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Determination of biogenic amines in fish implicated in food poisoning by micellar electrokinetic capillary chromatography

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Abstract

A micellar electrokinetic capillary chromatography (MECC) method for the simultaneous determination of seven biogenic amines in fish was developed. The peaks of all components were successfully separated within 11.5 min. MECC was performed with 0.06 *M* sodium deoxycholate in 0.02 *M* borate buffer (pH 9.2)–methanol (95:5, v/v) solvent. The average recoveries for all components ranged from 84.4 to 100.3%. The application of this method to detect amines in fried marlin fillet implicated in a food poisoning incident indicated that a high level (56.24 mg/100 g) of histamine was present in the sample. Another 10 fish samples collected from markets were also analyzed and did not contain detectable levels of histamine (<2.5 mg/100 g).

Keywords: Biogenic amines; Histamine

1. Introduction

Biogenic amines are found in a wide variety of foodstuffs [1,2]. Of them, histamine is the causative agent of scombroid fish poisoning [3]. Meanwhile, biogenic amines are useful as quality indices to assess the decomposition of fish [4,5].

In a previous paper [6], we developed a high-performance liquid chromatography (HPLC)-based method for simultaneous measurement of the levels of various biogenic amines. We have also reported three incidents of scombroid histamine poisoning in Taiwan [6–8]. The fishes implicated in these episodes were sailfish (*Istiophorus platypterus*) and marlin (*Makaira mazara*), both of which belong to the Istiopheridae and are commonly consumed as raw fish slices (sashimi) and fried fillets in Taiwan. However, fishes of the Scombridae, Scomberesocidae, Pomatomidae, Coryhaenidae, Carangidae, Clupeidae and Engraulidae are usually implicated in episodes of histamine poisoning in other regions [1].

In September 1999, a serious scombroid outbreak occurred in Pingtung County, southern Taiwan. More than 256 children in a public primary school suffered from allergy-like symptoms, including rashes, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin. The implicated fish was a marlin (*M. mazara*). To elucidate the causative agent, two samples of the fried fillets collected from the school were analyzed for amine levels. Although

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HPLC is popularly applied to the determination of the concentration of biogenic amines [2,5,6,9–11], attention has recently focused on capillary electrophoresis (CE) due to its availability [12–18]. In this study, micellar electrokinetic capillary chromatography (MECC) for the determination of biogenic amines in the two fried fish fillets implicated in the food poisoning incident was first developed, and the results were compared with those obtained with a previous HPLC method [6]. Then, another 10 samples including marlin, sea bream, tuna, mackerel and their products were collected from common markets and used for the determination of biogenic amines using the MECC method.

2. Materials and methods

2.1. Samples

Two fried fillets (132 and 138 g) which were left-overs from the public primary school were stored at −20°C until use. Another 10 samples were also collected from the common markets in Pingtung County in October 1999. These samples included a marlin fillet (136 g), a sea bream fillet (152 g), a tuna fillet (241 g), three salt mackerel fillets (53, 62 and 63 g), two tuna can meats (140 and 145 g) and two mackerel can meats (130 and 145 g) of different brands.

2.2. Reagents

Standard amines, including tryptamine hydrochloride (Tpm), 2-phenylethylamine hydrochloride (Phe), putrescine dihydrochloride (Put), cadaverine dihydrochloride (Cad), histamine dihydrochloride (Him), spermidine trihydrochloride (Spd) and spermine tetrahydrochloride (Spm), were obtained from Sigma (St. Louis, MO, USA). Sodium tetraborate, sodium cholate, sodium dodecyl sulfate (SDS), sodium deoxycholate, benzoyl chloride, NaOH and perchloric acid (PCA) were purchased from Nacalai Tesque (Kyoto, Japan). Methanol and diethyl ether (LC grade) were purchased from E. Merck (Darmstadt, Germany).

2.3. Preparation and benzoylation of standard amine solution

Tpm (61.4 mg), Phe (65.1 mg), Put (91.5 mg), Cad (85.7 mg), Spd (87.7 mg), Spm (86.0 mg) and Him (82.8 mg) were dissolved in 50 ml 0.1 *M* HCl and used as the standard stock (each amine 1.0 mg/ml). A series of diluted standard solutions were prepared from standard stock and used to obtain the standard curve.

The benzoyl derivatives of amines were prepared according to our previously described method [6]. A 1-ml volume of 2 *M* sodium hydroxide and 10 μl of benzoyl chloride were added sequentially to 2 ml standard amines solution. The resulting solution was mixed with a vortex mixer and allowed to stand at 30°C for 40 min. The benzoylation was stopped by adding 2 ml saturated NaCl solution, and the solution was extracted with 3 ml diethyl ether. After centrifugation, the upper organic layer was transferred to a test tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved with 1 ml 0.06 *M* sodium deoxycholate–methanol (70:30, v/v), filtrated through a 0.45-μm filter, and then used for MECC.

2.4. Sample preparation and amine extraction

Each fillet of the dip-fried fish and meat was ground in a Waring blender for 3 min. Ground samples (5 g) were transferred to 50-ml centrifuge tubes and homogenized with 20 ml 6% PCA solution for 3 min. The homogenates were centrifuged (10 000 *g*, 10 min, 4°C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, UK). The above procedure was performed in duplicate. The filtrates were then placed in volumetric flasks, and 6% PCA was added to a final volume of 50 ml. Samples of each extract (2 ml) were derivatized with benzoyl chloride using the same procedure as for the benzoylation of standard amine solution.

2.5. Separation of amines by MECC

A Beckman P/ACE MDQ CE system with a UV detector (Beckman Instrument, Fullerton, CA, USA) was used with Beckman eCap capillary tubing 60 cm (50 cm to detector window)×75 μm I.D. for

biogenic amine separation. The P/ACE MDQ CE system was used on-line at 214 nm for amines. During electrophoresis, the capillary was maintained at ambient temperature (usually 25°C) with circulating coolant surrounding the capillary. The tested samples were introduced by hydrodynamic injection (0.5 p.s.i.) for 5 s (1 p.s.i.=6894.76 Pa). The approximate volume of the sample injected was 25 nl. Electrophoresis was performed at 25 kV. Between runs, the capillary was sequentially washed with 0.1 *M* sodium hydroxide and water for 2.5 min, followed by reconditioning with run buffer for 2.5 min. The MECC procedure was performed with 0.06 *M* sodium deoxycholate in 0.02 *M* borate–methanol buffer (95:5, v/v).

2.6. Determination of amines by HPLC

The amounts of amines in two samples implicated in the food poisoning incident were also determined by a previously developed HPLC method [6]. The equipment used was an Hitachi liquid chromatograph (Hitachi, Tokyo, Japan), consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector (set at 254 nm), and a Model D-2500 chromato-integrator.

3. Results and discussion

The influence of separation conditions on the separation of seven biogenic amines (Phe, Put, Cad, Spd, Tpm, Spm and Him) derivatized with benzoyl chloride was investigated. The derivatives bear no charge, therefore MECC was applied for their separation. In MECC the optimization of the buffer composition plays the key role in the method development. A lot of buffer systems were tested for the suitability for amine determination. Borate buffer (0.2 *M*) exercising high electroosmotic flow in the capillary was found to be better than other media. Sodium deoxycholate was found to obtain better a resolution and shorter retention time than sodium cholate and SDS. The respective concentrations of sodium deoxycholate and methanol were changed ranging from 0.04 to 0.08 *M* and 0 to 10%, it was found that 0.06 *M* sodium deoxycholate and 5% methanol were the best to separate each amine.

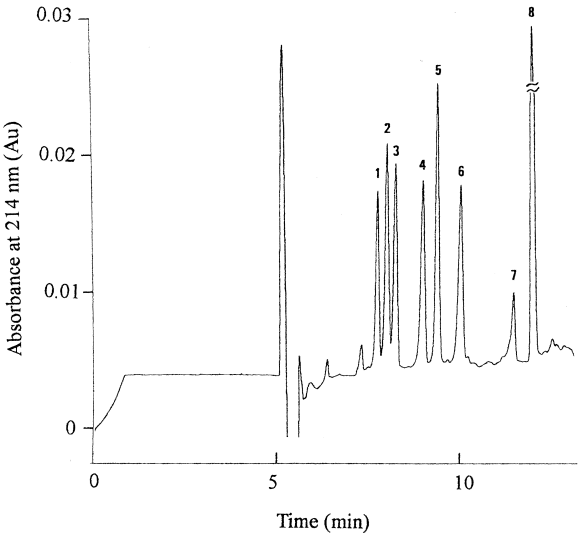

Fig. 1. Electropherogram of seven biogenic amines by MECC. Buffer: 0.06 *M* sodium deoxycholate in 0.02 *M* borate (pH 9.2)–methanol (95:5, v/v); capillary: fused-silica, 50 cm length to detector×75 μm I.D.; injection time: 5 s; voltage: 25 kV. Concentration: 20 μg/ml. Peaks:: 1=Phe; 2=Put; 3=Cad; 4=Spd; 5=Tpm; 6=Spm; 7=Him; 8=benzoyl chloride.

The chromatographic profile of seven authentic biogenic amines by the 0.06 *M* sodium deoxycholate in 0.02 *M* borate–methanol buffer (95:5, v/v) was developed (Fig. 1). All seven amines were well separated in a total duration of 11.5 min, with good peak resolution, sharpness, symmetry and reproducibility (Table 1). The remaining peak of benzoyl chloride in the MECC appeared after 12 min.

Table 1
Reproducibility of biogenic amines separation in CE

Analyte	Migration time (min)[a]	Peak area ($\cdot 10^4$)[a]
Phe	7.66 (1.20)[b]	8.83 (4.15)
Put	7.90 (1.32)	12.11 (3.14)
Cad	8.12 (1.38)	11.40 (4.74)
Spd	8.83 (1.63)	11.57 (2.77)
Tpm	9.23 (1.73)	16.70 (4.45)
Spm	9.82 (1.88)	12.41 (5.03)
Him	11.18 (2.33)	4.67 (5.27)

[a] Average of 10 determinations.
[b] Values in parentheses are the relative standard deviations (RSDs, %).

Table 2
Linear regression equations and correlation coefficients of calibration curves for biogenic amines

Analyte	Linear equation	Correlation coefficient
2-Phenylethylamine (Phe)	$y=3458x+5647$	0.9975
Putrescine (Put)	$y=4977x+12\,710$	0.9941
Cadaverine (Cad)	$y=4249x+6440$	0.9981
Spermidine (Spd)	$y=4053x+10\,636$	0.9955
Tryptamine (Tpm)	$y=5019x+10\,298$	0.9930
Spermine (Spm)	$y=3854x+12\,420$	0.9965
Histamine (Him)	$y=1968x+1030$	0.9936

Therefore, the peak of benzoyl chloride did not interfere with any of the biogenic amines tested. The benzoyl chloride peak interferes with that of Tpm, sometimes Him in the determination of biogenic amines using HPLC [6].

Standard curves of seven amines were prepared separately in the range of 2.5–50 μg/ml and peak area vs. amount of amine regression coefficients for standard curves were subjected to linear regression analysis. The correlation coefficients and linear regression coefficients for each amine were compared (Table 2). The correlation coefficient in every curve was better than 0.99. This indicated a definite linear relationship between amine concentration and detector response. We concluded that the 0.06 *M* sodium deoxycholate in 0.02 *M* borate–methanol buffer (95:5) was satisfactory. Except for Him (2.5 μg/ml), the detectable limit of the other six amines was 1.0 μg/ml. Recoveries of the amines by the sample extraction procedure were 84.4% for Phe, 99.0% for Put, 100.3% for Cad, 92.6% for Spd, 84.2% for Tpm, 92.0% for Spm and 89.3% for Him (Table 3).

The MECC profile of amines from a fried marlin fillet was developed (Fig. 2), and the amine levels of two fried marlin fillets implicated in the food poisoning incident are summarized in Table 4. Him and Cad were predominant in two samples; other amines were not detectable (<1 mg/100 g). The data obtained from the MECC method were almost the same as those obtained from the HPLC method. The respective ranges of Him and Cad were 53.86–56.24 and 3.89–4.21 mg/100 g. The results using the MECC method to determine the amine levels in scombroid fish and its products purchased from common markets are shown in Table 5. Only minor levels of Cad, Tpm and Spm were detected in some samples. Hence, the MECC method may be a good, simple and rapid method for screening the deterioration of scombroid fish and their products.

Table 3
Recoveries of biogenic amines spiked into marlin fish meat

Analyte	Initial content (mg/100 g)	Recovery[a] of spiked amine (%)		
		5 mg/100 g	20 mg/100 g	Average
Phe	N.D. [b]	82.8 (3.5)[c]	85.9 (2.5)	84.4
Put	N.D.	101.3 (2.3)	96.7 (3.9)	99.0
Cad	N.D.	104.9 (3.2)	95.6 (2.4)	100.3
Spd	N.D.	95.1 (4.7)	90.1 (3.7)	92.6
Tpm	N.D.	83.1 (3.5)	85.3 (5.0)	84.2
Spm	2.06	90.2 (4.1)	93.7 (0.9)	92.0
Him	N.D.	88.3 (2.3)	90.3 (4.5)	89.3

[a] Average of triplicate determinations.
[b] Not detected.
[c] Values in parentheses are the relative standard deviations (RSDs, %).

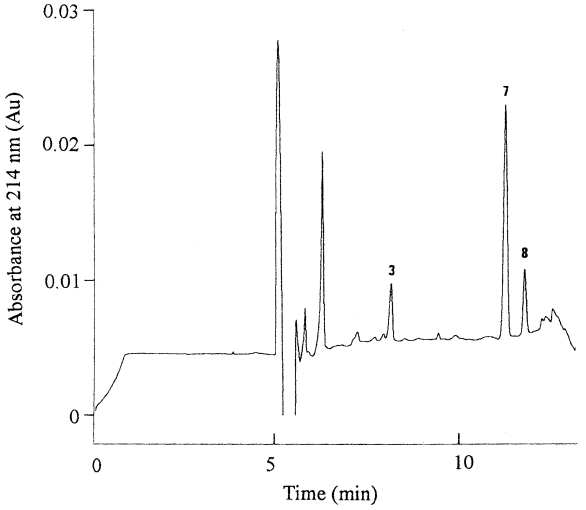

Fig. 2. Electropherogram of amines from a fried marlin fillet implicated in a food poisoning incident. Separation conditions and peak identification are as described in Fig. 1.

Comparing MECC and HPLC methods for amine determination, the following characteristics were found. (1) The detection limits of MECC and HPLC for each amine were 1 mg/100 g and 0.1 mg/100 g, respectively. (2) The HPLC method could simultaneously determine nine amines, but the MECC method determined seven amines which did not include tyramine and agmatine. (3) The peak from benzoyl chloride in the HPLC method appeared among those of amines and might interfere with the amine identification. Whereas it appeared at the end in the MECC method and did not interfere those of amines. (4) The MECC method had better resolution than HPLC separation and was faster, more automatic and less costly to operate than the HPLC method.

As mentioned above, the MECC method could not detect tyramine. However, tyramine is a very important biogenic amine known to cause some toxic effects and potentiate the toxicity of histamine. How to improve the MECC method for determining simultaneously tyramine needs further study. Furthermore, the interfering peak of benzoyl chloride (peak 8 in Fig. 2) in the standard electropherogram was higher than that of the sample. Other substances that interacted with benzoyl chloride in the sample might cause this symptom.

Normally, the level of Him in fresh fish fillets is <5 mg/100 g, and this high level is suggested as the maximum level for fish fillet as frying material [19]. Hence, the two fried marlin fillets implicated in the food poisoning incident appeared to have been somewhat decomposed by histamine-producing bacteria due to the fact that the histamine level was even higher in the fillets obtained from the school. This indicated that the fried marlin fillets were not appropriate to eat. Although scombroid poisoning may be caused by many factors other than histamine itself [3,20], the histamine level is considered as a good indicator of fish decomposition and scombroid poisoning [21,22]. The US Food and Drug Administration (FDA) has established a hazard action level of 50 mg of histamine per 100 g fish (500 ppm), based on data collected from numerous outbreaks [22]. In the present food poisoning case, the victims were all below 12 years old, and young children may be more sensitive to histamine than adults. The high level of

Table 4
Level of amines in the fried marlin fillets implicated in food poisoning

Sample No.	Weight (g)	Detection method	Amine level[a] (mg/100 g)						
			Phe	Put	Cad	Spd	Tpm	Spm	Him
1	132	MECC	N.D.[b]	N.D.	3.89 (2.56)[c]	N.D.	N.D.	N.D.	53.86 (3.71)
		HPLC	N.D.	0.40 (2.10)	4.01 (3.41)	0.25 (2.14)	0.26 (0.75)	0.18 (2.58)	54.30 (1.75)
2	138	MECC	N.D.	N.D.	4.21 (4.10)	N.D.	N.D.	N.D.	56.24 (2.67)
		HPLC	N.D.	0.59 (1.74)	4.18 (1.25)	0.36 (1.20)	0.18 (2.10)	0.21 (1.92)	55.91 (2.59)

[a] Average of triplicate determinations.
[b] Not detected (<0.1 mg/100 g for HPLC; <1 mg/100 g for MECC).
[c] Values in parentheses are the relative standard deviations (RSDs, %).

Table 5
Level of amines in the fresh fish fillet, salted fish fillet and fish can meat purchased from common markets

Sample	Weight (g)	Amine level[a] (mg/100 g)						
		Phe	Put	Cad	Spd	Tpm	Spm	Him
1. Marlin fillet	136	N.D.[b]	N.D.	N.D.	N.D.	N.D.	2.06 (2.14)[c]	N.D.
2. Sea bream fillet	152	N.D.	N.D.	N.D.	N.D.	N.D.	1.98 (2.58)	N.D.
3. Tuna fillet	241	N.D.	N.D.	N.D.	N.D.	1.20 (2.17)	N.D.	N.D.
4. Salted mackerel fillet	53	N.D.	N.D.	1.52 (0.75)	N.D.	N.D.	N.D.	N.D.
5. Salted mackerel fillet	62	N.D.	N.D.	N.D.	N.D.	1.22 (4.21)	N.D.	N.D.
6. Salted mackerel fillet	63	N.D.	N.D.	1.66 (3.05)	N.D.	N.D.	N.D.	N.D.
7. Tuna can meat	140	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
8. Tuna can meat	145	N.D.	N.D.	1.25 (2.71)	N.D.	N.D.	N.D.	N.D.
9. Mackerel can meat	130	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
10. Mackerel can meat	145	N.D.	N.D.	1.37 (5.21)	N.D.	N.D.	N.D.	N.D.

[a] Average of triplicate determinations.
[b] Not detected (<1 mg/100 g).
[c] Values in parentheses are the relative standard deviations (RSDs, %).

histamine (>50 mg/100 g) in the marlin fillets along with the allergy-like symptoms of the victims supported the conclusion that histamine was the cause of this food poisoning incident.

Clifford et al. [20] pointed out that histamine administered alone was less toxic than an equal amount of histamine consumed with fish and that other biogenic amines such as Cad and Put may act as synergistic cofactor on the toxicity of histamine [23]. In addition to histamine, Cad, Put and Tpm were found in the fried marlin fillets. The possible contribution of these amines on the potentiation of histamine toxicity should be considered for future studies.

The production of histamine is well known to be associated with the growth of bacteria possessing the enzyme histidine decarboxylase. In fish, several histamine-producing bacteria have been implicated as primary contributors to histamine formation. These bacteria are *Proteus (Morganella) morganii*, *P. vulgaris*, *Photobacterium histamnium*, *Ph. phosphoreum*, *Klebsiella pneumoniae*, *Escherichia*, *Clostridium*, *Salmonella* and *Shigella* [1,21,24–26]. These bacteria are capable of producing hazardous amounts of histamine in a very short period of time when fish is kept at elevated temperatures. As a result, contaminated fish may not appear spoiled but may still be hazardous to consume. Because scombroid food poisoning outbreaks have occasionally occurred in recent years in Taiwan [6–8], the association of histamine with histamine-producing bacteria is being investigated.

On the other hand, capillary electrophoresis has been developed as an usable tool for food analysis in the recent decade. There have been several reports on the development of capillary zone electrophoresis (CZE) and MECC methods [12–18] to determine histamine and/or biogenic amines. In two papers [14,15], ball-lens laser-induced fluorescence detection and xenon lamp-based fluorescence detection were used, respectively. In this study, we report the development a simple, rapid and specific MECC method for determining biogenic amines by benzoylating with benzoyl chloride and using UV detection. This method has been applied to real samples and was effective, usable and validated using a HPLC method.

References

[1] S.L. Taylor, CRC Crit. Rev. Toxicol. 17 (1986) 91.
[2] W.J. Hurst, J. Liq. Chromatogr. 13 (1990) 1.
[3] S.H. Arnold, W.D. Browd, Adv. Food Res. 24 (1978) 113.
[4] J.L. Mietz, E. Karmas, J. Food Sci. 42 (1977) 155.
[5] J.Y. Hui, S.L. Taylor, J. Assoc. Off. Anal. Chem. 66 (1983) 926.
[6] D.F. Hwang, S.H. Chang, C.Y. Shiau, T. Chai, J. Chromatogr. B 693 (1997) 23.
[7] D.F. Hwang, S.H. Chang, C.Y. Shiau, C.C. Cheng, J. Food Sci. 60 (1995) 926.

[8] D.F. Hwang, T.Y. Chen, S.H. Chang, S.S. Chou, J.F. Deng, T. Chai, Food Sci. Agric. Chem. 1 (1999) 223.
[9] C. Buteau, C.C. Duitshaever, J. Chromatogr. 284 (1984) 201.
[10] R.L. Heideman, K.B. Fickling, L.J. Walker, Clin. Chem. 30 (1984) 1243.
[11] K. Samejima, M. Kawase, S. Sakamoto, M. Okada, Y. Endo, Anal. Biochem. 76 (1976) 392.
[12] B. Mopper, C.J. Sciacchitano, J. AOAC Int. 77 (1994) 285.
[13] M. Nakashima, A. Sugiyama, J. Food Hyg. Soc. Japan 40 (1999) 285.
[14] G. Nouadje, M. Nertz, P. Verdeguer, F. Couderc, J. Chromatogr. A 717 (1995) 335.
[15] I. Rodriguez, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 745 (1996) 255.
[16] V.C. Trenerry, P.A. Marshall, K. Windahl, J. Cap. Electrophoresis 5 (1998) 27.
[17] A. Kovacs, L. Simon-Sarkadi, K. Granzler, J. Chromatogr. A 836 (1999) 305.
[18] M. Krizek, T. Pelikanova, J. Chromatogr. A 815 (1998) 243.
[19] S.H. Chang, C.Y. Shiau, T. Chai, D.F. Hwang, J. Fish. Soc. Taiwan 23 (1996) 253.
[20] M.N. Clifford, R. Walker, J. Wright, J. Sci. Food Agric. 47 (1989) 365.
[21] F.E. Russell, Z. Maretic, Toxicon 24 (1986) 967.
[22] S.L. Taylor, Clin. Toxicol. 27 (1989) 225.
[23] L.F. Bjeldances, D.E. Schutz, M.M. Morris, Food Cosmet. Toxicol. 16 (1978) 157.
[24] S.L. Taylor, L.S. Guthertz, M. Leatherwood, F. Tillman, E.R. Leiber, J. Food Safety 1 (1978) 173.
[25] M. Okuzumi, S. Okuda, M. Awano, Bull. Jpn. Soc. Sci. Fish. 47 (1981) 1591.
[26] M. Okuzumi, A. Hiraishi, T. Kobayashi, T. Fujii, Int. J. System. Bacteriol. 44 (1994) 631.