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Formation of volatile halogenated compounds in fresh-cut cabbage treated with sodium hypochlorite

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Abstract

This study was conducted to investigate factors affecting the formation of disinfection by-products in fresh-cut cabbage during sodium hypochlorite treatment. Fresh cabbage was disinfected with a sodium hypochlorite solution (100 mg/L) for 10 min, with and without organic acids. Volatile organic compound residues in the fresh-cut cabbage were analyzed using headspace GC/MS. Chloroform was detected as the main by-product. Chloroform formation was dependent on contact time, pH, temperature and initial concentration of sodium hypochlorite solution. The use of sodium hypochlorite solution in combination with hydrochloric acid or some organic acids did not affect chloroform formation, except that citric acid reacted with hypochlorite to produce large amount of chloroform. When the citric acid was coupled with sodium hypochlorite solution, the chloroform level in the sample was dependent on the pre-mixing time of the solution, but was independent on the contact time of the mixed solution with the sample. Rinsing with water effectively reduced chloroform contaminants in the fresh-cut cabbage to the levels of chlorinated drinking water.

Keywords : trihalomethanes, chloroform, sodium hypochlorite, cabbage, citric acid

I Introduction

Sodium hypochlorite is used as a disinfectant in the processing of fruits, vegetables, and other fresh foods. Chlorine is also widely used as a disinfectant for drinking water, and it is well known that chlorine reacts with natural organic compounds in water, resulting in the formation of trihalomethanes (THM)[1, 2]. THM are a group of chemicals that commonly include chloroform, bromodichloromethane (BDCM), dibromochloromethane (DBCM) and bromoform. The presence of THM in drinking water is a human health concern. The US Environmental Protection Agency (USEPA) has set the maximum contaminant level for total THM at 80 μg/L as the locational running annual average[3]. The European Union drinking water directive (98/83/EC) has set the maximum standard for total THM at 100 μg/L[4], while the World Health Organization (WHO) has announced the drinking water guidelines for chloroform (300 μg/L), BDCM (60 μg/L), DBCM (100 μg/L) and bromoform (100 μg/L)[5]. The Ministry of Health, Labour and Welfare of Japan has set the maximum contaminant level of total THM at 100 μg/L, for chloroform at 60 μg/L, for BDCM at 30 μg/L, for DBCM at 100 μg/L, and bromoform at 90 μg/L[6].

The U.S. Food and Drug Administration (FDA) has monitored residual chemical contaminants in foodstuffs through the total diet programs for over 40 years. THM and other chlorinated volatile organic compounds (VOC) have been detected in several foodstuffs, including butter, cheese, margarine and soft drinks[7-11]. Several studies have reported that treatment with chlorine solution on poultry, milk and fresh-cut vegetables may induce the formation of reaction by-products[12-16]. Huang *et al.* investigated the THM formation potentials of foods and beverages during production with chlorinated drinking water and observed that THM were formed in the preparation and cooking of foods[17]. Recently, Klaiver *et al.* and Lopez-Galvez *et al.* investigated effective washing procedures for microbe removal, and residual THM content in fresh foods[18, 19].

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The Joint FAO/WHO Expert Committee met in 2008 to conduct a risk and benefit assessment of chlorine-based disinfectants in food production and food processing[20]. The chemical hazard risk of disinfection by-products in foods was discussed in the context of microbial control. Referring to several case studies on the use of chlorine-based disinfection in foods, it was concluded that they posed no health concerns. However, there is a lack of quantitative information on the formation and presence of by-products in foods during disinfection. Therefore, the dietary exposure assessment of these by-products is required for food safety. The meeting recommended further scientific study on the formation, identification and quantification of disinfection by-products in foods at the point of consumption.

The antimicrobial efficacy of sodium hypochlorite solutions can be enhanced by acidification, shifting the predominant chlorine species from hypochlorite ion to hypochlorous acid[21]. Therefore, a sodium hypochlorite solution has often been used with hydrochloric acid or organic acids to adjust the pH value between 6.0 and 7.5[22]. However, it remains unclear whether any significant risks are associated with the use of acidified hypochlorite treatment on foods.

Our research objective was to investigate the factors that affect the formation of disinfection by-products in fresh-cut cabbage. The effects of sodium hypochlorite concentration, contact time, pH, and acids on the formation potential of THM in fresh-cut cabbage were assessed using Headspace-Gas Chromatography/Mass Spectrometry (HS-GC/MS). Moreover, we investigated the effect of a washing procedure on residual chloroform concentrations in fresh-cut vegetables.

Ⅱ Materials and Methods

1. Sample preparation

Raw shredded cabbage was used as an example of fresh-cut vegetables. Whole-cabbage was purchased from a local market in Tokyo, Japan. The outside leaves and core of the raw cabbage were removed by hand; internal leaves were cut into 1-mm pieces with a peeler to make the shredded cabbage. The shredded cabbage was placed in a bowl filled with distilled water for a few minutes and then removed and dried for approximately 1 h at the room temperature.

2. Reagents

A stock standard solution mixture of 23 VOC and internal standards solution were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol and sodium chloride (water quality test grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2, 4-^{13}C-Citric acid (99.9% purity) was purchased from ISOTEC, Inc. (Miamisburg, OH, USA). Food additive grade citric acid, S.C.-malic acid and tartaric acid of food additives grade were used. A food additive-grade sodium hypochlorite solution was purchased from Wako Pure Chemical Industries and analyzed by iodometric titration. The mineral water evian (Évian-les-Bains, France) was used as reagent water, which confirmed the absence of VOC using HS-GC/MS. All other reagents used were of analytical grade.

3. HS-GC/MS analysis

VOC were analyzed using HS-GC/MS according to standard methods for the examination of drinking water in Japan[23]. GC/MS analyses were carried out using a HP 5973 Mass Selective Detector (Hewlett-Packard Co., Palo Alto, CA, USA) interface to HP 6890 gas chromatography (Hewlett-Packard) with a HP 7694 automated headspace sampler. Samples were injected splitless, and VOC were separated using a 25% phenyl and 75% dimethyl polysiloxane capillary column (60 m × 0.25 mm diameter with a 1.0 μm film thickness; Aquatic, GL Sciences, Tokyo, Japan). Helium was employed as the carrier gas at a flow rate of 1.0 mL/min. The GC injector temperature was 200°C. The oven temperature was maintained at 40°C for 2 min, then increased at 4°C/min to 200°C. The headspace conditions were as follows: oven, 60°C; sample loop, 130°C; transfer line, 170°C; 25 min thermal equilibration with shaker on; source temperature, 200°C; quadrupole temperature, 150°C. The detected compounds were compared with the NIST/Willey mass spectra library and were further confirmed by comparing with the mass spectra and retention times of standards.

4. Experimental procedure

1) Disinfection protocol for fresh-cut cabbage

Cabbage (2 g) was weighed into a 50 mL screw cap vial. Samples were dipped with a 100 mg/L sodium hypochlorite solution (pH 10) for 10 min at room temperature. At the end of the dipping time, residual chlorine was quenched with adding 3-fold molar excess solution of ascorbic acid to the chlorinated sample mixture. After being quenched, the treated sample was transferred to tea strainer to removed excess solution, and applied to headspace analysis.

2) Headspace sampling procedure

Treated samples were transferred to a 20-ml headspace vial containing 8 ml of water and 3 g of sodium chloride. Following the addition of 20 ng fluoro-benzene and 4-bromofluorobenzene-methanol solution mixture as internal standards, the vials were immediately sealed using crimp top vial caps with a PTFE-lined silicone septum. Quantification of the 23 VOC was performed using HS-GC/MS as described above. All glassware was heated at 105°C for 3 hours prior to

use to remove volatile contaminants.

3) The chloroform formation potential experiments in acidified hypochlorite solution under the different organic acid treatments

An aliquot of sodium hypochlorite solution (10 ml; 100 mg/L) was placed in a 20-mL headspace vial. The solution was mixed with hydrochloric acid or organic acids (citric acid, malic acid, tartaric acid, succinic, acid and fumaric acid) and the pH was adjusted to between 6.0 and 7.0. The acidified solution was pre-mixed at room temperature for various time periods (0, 10, 30, 60 min). At the end of the reaction time, residual chlorine was quenched with adding a 3-fold molar excess solution of ascorbic acid to the mixture. Following the addition of internal standards, the vials were immediately sealed using crimp top vial caps with a PTFE-lined silicone septum. The chloroform concentration in samples was determined using HS-GC/MS as described above.

Ⅲ Results

1. Development and validation of analytical method

VOC were assessed using HS-GC/MS. All 23 compounds were eluted within 35 min. The isomers *m*- and, *p*-xylene co-elute and have essentially identical mass spectra; therefore, they are quantified as one peak. Benzene also co-eluted with 1,2-dichloroethane. However, the MS detection system could selectively determine these compounds using the specific mass fragments for each compound. The lower limits of quantification (LOQs) were 1 μg/kg for all analyses. The recovery experiments were carried out using fresh-cut cabbage spiked with the compounds at two different analyte concentrations (10 and 100 μg/kg). The mean recovery of spiked standard from samples varied between 72.6 and 109%, and the relative standard deviation ranged from 0.2 to 3.3% (Table 1). These results suggest that all compounds are determined with good accuracy and precision in the samples.

2. Effect of contact time on VOC formation

Figure 1 shows the formation of VOC as a function of contact time during chlorination (at pH 10 and room temperature). Chloroform was detected as the predominant VOC in the chlorinated samples, while BDCM, and tetrachloroethylene (TCE) were observed at lower levels. VOC levels rapidly increased for 20min, and plateaued after 30 min. The other VOC were not detected in the chlorinated samples.

3. Effect of initial sodium hypochlorite concentration on VOC formation

The effect of sodium hypochlorite concentration on VOC

Table 1. Average recoveries (Avg.) and CV resulting from the fortification of the fresh-cut cabbage with 23 volatile organic compounds at 100 μg/kg and 10 μg/kg

No.	Compounds	Amount of added VOCs			
		10 μg/kg		100 μg/kg	
		Avg.* (μg/kg)	CV (%)	Avg. (μg/kg)	CV (%)
1.	1.1-Dichloroethylene	10.0	0.8	101.3	0.7
2.	Dichloromethane	10.7	1.4	106.5	0.6
3.	*trans*-1,2-Dichloroethylene	10.0	0.9	100.5	0.5
4.	*cis*-1,2-Dichloroethylene	10.3	0.8	103.4	0.4
5.	Chloroform	10.2	0.9	102.4	0.5
6.	1,1,1,-Trichloroethane	9.7	0.6	99.0	0.7
7.	Carbon Tetrachloride	9.4	1.1	97.1	1.7
8.	1,2-Dichloroethane	10.9	1.0	107.6	0.7
9.	Benzene	10.2	0.5	101.4	0.4
10.	Trichloroethylene	9.7	0.5	96.5	0.2
11.	1,2-Dichloropropane	10.6	1.2	104.3	0.5
12.	Bromodichloromethane	10.3	1.1	101.8	0.9
13.	*cis*-1,3-Dichloropropene	9.7	2.0	95.4	1.5
14.	Toluene	9.6	0.7	94.2	0.3
15.	*trans*-1,3-Dichloropropene	9.9	1.8	98.8	0.8
16.	1,1,2-Trichloroethane	10.7	2.0	104.5	0.7
17.	Tetrachloroethyhlene	8.1	2.5	83.5	1.8
18.	Dibromochloromethane	10.1	1.1	99.1	1.7
19+20.	*m,p*-Xylene	8.2	2.9	77.8	2.0
21.	*o*-Xylene	8.1	2.9	76.1	1.7
22.	Bromoform	9.5	2.0	95.1	3.3
23.	*p*-Dichlorobenzene	7.6	1.8	72.6	1.5

* The analyses were replicated five times for each condition.

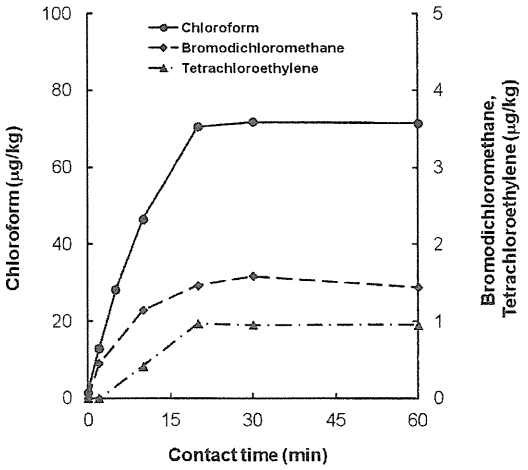

Fig. 1. Effect of contact time on VOC formation in chlorinated fresh-cut cabbage
Samples were disinfected with a 100 mg/L sodium hypochlorite solution at pH 10 and room temperature. VOC concentration in the samples was determined at 0, 5, 10, 20, 30 and 60 min.

formation was investigated (Fig. 2). Samples were disinfected with various sodium hypochlorite concentrations (0, 50, 100, 200 and 500 mg/L as chlorine) and the results indicated that chloroform concentrations increased linearly from 10 to 157.5 μg/kg,. BDCM and TCE were detected as minor products above a dose of 50 and 100 mg/L sodium hypochlorite, respectively. The formation of TCE increased with increasing chlorine dose, whereas BDCM formation was not dependent on chlorine dose.

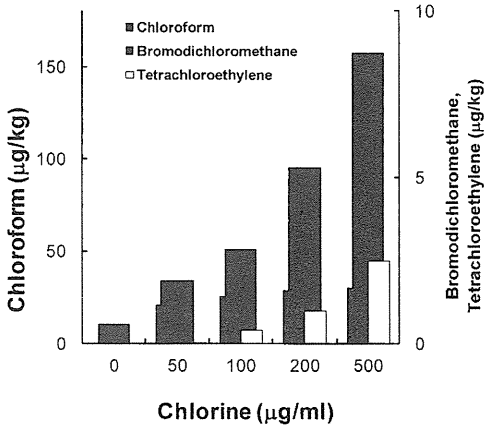

Fig. 2. Effect of sodium hypochlorite concentration on VOC formation in chlorinated fresh-cut cabbage
Samples were disinfected with various sodium hypochlorite concentration (0, 50, 100, 200 and 500 mg/L as chlorine) for 10 min at room temperature.

4. Effect of temperature on chloroform formation

Figure 3 shows the effect of temperature on chloroform formation during disinfection. The samples were disinfected with a 100 mg/L sodium hypochlorite solution at pH 10 at three different temperatures (2, 10 and 21°C). BDCM and TCE were detected at a trace level in chlorinated samples at 21°C. However, it was difficult to constantly monitor these compounds at lower levels. The other VOC were not detected in any of the chlorinated samples. Therefore, we focused on the chloroform formation hereafter. After 10 min of disinfection, the chloroform concentration was increased to 197 and 245%, respectively, when the temperature was raised from 2 to 10 and 21°C. The results suggest that the rate of chloroform formation increases with temperature. At higher temperature (21°C), the chloroform concentration rapidly increased, and plateaued after 30 min. In contrast, chloroform concentration gradually increased with increasing contact time at low temperature (2 and 10°C).

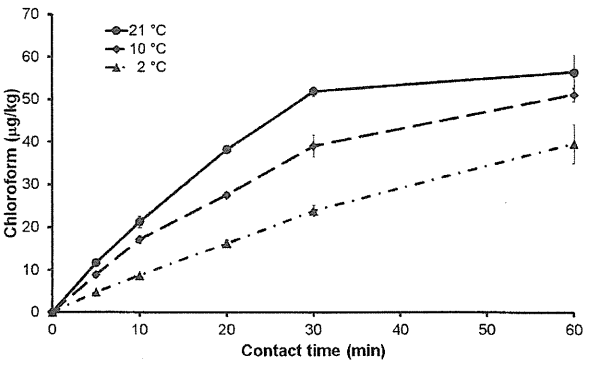

Fig. 3. Effect of temperature on chloroform formation in chlorinated fresh-cut cabbage
Samples were disinfected with a 100 mg/L sodium hypochlorite solution (pH 10) for between 0 and 60 min under various temperature conditions (2, 10, 21°C).

5. Effect of pH on VOC formation

Figure 4 shows the detected VOC concentrations after disinfection with a 100 mg/L sodium hypochlorite solution in fresh-cut cabbage at various pHs (pH 2, 4, 6, 8, 10 and 12). Only chloroform was detected at a high pH. The detected chloroform concentration was 19.7, 33.0, 54.9 and 76.0 μg/kg at pH 6, 8, 10 and 12, respectively. In contrast, there was no significant difference in detected chloroform concentrations at low pH values. In additions, a low-level concentration of carbon tetrachloride was detected only at pH 2.

6. Effect of organic acids on chloroform formation

Since sodium hypochlorite is often coupled used with inorganic and organic acids to enhance its anti-microbial

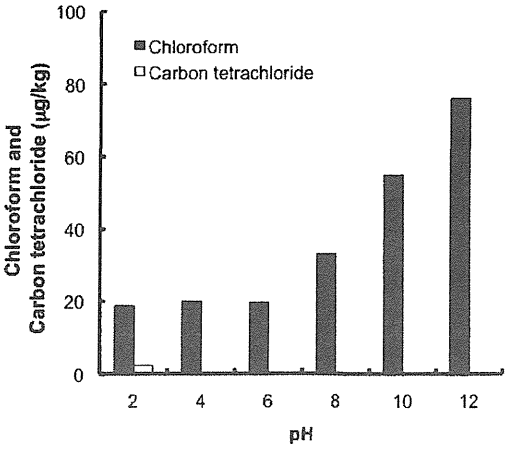

Fig. 4. Effect of pH on VOC formation in chlorinated fresh-cut cabbage
Samples were disinfected with a 100 mg/L sodium hypochlorite solution for 10 min at various pH (pH 2, 4, 6, 8, 10 and 12).

efficacy, effect of organic acids on chloroform formation was assessed (Fig. 5). Sodium hypochlorite solutions (100 mg/L) were mixed with hydrochloric acid or organic acids (citric acid, malic acid, tartaric acid, succinic, acid and fumaric acid) and the pH was adjusted to 6.0 - 7.0. The acidified hypochlorite solution was left to stand 0 - 180 min at 10°C. Fresh-cut cabbage were disinfected with a sodium hypochlorite solution for 10 min in the presence and absence of acids. Most organic acids, such as malic acid, tartaric acid, succinic acid and fumaric acid, did not affect chloroform formation. In contrast,

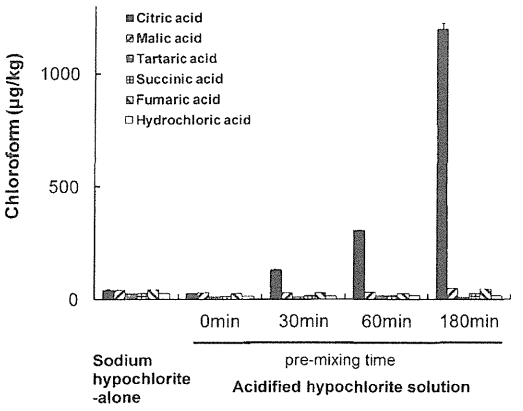

Fig. 5. The combination effect of sodium hypochlorite and organic acids on chloroform formation in chlorinated fresh-cut cabbage
Sodium hypochlorite solution (100 mg/L) was mixed with various acids at 10°C and left to stand 0 - 180 min. Sample was disinfected with the acidified hypochlorite solution or sodium hypochlorite solution-alone for 10 min at pH 6 - 7 at room temperature.

when citric acid was used as a pH buffers, the chloroform levels increased with pre-mixing time. The chloroform levels in fresh-cut cabbage treated with citric acid-activated sodium hypochlorite solution (pre-mixed for 180 min) was 28.7 times greater than with chlorine treatment alone.

7. Effects of pre-mixing time of acidified hypochlorite solution on chloroform formation

The influence of pre-mixing time with citric acid was examined (Fig. 6). A sodium hypochlorite solution was mixed with citric acid and the pH adjusted to 6.8. The mixture was left to stand 0 - 60 min as pre-mixing time at 10°C and was used for the treatment of sample. The concentration of chloroform in the cabbage sample was increased with the contact time, but the initial concentration and the increasing rate of chloroform were different among the pre-mixing time of sodium hypochlorite and citric acid. This fact suggests that citric acid reacts with sodium hypochlorite to form chloroform and the amount of formed chloroform is correlative to the pre-mixing time. On the other hands, the citric acid was found to act suppressively for the formation of chloroform when the pre-mixing time is zero. From these results, we considered that chloroform detected in cabbage is dependent on the pre-mixing time rather than the contact time to disinfectant when the sodium hypochlorite solution is acidified by citric acid.

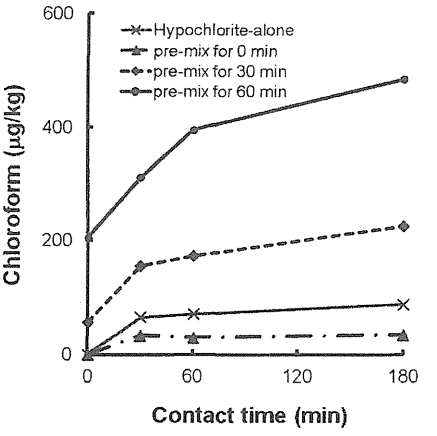

Fig. 6. The combined effect of sodium hypochlorite and citric acid on chloroform formation in chlorinated fresh-cut cabbage
Sodium hypochlorite solution (100 mg/L) was mixed with citric acid adjusted to pH 6.8 and left to stand for 0 - 60 min at 10°C. Sample were disinfected with the acidified hypochlorite solution or sodium hypochlorite solution-alone for 0 - 180 min at room temperature.

8. Chloroform formation potentials in acidified hypochlorite solution using various organic acid

The chloroform formation potentials of organic acids

were assessed under chlorination (Fig. 7). The organic acid solutions were treated with sodium hypochlorite solution at pH of 6.0 - 7.0 and room temperature. Most organic acids, such as malic acid, tartaric acid, succinic acid and fumaric acid, did not affect chloroform formation. In contrast, only citric acid reacted with sodium hypochlorite to form chloroform. Chloroform formation was correlated with reaction time.

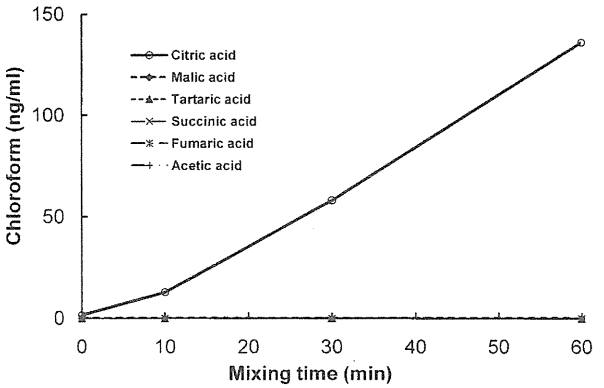

Fig. 7. Chloroform formation potentials in the chlorination of organic acids

A sodium hypochlorite solution (100 mg/L) was mixed with various organic acids at pH 6 - 7 and room temperature.

9. Isotopic study of the mechanism of chloroform formation

To elucidate the reaction pathway of chloroform formation, sodium hypochlorite solution was reacted with 2, 4-^{13}C-citric acid or 2, 4-^{12}C-citric acid (unlabeled citric acid), and resulting mass spectra of each chloroform were compared using HS-GC/MS (Fig. 8). The mass spectrum of chloroform from 2, 4-^{12}C-citric acid has a base ion peak at *m/z* 83. However, the mass spectrum of chloroform from 2, 4-^{13}C-citric acid showed a base ion at *m/z* 84. The one mass unit shift from 83 to 84 suggests that the 2 and 4-position carbon atoms in citric acid have been chlorinated with sodium hypochlorite.

10. Effect of water rinse treatment on residual chloroform concentration in fresh-cut cabbage

A sodium hypochlorite solution is used in washing vegetables or in the peeling of fruits and vegetables, and rinsing with potable water to remove the residual chemicals normally follows this treatment. The effect of water rinsing on removal of chloroform was examined (Table 2). The sample were treated with a sodium hypochlorite solution for 10 min with and without citric acid. Chloroform levels were 31.9 μg/kg for the treatment without citric acid and 366 μg/kg for the treatment with citric acid. However, after rinsing with distilled water for 1 min, the levels of chloroform contaminants in the fresh-cut cabbage were effectively decreased to 16.2 μg/

Table 2. The effect of water rinsing on chloroform residue in fresh-cut cabbage

Process[*1]	Sodium hypochlorite solution alone		Acidified hypochlorite solution	
	Avg.[*2] (μg/kg)	CV (%)	Avg. (μg/kg)	CV (%)
A. Blank	8.8	0.0	–	
B. Chlorination	31.9	1.8	366.0	15.8
C. Washing	16.2	0.8	17.9	0.8

*1 Process A: Control, (no chlorination),
Process B: Chlorinated fresh-cut cabbages,
Process C: Washed fresh-cut cabbages after chlorination.

*2 The analyses were replicated three times for each condition.

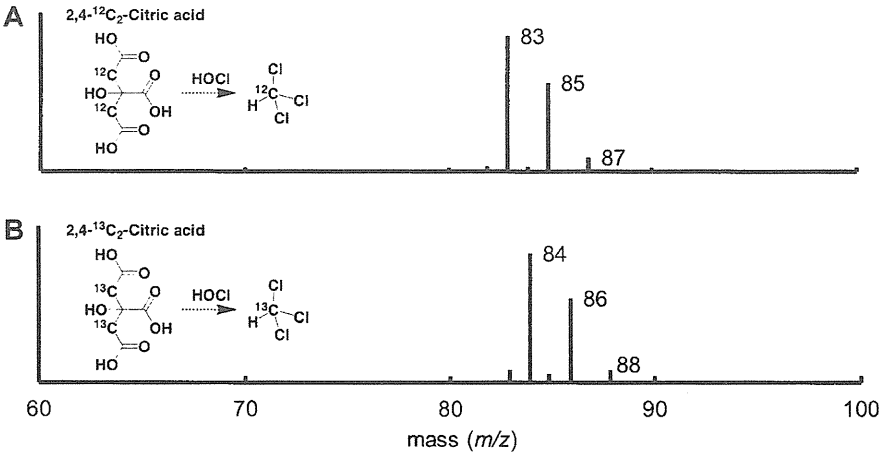

Fig. 8. Mass spectra of chloroform production after the reaction of sodium hypochlorite with 2,4-^{12}C-citric acid (A) and 2,4-^{13}C-citric acid (B)

A sodium hypochlorite solution (100 mg/L) was reacted with non-isotopically or isotopically labeled citric acid compounds for 10 min at pH 6.8 and room temperature.

kg for the treatment without citric acid and 17.9 μg/kg for the treatment with citric acid.

Ⅳ Discussion

1. Factors affecting VOC formation during chlorination of fresh-cut cabbage

After treatment of fresh-cut cabbage with sodium hypochlorite solution, chloroform was produced as major product. This finding is consistent with the previous studies of Ichikawa et al., in which the formation of chloroform and carbon tetrachloride was observed after the treatment of fresh vegetable homogenates with sodium hypochlorite solution[14]. It is well known that THM are formed in water after the reaction of chlorine with humic substances. Typical structures of humic substances are thought to be a mixture of large molecules composed of phenolic and carboxylic substances[24, 25]. Rook et al. demonstrated the ability of flavonoids to form THM and showed that their *m*-dihydroxybenene moieties are responsible for THM formation during chlorination[26]. Since fruits and vegetables contain various flavonoids and other phenolic compounds, they may serve as THM precursors during disinfection.

The chloroform concentration was increased with increase of the contact time, initial chlorine concentration and temperature. These results are in agreement with previous studies in drinking water experiments[27-29]. The contact time, sodium hypochlorite concentration and temperature are considered to be important factors controlling the THM formation. Sodium hypochlorite solution was generally used in the 50 to 200 mg/L concentration in short contact time (5 to 10 min) to sanitize fresh foods at cool temperature. Therefore, in such case, THM concentrations would be controlled at a lower level in fresh foods.

We detected BDCM at lower levels than that of chloroform. The chlorination of water containing inorganic bromide leads to the formation of brominated THM. Bromide is readily oxidized to hypobromite by sodium hypochlorite, and reacts with phenolic compounds to form brominated-THM[30]. However, disinfectant contains little bromide, and brominated-THM was not detected by the reaction of organic acids with the sodium hypochlorite solution. Therefore, brominated-THM might not be produced from bromide in disinfectant. Since bromide has also been found in foods, this might be the source of BDCM formation. The other VOC were not detected in the chlorinated samples. Some of these compounds, such as *p*-dichlorobenzene, were known as disinfection by-products in drinking water[31]. However, these compounds may not be formed from fresh foods by chlorination.

At basic pH, higher concentrations of THM occurred with increasing pH values; a number of researchers have reported similar finding in chlorinated drinking waters[32-35]. The haloform-type reaction mechanism was proposed to be the major pathway for THM formation[21]. This reaction involves a replacement of the three hydrogen atoms with halide on the alpha-carbon of the carbonyl group. The trihalocarbonyl group is then attacked by hydroxide to produce THM and carboxylic acid. Under a basic pH condition, the hydrolysis reaction should be accelerated, thereby increasing the THM concentration.

2. The effect of organic acid on chloroform formation during chlorination

When fresh-cut cabbage were disinfected with a combination of sodium hypochlorite solution and citric acid, chloroform levels increased with increasing pre-mixing time of the acidified hypochlorite solution. Larson and Rockwell reported that citric acid acted as a chloroform precursor in chlorinated water under neutral pH conditions[36]. Streicher and Zimmer further investigated the interaction of aqueous chlorine solutions at various pHs with citric acid and frozen condensed orange juice[37]. It was found that citric acid reacted with sodium hypochlorite, producing a number of chlorinated compounds. They also proposed the reaction pathway. In the first step, citric acid is oxidatively decarboxylated to form 3-ketoglutaric acid in hypochlorite solution. 3-Ketoglutaric acid is subsequently chlorinated with sodium hypochlorite and decarboxylated to produce polychlorinated propanone, due to the activation of methylene group of 3-ketoglutaric acid by the neighboring carbonyl and carboxyl groups. It is enolized and readily attacked by hypochlorite. Finally, pentachloropropanone is hydrolyzed to form chloroform and dichloroacetic acid. Our stable isotope experiments clearly show that the 2 and 4-position carbon atoms in citric acid have been selectively chlorinated with hypochlorite. The present study proved the hypothetical reaction mechanism previously reported is correct and that citric acid can be the precursor for chloroform formation.

3. The effect of water rinsing on residual chloroform concentrations in fresh-cut cabbage

Chloroform contaminants levels in fresh-cut cabbage were effectively decreased after rinsing with water, falling to those found in drinking water. There was no significant difference in residual chloroform levels after rising with water between treatment with sodium hypochlorite alone and the acidified hypochlorite solution. López-Gálvez et al. reported that residual THM were not detected (<5 g/L) in lettuce leaves washed with a 100 mg/L sodium hypochlorite solution. However, when lettuce was treated with a higher concentration (1800 mg/L sodium hypochlorite for 60 min),

and then rinsed with water, large amount of THM remained. Sodium hypochlorite is commonly used in the concentration range of 50 to 200 mg/L, except in special cases, such as with alfalfa seeds intended for sprout production[19]. Therefore, in most cases, the washing process could remove the THM contaminants. The levels of THM contaminants could decrease down to as low as those in drinking water. We suggest that the THM levels found in fresh produce are too low to cause adverse health effects.

V Conclusion

We investigated factors that affect the formation of VOC in fresh-cut cabbage. Chloroform was detected as the predominant THM in the chlorinated samples. The formation of THM was dependent on pH, temperature and the initial sodium hypochlorite concentration. Citric acid reacted with hypochlorite to produce large amount of chloroform. When the sodium hypochlorite solution was acidified by citric acid, chloroform level was depending on the pre-mixing time with citric acid rather than the contact time with fresh-cut cabbage. Rinsing with water was useful enough to reduce the residual chloroform in cabbage to the same level as chlorinated drinking water. Since fresh-cut vegetables ware usually rinsed with water after disinfection, we consider such vegetables in the market to be safe regarding the health risk of THM.

However, another group of disinfection by-products are formed by the chlorination of drinking water. Wu *et al.* reported that haloacetic acids and haloacetonitriles were formed in instant tea prepared with hot water containing the free residual chlorine (4 mg/L)[38]. As there is limited information available regarding the occurrence of these compounds on fresh foods, further studies are necessary to investigate their formation. These studies would assist in establishing a risk assessment of chlorine-based disinfectant on foods and contribute to formulating effective sanitizing procedures for fresh foods.

VI Acknowledgments

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論　　文

次亜塩素酸ナトリウム処理によるカットキャベツからの揮発性ハロゲン化合物の生成

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キーワード：トリハロメタン、クロロホルム、次亜塩素酸ナトリウム、キャベツ、クエン酸

概　要

カットキャベツを次亜塩素酸ナトリウムにより殺菌処理したときに生成する消毒副生成物の生成影響因子について検討を行った。カットキャベツは、次亜塩素酸ナトリウム（100 mg/L）単独あるいは、有機酸と共に10分間殺菌処理を行い、カットキャベツに残存する揮発性ハロゲン化合物をヘッドスペースガスクロマトグラフ質量分析装置で測定した。主要な副生成物としてクロロホルムが検出された。クロロホルムは、殺菌時間、pH、温度、初期次亜塩素酸ナトリウム濃度に依存して増加した。次亜塩素酸溶液を種々の無機酸及び有機酸と併用してもトリハロメタンは生成しないが、クエン酸は次亜塩素酸と反応し、クロロホルムを生成した。クロロホルム濃度は、次亜塩素酸・クエン酸混液の混和時間に対応して増加し、カット野菜の殺菌時間には影響しなかった。また、カット野菜に残存したクロロホルムは水洗浄により、水道水中のトリハロメタン濃度レベルまで減少した。

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Determination of sodium stearoyl lactylates in foods using HPLC after derivatization with 2-nitrophenyl hydrazine

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Abstract

A high-performance liquid chromatographic method, following saponification and derivatization with 2-nitrophenyl hydrazine, was developed for determination of lactic acid derived from sodium stearoyl lactylates (SSL) in processed foods. Recoveries of SSL from ten kinds of processed foods spiked with SSL (2 g/kg) ranged from 79 to 102%, while the error associated with repeatability and intermediate reproducibility was less than 6.8% and 7.2%, respectively. This study showed that the proposed method can be applied for analysis of SSL in processed foods. The method is useful and reliable.

Keywords : sodium stearoyl lactylate, derivatization, 2-nitrophenylhydrazine, food, HPLC

I Introduction

Sodium stearoyl lactylate (SSL) and calcium stearoyl lactylate (CSL) are used worldwide as emulsifiers for the facilitation of food and beverage processing. The latter has been designated as a food additive in 1964. The Minister of Health, Labour and Welfare of Japan (MHLW) approved SSL as a food additive in 2010[1]. The standards for use of SSL in processed foods are also established by the MHLW. The permitted levels of use of SSL are defined as CSL. The levels are 8.0 g/kg in mixed-powder for sponge cakes, butter cakes and steamed breads; 5.5 g/kg in butter cakes, sponge cakes and steamed bread; 4.5 g/kg in noodles (raw noodles and instant noodles, excluding other dry noodles); 4.0 g/kg in bread, confections and macaroni; 2.0 g/kg in steamed bean-jam bun (manjyu).

To date, a number of methods have been developed to identify SSL in foods. Regula *et al.* showed that SSL is a mixture of various components using TLC with visualization by bromocresol green spray[2]. A qualitative analytical method for SSL and CSL using HPLC with off-line mass spectrometry was reported by Sudraud *et al*[3]. A semi-quantitative analytical method for SSL determination in wheat flour using TLC was developed by Wheeler[4]. Yukawa and Hanada reported a GC derivatization method for determining CSL in bread[5]. However, this method requires the purified SSL standards, which must be prepared by chemical synthesis. SSL standards are not commercially available. Meanwhile, the MHLW had announced a reference method (a now-defunct method) for CSL determination in bread and confections[6]. In this method, stearoyl lactylate is extracted from food and subjected to saponification to obtain lactic acid, which is then analyzed using enzymatic methods. However, the disadvantages of this method are that they are time-consuming and using a large amount of hazardous organic solvents such as chloroform. Therefore, it is necessary to develop an efficient method for the determination of SSL in processed foods.

Recently, we investigated the components of commercial SSL products in Japanese manufacture using thin layer chromatography (TLC) and liquid chromatography with mass spectroscopy (LC-MS). Sodium stearoyl-2-lactylate is the major component of commercial SSL products (Fig. 1).

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Additionally, sodium palmitoyl-2-lactylate, sodium stearoyl-2-lactoyl lactylate, free fatty acids (principally palmitic and stearic) and free lactic acid are contained as minor constituents. The SSL product contains approximately 20% (19.5 – 20.5) for the content of lactic acid combined fatty acid, which composed of stearoyl-2-lactate, sodium palmitoyl-2-lactylate and sodium stearoyl-2-lactoyl lazctylate, and does not include free lactic acid[7].

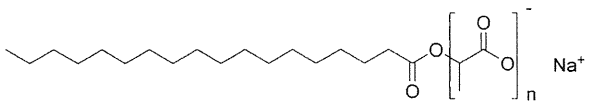

Fig. 1. The structure of sodium stearoyl lactylates
The value of "n" is normally 1.
Sodium stearoyl-2-lactylate (n=1) is contained as the major component of the SSL.

Therefore, we attempted to indirectly determine the SSL and CSL content in process foods by the determination of the lactic acid combined fatty acid. The commercial SSL product has been used as recovery standards, which was determined from our previous work. The lactic acid combined fatty acid in stearoyl lactylate was determined using HPLC method followed by extraction with ethylacetate, saponification and derivatization of resulting lactic acid with 2-nitrophenylhydrazine (2-NPH). SSL content was calculated from the content ratio of the lactic acid combined fatty acid to SSL (20%). This determination method can reduce operation time, as well as the consumption of hazardous solvents. The MHLW has announced a new analytical method for SSL and CSL determination in 2010[8]. The result of this work has been partly used to establish new official method.

Ⅱ Materials and Methods

1. Reagents

SSL (Musashino Chemical Laboratory, Ltd., Lot. No. SLN71015) was kindly provided by the Food Additives Association (Tokyo, Japan). Lactic acid lithium salt was purchased from ACROS (Geel, Belgium), 2-nitrophenylhydrazine (2-NPH) from Kanto Chemical (Tokyo, Japan), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents were of analytical grade, and solvents were of HPLC quality. The ultra pure water used for sample preparation was obtained from a Milli-Q (Millipore, MA, USA) purification system.

2. Reagent solutions

A solution of 2-NPH-HCl (0.02 mol/L) was prepared by dilution of 2-NPH in 0.3 mol/L hydrochloride acid-ethanol (1:1, v/v). A solution of EDC-HCl (0.25 mol/L) was prepared by dilution of the reagent in 3% (v/v) pyridine in ethanol. A solution of KOH (1%, w/v) was prepared by dissolving KOH in ethanol. A solution of KOH (5%, w/v) was prepared by dilution of KOH in ethanol-water (1:1, v/v). All reagent solutions were stable for at least 4 weeks when kept below 4°C.

3. Samples

Since commercial products usually contain emulsifiers, emulsifier-free cookies, bread, doughnuts, sponge cake, and steamed buns, were prepared by ourselves. All prepared samples were stored at -20°C until use.

Emulsifier-free macaroni, dried noodle, mixed flour (wheat flour, sugar, starch, egg yolk powder, fat and oil, glucose, vegetable protein, skim milk-powder, salt, baking powder, vitamin B_2, flavors, spices), dango (rice dumpling), and udon (Japanese wheat noodles, raw type) were purchased from a local market in Tokyo, Japan.

4. Preparation of test solution

1) Extraction

The samples: cookie, bread, doughnut, sponge cake, steamed bun, macaroni, and dried noodle were ground in a mill (National Speed Cuter MK-K57, Matsushita Electric Industrial Co., Osaka, Japan), while the dango and udon were finely cut using a kitchen knife. The dango and udon were homogenized for 1 min using a homogenizer (Nissei Biomixier BM 2, NIHONSEIKI KAISHA LTD., Tokyo, Japan). The prepared samples (2.5 g) were transferred to 50-mL glass centrifuge tubes, and then 20 mL of a 0.1 mol/L HCl solution, 15 mL of ethylacetate and 2 g of NaCl were added to the tubes. The samples were mixed thoroughly and centrifuged for 5 min at 3000 rpm. The obtained upper-layer was transferred to a 50-mL volumetric flask.

A further 15 mL of ethylacetate was added to the mixture in the original tube and the contents were vortexed using a vortex mixer (TTM-1, SIBATA, Saitama, Japan) for 1 min, centrifuged and the upper-layer pooled with that obtained from the first extraction. This step was repeated two times.

The combined solution was brought up to 50 mL with additional ethylacetate. Five milliliters of the solution was transferred to a 10-mL screw cap tube and blow-dried under nitrogen gas, and the residue was obtained.

2) Saponification

One milliliter of the KOH solution (1%, w/v) was added to the residue. The sample tube was capped and vortexed thoroughly. After vortexing, the tube was placed in a water bath at 95°C for 60 min. The mixture was kept warm on the

water bath to evaporate the ethanol, while taking care to avoid evaporation to dryness by the addition of distilled water (0.5 ml). After evaporation of the ethanol, the mixture was cooled at room temperature for 10 min.

Next, 2 mL of 0.1 mol/L HCl solution was added to the tube, and the solution was loaded onto an InertSep-C18 cartridge (500 mg/6 mL) (GL Sciences, Tokyo, Japan), and the eluate was discarded. The cartridge was rinsed with 10 mL of methanol followed by 10 mL of 0.1 mol/L HCl solution before use. The cartridge was eluted with 4 mL of 0.1 mol/L HCl solution. The eluent was brought to a final volume of 5 mL with an addition of 0.1 mol/L HCl solution.

3) Derivatization

The eluent (1 mL) was transferred to a 10-mL screw-cap tube. Two hundred microliters of 0.02 mol/L 2-NPH solution and 200 μL of 0.25 mol/L EDC-HCl solution were added to the tube, and incubated in a water bath at 60°C for exactly 20 min. After immediately adding 200 μL of the KOH solution (5%, w/v) to stop the reaction, the tube was placed in a water bath at 60°C for 15 min, and then cooled at room temperature for approximately 10 min. The final volume in the tube was brought up to 5 mL with an addition of 0.1 mol/L HCl solution. The solution was then filtrated using a membrane filter (Millex-LG 0.45 μm, Millipore). The filtrate was used as the sample solution for HPLC analysis.

5. Quantitation

A standard stock solution of lactic acid (1 mg/mL) was prepared by accurately weighing 106 mg of the lithium lactate standard and dissolving it in water to a final volume of 100 mL. Working standard solutions of 0.5 – 200 μg lactic acid/mL were prepared by diluting the standard stock solution with water. A calibration curve was prepared by analyzing the working standard solutions. The derivatized lactic acid standard solutions (20 μL) and sample solutions were injected into the HPLC system. Peak areas were determined from the obtained chromatogram of the derivatized lactic acid.

6. LC-MS Apparatus and conditions

The sample solutions were analyzed using a Waters UPLC Acquity system, coupled to a Waters Quattro Premier XE mass spectrometer (Waters Corp., MA, USA) with an Inertsil C8 column (5 μm, 2.1 × 150 mm, I.D.) (GL Sciences, Tokyo, Japan). The mobile phase consisted of 10% water containing formic acid (0.1%, v/v) and 90% acetonitrile. The column temperature was maintained at 40°C. The separation was performed isocratically at a flow rate of 0.2 mL/min. A two micro liter aliquot of the sample solution was injected into the LC-MS system.

ESI (electronspray ionization) operated in the negative ion mode was used as the ionization source. The probe voltage was held at 3.0 kV and the cone voltage was set to 30 V. The source temperature was 120°C and desolvation temperature was 350°C. The cone gas flow was 50 L/hr and desolvation gas flow was 350 L/hr. Mass spectra were acquired by scanning from *m/z* 80 to 500 and data were processed using the software of Waters MassLynx (Waters Corp.). Selected ion monitoring (SIM) of *m/z* 98 for the $[M-H]^-$ ion of lactic acid. LC-MS analysis was used to examine the optimization of saponification time.

7. HPLC Apparatus and conditions

The derivatized sample solutions were analyzed using an Agilent1100 series HPLC system, coupled to an Agilent1200 series diode array detector (Agilent Technologies Inc., CA, USA) with an Inertsil C8-4 column (5 μm, 4.6 × 150 mm, I.D.) (GL Sciences). The mobile phase consisted of 25% water containing formic acid (0.1% v/v) and 75% methanol. The column temperature was maintained at 40°C. The separation was performed isocratically at a flow rate of 1.0 mL/min. The detection wavelength was set at 400 nm. The sample solution (20 μL) was injected into the LC column using an autosampler. The data was processed using the software of ChemStation (Agilent Technologies).

8. Calculation of SSL content

SSL content was calculated from the ratio of the content of the lactic acid derived from stearoyl-2-lactate, sodium palmitoyl-2-lactylate and sodium stearoyl-2-lactoyl lactylate to SSL content. Since SSL product contains approximately 20% (19.5 – 20.5)[7] for the content of lactic acid derived from stearoyl-2-lactate, sodium palmitoyl-2-lactylate and sodium stearoyl-2-lactoyl lactylate, SSL content (D) can be calculated using the following equation:

$$D = L \times (100/20) \quad \text{Eq.(1)}$$

D: SSL content (g/kg)

L: the content of lactic acid derived from stearoyl-2-lactate, sodium palmitoyl-2-lactylate and sodium stearoyl-2-lactoyl lactylate in SSL product (g/kg)

L (g/kg) was calculated using the following equation,

$$L = (C \times V \times F \times 10^{-3}) / W \quad \text{Eq.(2)}$$

C: the lactic acid concentration in the test solution (μg/mL)

V: the volume of test solution used for the derivatization reaction (mL)

F: dilution factor (F = 50)

W: sample weight (g)

Based on both Eq.1 and Eq.2, the SSL content was calculated according to the following formula:

$$\text{SSL content (g/kg)} = (C \times V \times 50 \times 10^{-3} / W) \times (100/20)$$
$$= C \times V / W \times 4 \quad \text{Eq.(3)}$$

C: the lactic acid concentration in the test solution (μg/mL)

V: the volume of test solution used for the derivatization reaction (mL)
W: sample weight (g)

9. Calculation of recovery rate from food

The recovery of SSL content was calculated using Eq.(4) as follows.

Recovery (%) = (D/A) × 100 Eq.(4)

D: SSL content determined by Eq (3)
A: spiked SSL content (g/kg)

10. Determination of LOQ

The limit of quantification (LOQ) for SSL content in cookie was calculated from the lactic acid concentration obtained by the derivatized lactic acid peak with S/N = 10 using Eq. (3).

11. Method validation

The in-house method validation studies were performed according to the MHLW pesticide assessment guidelines[9]. The samples were analyzed in duplicate each day for 5 days. A one-way analysis of variance (ANOVA) was used to determine the reproducibility and repeatability. Data analysis was performed using Microsoft Excel software (Microsoft Corp, Seattle, WA, USA).

Ⅲ Results and Discussion

1. Selection of stearoyl lactylate extraction procedure in processed foods

For the extraction of stearoyl lactylate from the processed foods, some extraction solvents (ethyl acetate, hexane and chloroform) were assessed. Among these, ethyl acetate was chosen to be the best solvent to extract stearoyl lactylate from foods due to the results of the extraction efficiency (data not shown) and the toxicity. We also confirmed that no free lactic acid was detected in extracts with ethyl-acetate

2. Optimization of saponification procedure in processed foods

Quantitative analysis for the lactic acid derived from stearoyl lactylate after saponification was performed by the derivatization of lactic acid with 2-NPH[10, 11], followed by the HPLC analysis. However, stearoyl lactylate is used in bread and baked processed foods which contain a large amount of butter and fats composed of glycerol esters of fatty acids. 2-NPH would react with the fatty acids derived from both stearoyl lactylate and food products[10]. Therefore, we considered it necessary to examine the optimization of the saponification, the lactic acid purification step and the derivatization for the analysis of stearoyl lactylate in processed foods.

We optimized the saponification time by measuring the lactic acid derived from SSL using cookie spiked with 2.0 mg SSL/g cookie. SSL product contains approximately 20% (19.5 – 20.5) for the content of lactic acid combined fatty acid[7]. Therefore, 2.0 mg SSL/g cookie contains 400 μg/g lactic acid combined fatty acid. We examined the effect of various saponification times (0, 20, 40, 60, 80, and 100 min) on the recovery of lactic acid derived SSL using LC-MS. The linearity of calibration between 5 and 50 μg/mL gave an appropriate coefficient of determination of R^2 = 0.993. The recoveries appeared to plateau after 60 min of saponification. Consequently, we found that 60 min was an appropriate saponification time, resulting in the highest saponification efficiently (Fig. 2). The reaction time of the examinations was thereafter set to 60 min.

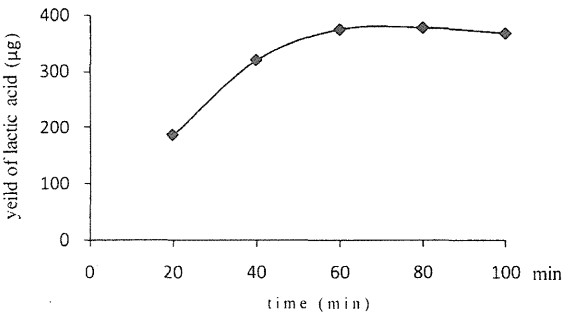

Fig. 2. Effect of incubation time on saponification of SSL

3. Optimization of clean-up procedure of lactic acid using solid phase extraction

When the sample extracted solution after saponification was derivatized with 2-NPH (as described in the Materials and Methods section) and subsequently analyzed using LC-MS, the SSL recoveries were observed to be less than 70%. The sample solution was derivatized with 10 molar excess of 2-NPH solution according to the procedure of Miwa *et al.*[11]. We considered that recovery of lactic acid combined fatty acid in SSL could inhibit the derivatization of other fatty acids such as stearic acid after saponification. Therefore, we examined the purification step to remove fatty acids using a solid phase extraction column from the sample solution after saponification.

The cookie sample spiked with SSL (5 mg) was extracted with ethylacetate and then the extract was subjected to saponification. The entire sample solution was applied to the InertSep-C18 cartridge (500 mg/6 mL) and eluted with 0.1 mol/L HCl solution (10 mL). Fractions (1 mL) were collected and analyzed using LC-MS.

Lactic acid was detected in the first three fractions. In contrast stearoyl acid was not detected until the 10th fraction because steraroyl acid is retained on the InertSep-C18

cartridge until the 10th fraction. These results suggest that lactic acid can be separated from steraroyl acid using the procedure. After the examined solid-phase extraction (SPE) procedure, the recoveries of SSL using the SPE procedure was clearly higher (>87%) than those without the SPE procedure (data not shown).

4. Determination of lactic acid using HPLC-UV

We derivatized lactic acid with 2-NPH according to the previously developed method[10] with minor modifications for the scale of sample volume. In addition, to standardize the analytical method for universal determination of SSL and CSL in processed foods, we sought that the use of HPLC with a UV detector is generally well-used compared to that of LC-MS, because LC-MS equipment is still expensive. Therefore, the lactic acid was derivatized with 2-NPH and then determined by HPLC with UV detector.

We next examined the HPLC conditions based on the report of Miwa *et al.*[11]. Since ethylacetate as extraction solvent was hydrolyzed to acetic acid under the alkaline saponification and the peak of derivatized acetic acid was close to that of the derivatized lactic acid in the HPLC analysis with UV detecion, the separation of derivatized acetic acid peak and derivatized lactic acid peak was required in the HPLC analysis with UV detection. After the examination of the column and the mobile phase conditions, the derivatized lactic acid peak and derivatized acetic acid peak can be clearly separated in the HPLC condition described in Material and Methods section as shown in Fig. 3.

The working standard solution of lactic acid (0.5 – 200 μg/mL) was also derivatized with 2-NPH according to the procedure of Miwa *et al.*[11]. They showed that the 2-NPH could be stoichiometrically reacted with lactic acid. Consequently, we confirmed that the calibration curves of the derivatized lactic acid were linear over a wide concentration range (0.5 – 200 μg/mL), with a good coefficient of determination ($R^2 = 0.999$).

Since the curve obtained by the developed method using commercial SSL product (100, 200, 400, 1000 and 2000 ppm corresponding to 20, 40, 80, 200 and 400 ppm as an amount of lactic acid combined fatty acid, respectively) is well correlated with that obtained using standard of the lactic acid (Fig. 4), we confirmed that the ratio of the content of lactic acid derived

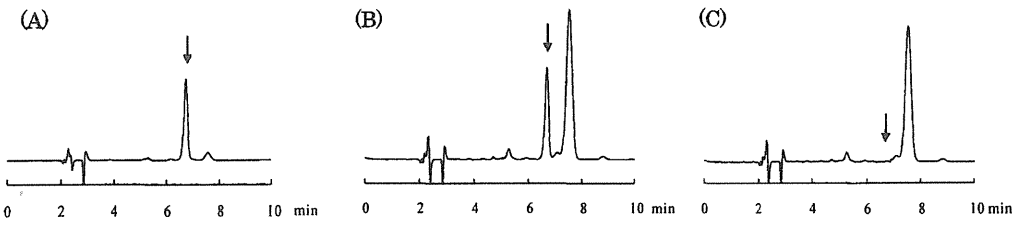

Fig. 3. Chromatograms of SSL analysis from cookie samples spiked with and without SSL
(A) Derivatized lactic acid standard solution (40 μg/mL)
(B) Cookie sample spiked with SSL
(C) Unspiked cookie sample (blank)
HPLC conditions are as follows ; Column : Inertsil C8-4 (4.6 mm × 150 mm, I.D.), mobile phase : methanol-formic acid (0.1 v/v)% solution (25:75), column temperature : 40°C, flow rate : 1.0 mL/min, detected wavelength : 400 nm

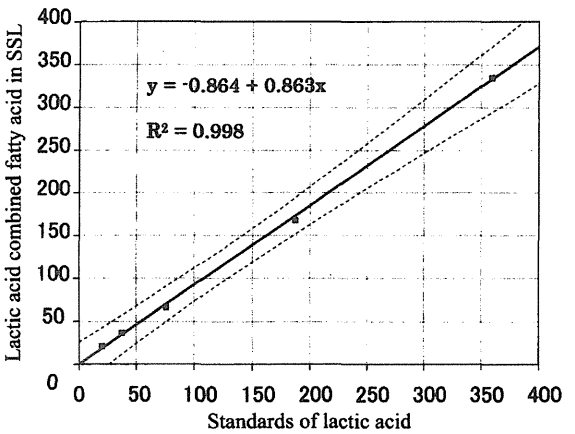

Fig. 4. Comparison of the lactic acid combined fatty acid content in commercial SSL and standard of the lactic acid
The lactic acid combined fatty acid was determined using HPLC followed by extraction with ethylacetate, saponification and derivatization of resulting lactic acid with 2-nitrophenylhydrazine (2-NPH). The SSL content was calculated from the ratio (20%) of the content of the lactic acid combined fatty acid to SSL content.

from stearoyl-2-lactate, sodium palmitoyl-2-lactylate and sodium stearoyl-2-lactoyl lactylate after saponification and purification to SSL content is 20%.

5. SSL recovery test

To perform the SSL recovery test, we prepared three model processed foods (cookies, bread and doughnuts) spiked with 2 mg SSL/g food. The concentration employed was equal to the permitted use levels in foods[1]. Unspiked and spiked model processed foods were analyzed as described in the Materials and Methods section. As shown in Table 1, the SSL recoveries from cookies, bread and doughnuts were 87 ± 4.3%, 83 ± 4.2% and 96 ± 5.9%, respectively. The limit of quantification (LOQ) for the cookie sample were 0.2 g/kg (S/N = 10), respectively.

Table 1. Recovery of SSL from processed foods spiked with SSL

Sample	%Recovery (2 replicates ×5) [a]		
	Mean	RSD_r % [b]	RSD_R % [c]
Cookie	87	4.3	5.3
Bread	83	4.2	4.8
Doughnut	96	5.9	6.5
Sponge cake	102	5.5	6.1
Steamed bun	97	3.8	4.5
Dango	94	2.6	2.8
Udon	92	2.5	3.0
Dried noodle	97	3.3	4.1
Macaroni	88	4.8	5.8
Mixed powder	79	6.8	7.2

a) Samples were spiked with 2 g/kg of SSL
b) RSD_r = repeatability relative standard deviation
c) RSD_R = intermediate reproducibility relative standard deviation

6. Evaluation of the proposed method

The proposed method was validated for accuracy and precision using the 10 processed foods spiked with 2 mg SSL/g food (cookie, bread, doughnut, sponge cake, macaroni, dried noodle, steamed bun, mixed powder, dango, and udon).

As shown in Table 1, the recoveries were 79 – 102%, while the error associated with repeatability and intermediate reproducibility was less than 6.8% and 7.2%, respectively. Consequently, we showed that the proposed method has satisfactory accuracy and precision for the determination of SSL at the permitted use levels.

In this study, SSL content was calculated from the content ratio of the lactic acid combined fatty acid to SSL products. The content ratio of lactic acid combined fatty acid was determined from Japanese SSL products. The SSL and CSL-containing processed foods are also imported from foreign countries. It is necessary to investigate the average and dispersion of the lactic acid content in made of the foreign countries SSL products. However, it is difficult to obtain the SSL and CSL products from foreign countries. While there are a number of limitations to our result, further investigation would be required. To the best of our knowledge, this is a first-time study to determine SSL and CSL products in process foods using a pre-column derivatization HPLC method. This method is a good alternative to others in terms of simple and safety preparation. It can be applicable for the determination of SSL and CSL in processed foods.

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論　　文

2-ニトロフェニルヒドラジン誘導体化法を用いたHPLCによる食品中のステアロイル乳酸ナトリウムの定量

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キーワード：ステアロイル乳酸ナトリウム、誘導体化、2-ニトロフェニルヒドラジン、食品、HPLC

概　要

加工食品中のステアロイル乳酸ナトリウム（SSL）を2-ニトロフェニルヒドラジン（2-NPH）で誘導体化し、HPLCで定量する方法を開発した。本法は、酢酸エチルによる抽出、けん化、2-NPHによる誘導体化から構成される。本法は簡便かつ正確で、選択的な方法である。ステアロイル乳酸ナトリウム製剤（C-SSL）を添加した10種の加工食品からのSSLの回収率は79～102%であり、併行精度及び室内精度はそれぞれ6.8%、7.2%であった。本研究は、本法が加工食品中のSSLの定量に適応可能であることを示した。

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Short communication

Sensitive and simple determination of bromate in foods disinfected with hypochlorite reagents using high performance liquid chromatography with post-column derivatization

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ABSTRACT

A novel analytical method for the quantification of bromate in fresh foods using high performance liquid chromatography (HPLC) with a post-column reaction has been developed. The fresh food sample solutions were pretreated with homogenization, centrifugal ultrafiltration and subsequent solid phase extraction using a strong anion-exchange resin. After separation on a strong anion-exchange chromatography column using a highly concentrated NaCl solution (0.3 M) as the eluent, the bromate was quantified by detection using a post-column reaction with a non-carcinogenic reagent (tetramethylbenzidine). The developed HPLC technique made it possible to quantify bromate in salt-rich fresh foods. The recoveries from fresh foods spiked with bromate at low levels (2 or 10 ng/g) satisfactorily ranged from 75.3 to 90.7%. The lowest quantification limit in fresh foods was estimated to be 0.6 ng/g as bromic acid. The method should be helpful for the quantification of bromate in fresh foods disinfected with hypochlorite solutions.

1. Introduction

Bromate is classified as possibly carcinogenic to humans (Group 2B) [1]. A decade ago, it was reported that bromate was present in the sodium hypochlorite solutions historically employed as disinfectants for drinking water [2]. Sodium hypochlorite is prepared from the reaction of chlorine and sodium hydroxide, which are produced from the electrolysis of brine which is a solution of sodium chloride. While, bromide ions are found to a very varying extent in the sodium chloride. Accordingly, the bromine in chlorine has been thought to be related to bromate production [2]. Therefore, a provisional guideline value of 10 ng/mL in drinking water is recommended by the WHO [3]. In general, sodium hypochlorite solutions have also been employed for the disinfection of fresh foods, including vegetables, meats and fishes, in many countries. However, residual bromate remaining in fresh foods treated with sodium hypochlorite solution remains obscure.

To date, several analytical methods for bromate detection have been reported [4–8]. Initially US Envirinmental Protection Agency (EPA) 300.1 method was established by using ion chromatography with conductivity detection. As more information concerning bromate toxicity became available, lower regulatory limits were imposed, resulting in demands for lower detection limits. This led to development of post-column derivatization and visible detection method EPA 317. In this method, bromate is specifically reacted with a post-column reagent, o-dianisidine, and detected by visible absorbance detection (450 nm). Separation is basically the same as in EPA 300.0. The ion chromatography method with a post-column reaction was reported for the quantification of residual bromate in bread [9] and announced as the official method by the Japanese Ministry of Health and Welfare (published in 1997). However, the use of o-dianisidine is a safety concern because of its carcinogenicity. In addition, the method requires time-consuming clean-up steps for the bread sample extract, including a C18 cartridge for defatting, a silver cartridge for removal of chloride ions, centrifugal ultrafiltration for deproteinization and a cation-exchange cartridge for removal of silver ions [7]. Furthermore, the method is difficult to apply to fresh foods, because excess amounts of byproducts derived from hypochlorite prevent the quantification of bromate. For the quantification of bromate in fresh foods using HPLC, we considered it necessary to develop a separation method and a detection system, as well as a pretreatment method that is closely linked to the separation and detection systems. In the present study, we report a novel analytical method for the quantification of bromate in fresh foods that is based on HPLC with post-column derivatization using tetramethylbenzidine (TMBz) [10,11].

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2. Materials and methods

2.1. Materials

TMBz was purchased from Tokyo Kasei (Tokyo, Japan). Potassium bromate and potassium bromide were purchased from Wako Pure Chemicals (Osaka, Japan). TSKgel SAX (particle size, 5 μm) was purchased from Tosoh Co. (Japan) and packed in a stainless empty column (4.6 mm i.d. × 50 mm). Amicon Ultra-15 Centrifugal Filter device (volume, 15 mL; MWCO, 30 kDa) was purchased from Millipore Co. Ltd. (USA). Muromac AG 1X8 (200–400 mesh, Cl^- form) was purchased from Muromachi Chemical Inc. (Japan).

2.2. HPLC with post-column derivatization for bromate quantification

The HPLC assembly consisted of HPLC pumps (LC-10AD; Shimadzu), a sample injector (7725; Rheodyne), a column oven (CTO-10A; Shimadzu), a UV–vis detector (L-7485; Hitachi), a dry reaction bath (L-5050; Hitachi) and a chromatointegrator (Chromatocorder 21; SIC). Chromatographic conditions for the quantification of bromate were as follows: a separation column, TSKgel SAX (4.6 mm i.d. × 50 mm, particle size 5 μm); column temperature, 40 °C; eluent, 0.3 M NaCl (flow rate, 0.8 mL/min); TMBz reagent, 0.15 M sulfuric acid containing 10 g/L potassium bromide, 250 mg/L TMBz and 20% (v/v) methanol (flow rate, 0.4 mL/min); reaction temperature, 80 °C; cooling temperature, room temperature in water bath; reaction coil, Teflon tube (0.5 mm i.d. × 8 m); cooling coil, Teflon tube (0.5 mm i.d. × 2 m); detection, 450 nm and sample volume, 200 μL for quantification.

3. Results and discussion

3.1. Post-column detection for bromate quantification

TMBz has generally been used as a chromogen for horseradish peroxidase in enzyme immunoassays, and is also widely used as an Ames test negative reagent [10]. Therefore, this non-mutagenic chromogen was used for bromate detection. When a potassium bromate solution was mixed with an acidic solution containing potassium bromide and TMBz, the TMBz was oxidized to a yellow product with a λmax at 450 nm. In this reaction, hydrochloric and sulfuric acids were found to be appropriate as acidic reagents for colour development, whereas nitric acid was not because it caused precipitation of TMBz (data not shown).

3.2. Chromatographic separation of bromate

Inorganic anions are generally separated by ion chromatography, in which both a special column with low capacity and detection by a conductivity detector are employed. Therefore, the concentration of salts in the eluent should be lowered to enable detection. As a result, the ion concentration in the sample solution is also required to be as low as possible. On the other hand, the solid phase extraction of bromate in sample solutions using an ion-exchange resin in the pretreatment steps requires a high concentration of the salt for the elution of the bound bromate on the ion-exchange resin. To solve these problems, the use of a high capacity, strong anion-exchange resin for HPLC, TSKgel SAX (exchange capacity, 1.0 equiv./L; particle size, 5 μm), was investigated. Using this column, a comparatively high concentration of sodium chloride (0.3 M) could be used in the eluent to separate the bromate.

To achieve the lowest quantification limit of 1.0 ng/mL as bromic acid, a high injection volume of up to 200 μL was required. Because a sample solution containing a high concentration of salt was subjected to ion-exchange HPLC with a high injection volume, it was presumed that the bromate might be eluted as a broad peak. Theoretically, the concentration of the salts in the sample solution should be similar to that in the eluent. As shown in Fig. 1A, when the concentration of salt in the sample solution is significantly higher than that in the eluent, the correlation between the peak height and injection volume is not linear. Therefore, the concentration of NaCl in the sample solution was adjusted to 0.3 M, which is identical to the concentration of NaCl in the eluent. Consequently, linearity was observed for the correlation between the peak height and injection volume (Fig. 1B). A chromatogram of bromate in 0.3 M NaCl and a calibration curve for the quantification of bromate in the range from 0.64 to 12.9 ng/mL (as bromic acid) are shown in Fig. 2. The lowest quantification limit (S/N = 5) was estimated to be 0.6 ng/mL as bromic acid. The relative standard deviation at 0.64 ng/mL bromate (approximately the lowest quantification limit) was 10.3% ($N = 3$).

Hypochlorite solutions (100 μg/mL as chlorite) were prepared from a sodium hypochlorite feedstock solution and dry calcium hypochlorite disinfectant by diluting with or dissolving in water and then subjected to HPLC (data not shown). There is no peak interfering with the quantification of bromate in the hypochlorite solution.

3.3. Pretreatment of homogenates prepared from fresh foods

Fresh foods including vegetables, meats and fish were homogenized to 10% (w/v) homogenates in water. The homogenate of cabbage, to which potassium bromate was spiked (final content was 10 ng/mL), was centrifuged at 5000 × *g* for 15 min at 4 °C, and then the supernatant was subjected to centrifugal ultrafiltration using an Amicon Ultra-15 Centrifugal Filter device (volume, 15 mL; MWCO, 30 kDa; 5000 × *g* for 90 min). When the filtrate was subjected to HPLC, the peak for bromate was detected and the recovery was estimated to be 102% (data not shown). This result demonstrates that the present HPLC method requires no special pretreatment techniques, except centrifugal ultrafiltration.

For the solid phase-extraction of the bromate in the filtrate, anion-exchange chromatography using Muromac AG 1X8 (Muromachi Chemical Inc.; 200–400 mesh, Cl− form) was examined. A Pasteur pipette (5 mm i.d.) was used as a disposal column tube, to which 120 μL of Muromac AG 1X8 was packed (5 mm i.d. × 6 mm). Defatted cotton was used as a filter to prevent outflow of the resin. After equilibration with water, 10 mL of the filtrate prepared from the cabbage homogenate spiked with potassium bromate to 1.28 ng/mL was passed through the column. The column was washed with 5 mL of water, and then the bound bromate was eluted by 0.3 M NaCl. An initial 0.15 mL corresponding to the dead volume of the column was discarded, and then the eluent was fractionated into 0.5 mL aliquots. The bromate concentration in each fraction was quantified using HPLC (data not shown). The bromate was eluted in a total of 1 mL of 0.3 M NaCl; therefore, the bromate in 10 mL of ultrafiltrate obtained from a 10% homogenate was concentrated to 1 mL of a 0.3-M NaCl solution.

The recoveries for bromate from cabbage, poultry and horse mackerel were then examined. Bromate was spiked into each homogenate at a concentration of 2 or 10 ng/mL, and the homogenates were analysed after pretreatment of the sample solution. The results are shown in Table 1. The recoveries ranged from 75.3% to 90.7%. Chromatograms of the non-spiked sample solutions prepared from cabbage, poultry and horse mackerel are shown in Fig. 3. Interestingly, although residual bromate was not detected in the non-spiked sample solutions of cabbage and horse mackerel, a trace amount of bromate was detected in the non-spiked sample solutions prepared from commercially available poultry. It is, however, difficult to confirm whether this poultry was disinfected

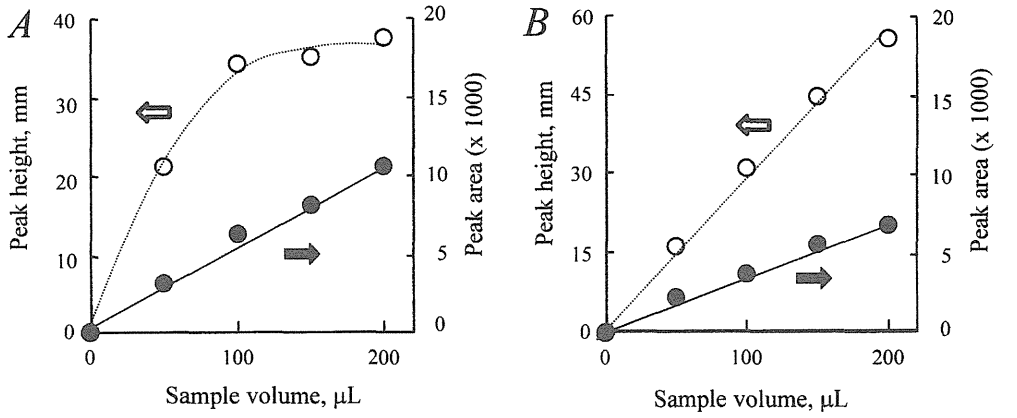

Fig. 1. Effects of sample volume and NaCl concentration in the eluent and sample solution on the elution profile. (A) Eluent, 0.25 M NaCl; sample solution, 100 nM potassium bromate solution (16.7 ng/mL as bromic acid) containing 1.0 M NaCl. (B) Eluent, 0.3 M NaCl; sample solution, 100 nM potassium bromate solution (16.7 ng/mL as bromic acid) containing 0.3 M NaCl. Symbols: open circle, peak height; closed circle, peak area.

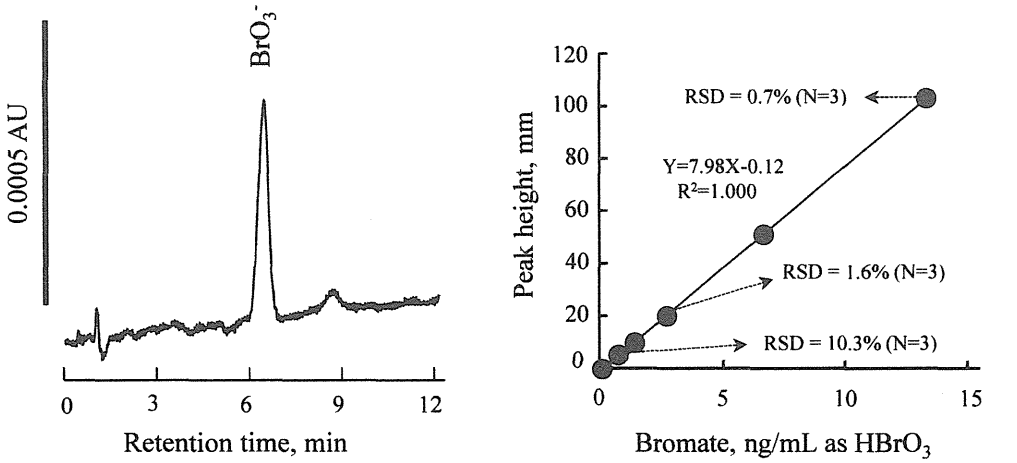

Fig. 2. Quantification of bromate in a solution. Left panel: chromatogram of standard potassium bromate. Two hundred microlitres of 0.3 M NaCl solution containing 10 ng/mL bromate as bromic acid were subjected to HPLC. The chromatographic conditions are described in Section 2. Right panel: calibration curve for the quantification of bromate. Each point indicates the average of individual triplicate analyses.

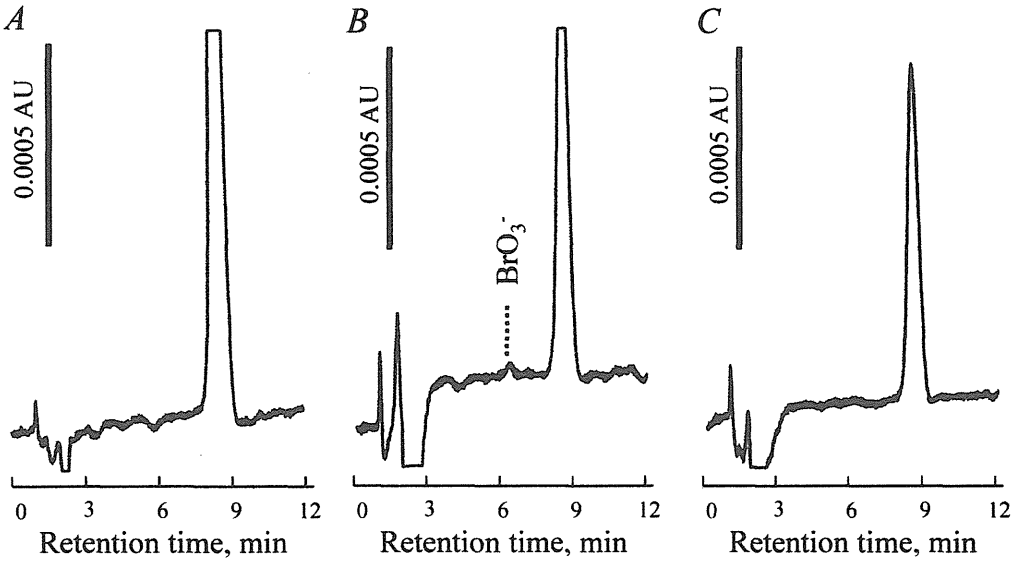

Fig. 3. Chromatograms of (A) cabbage, (B) poultry and (C) horse mackerel samples. Pretreated, non-spiked sample solutions were subjected to HPLC. The chromatographic conditions are described in Section 2.