due to the derivatization of the amines. However, the HPLC method enables the determination of putrescine, cadaverine, and spermidine in addition to histamine and tyramine which is not possible by CE without derivatization.

There are a lot of reagents which could be used for derivatization of biogenic amines [21–23]. Pre- or post-column derivatization of primary amines with OPA/RSH is often applied. Post-column derivatization with OPA/RSH is recommended due to lack of the derivatives although this could not be observed in our case. A disadvantage of post-column derivatization would be a prolongation in separation times (80–130 min) and peak broadening [24].

By application of CE method for the determination of biogenic amines the overall analysis time could be reduced as time-consuming cleanup (LLE or SPE) and derivatization of the analytes could be omitted. However, bigger differences in migration times

Table 6
Method comparison HPLC versus CE

Aspect	CE	HPLC
Sample pretreatment	Extraction required (ca. 20 min)	Extraction+neutralization+ LLE or SPE required (ca. 1 h)
Correlation with HPLC	Coefficient 0.994 (histamine) Coefficient 0.987 (tryamine)	
Analysis time (including sample pretreatment)	15 min (35 min)	30 min (90 min)
Separation time	9 min	20 min
Lower detection limit	2–6 mg/kg	1.0–8.5 mg/kg (with DAD)
Linear range	Up to 1000 mg/kg	Up to 100 mg/kg
Analytes	Histamine, tyramine	Histamine, tryamine, cadaverine, putrescine, spermidine, agmatine
Validity of analysis data	2% false-negative results	

occur if the capillary is used for a longer time-period without any equilibration. Migration times for histamine varied in different solvents as much as 1 min or even longer. Histamine standards prepared with 50% methanol showed longer migration times as compared to a histamine standard made up in distilled water. Therefore, the standards were prepared either in 5% TCA or 0.1 *M* HCl in analogy to the solvent used for the amine extraction from the respective food sample.

In conclusion, the CE method was generally favoured by the food industry, e.g., to control fish products regarding the regulation limit for histamine. The HPLC method is applied to obtain an information on the composition of the biogenic amines in the food samples. This is of importance as the presence of other amines besides histamine might intensify symptoms in the case of poisoning.

Acknowledgements

Financial support from BMBF (grant: 1700498) is gratefully acknowledged. We want to thank the local food industry for providing us with food samples for HPLC and CE measurements.

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