35) 280, 283, 284合わせて，摂取量はプロピオン酸として
36) 335, 336合わせて乾燥物として
37) ADI はアルミニウムとして，PTWI
38) 無水物として
39) 3水塩として
40) リンゴ酸として

以　上

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Analysis of residual solvent in thickeners by headspace gas chromatography using a standard addition method

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Abstract

Headspace gas chromatography (HS-GC) is an accepted method for analysis of residual solvents in pharmaceuticals, food additives and food. The amounts of residual solvent present in various food thickeners were analysed by HS-GC standard addition method. Conditions for the HS-GC were optimised, and equilibration time was determined to be 40 min at 60°C for the determination of residual solvent. The results were very similar to those obtained by distillation and gas chromatography (Distillation–GC). We conclude that both methods are equally efficient for the determination of residual solvent in thickeners. In addition capillary column (Aquatic-2 GL Sciences Co.) was used to analyze by headspace or distillation.

Keywords : residual solvent, food additive, headspace gas chromatography (HS-GC), standard addition method

I Introduction

The production of natural food additives often involves the use of organic solvents. The eighth edition of Japan's Specifications and Standards for Food Additives[1], restricts the types of organic solvents that can be used in the manufacturing of food additives—extracts and colours—and regulates the levels of residual solvents in these food additives.

The level of residual solvents in food additives in Japan including thickeners and stabilizers, such as carob bean gum, xanthan gum, guar gum, gellan gum, purified carrageenan, pectin, processed eucheuma algae, macrophomopsis gum and rhamsan gum, need to be determined to ensure international consistency with the Joint FAO/WHO Expert Committee on Food Additives (JECFA) standards. In the eighth edition of Japan's Specifications and Standards for Food Additives, the standard residual solvent levels for 2-propanol or 2-propanol plus methanol were set for nine different thickeners.

There are several methods available for the quantification of residual solvents in thickeners. Distillation with water is the most widely used for collecting residual solvents from thickeners, and this is the method adopted in the eighth edition of Japan's Specifications and Standards for Food Additives. This method uses gas chromatography (GC) with a packed column, because the distillate includes large amounts of water. Headspace (HS)-GC using packed columns is also the method of choice for purity testing, in which the residual solvent is converted into corresponding nitrite esters, according to the JECFA monograph for carob bean gum and guar gum*1. At present, HS-GC using capillary columns for determining levels of volatile substances is used in laboratories because of its high resolution. The HS-GC method is widely used to determine volatile substances in pharmaceutical drugs[2], and the standard addition method primarily helps avoid influences of the sample matrix[3]. Comparability between HS-GC using the standard addition method and distillation-GC has not been assessed. Moreover, the eighth edition of Japan's Specifications and Standards for Food Additives and JECFA have not established the use of capillary columns for detecting residual solvents in thickeners. It would be desirable to have information on the usefulness of capillary columns when analysing samples containing large amounts of water with gas chromatography.

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*1 JECFA (Joint FAO/WHO Expert Committee on Food Additives), Online Edition: "Combined Compendium of Food Additive Specifications" http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en

This study determines residual solvents in food thickeners such as carob bean gum, guar gum and gellan gum by HS-GC, using the standard addition method and distillation-GC. We compared the results of both methods, and investigated the use of capillary columns for determining residual solvents in thickeners.

Ⅱ Materials and methods

1. Chemicals and instruments

Methanol (HPLC grade) was obtained from Merck (Damstadt, Germany), 2-propanol (HPLC grade), tertiary-butanol (*tert*-butanol), guar gum, carob bean gum, and gellan gum were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Headspace vials (10 ml) and aluminium crimp seals with PTFE septa, an Aquatic-2 capillary column (60 m × 0.25 mm i.d., 1.4 μm film thickness) and a glass column (2.1 m × 3.2 mm i.d.) packed with Gaskuropak54 60/80 were supplied by GL sciences Inc. (Tokyo, Japan). Gas chromatography (HP 5890) using a flame ionisation detector (FID) and headspace sampler (HP 7694) for the standard addition method were supplied by Hewlett Packard Inc.. Gas chromatography using a FID (GC 14B) for the distillation method was supplied by Shimadzu Corp. (Kyoto, Japan).

2. Solvent additional sample preparation

The regulation of residual solvent limits 2-propanol to 1% in carob bean gum and guar gum, to 0.075% in gellan gum in the eighth edition of Japan's Specifications and Standards for Food Additives. Because there are no residual solvents in commercial versions of these gums, 2-propanol was added to guar gum, carob bean gum, and gellan gum. Since methanol was limited in other some thickeners, methanol was added to them to obtain additional information. A hundred millilitres of methanol and 2-propanol were added to 200 g of each gum. The gums were placed in a fume cupboard for a week at room temperature. The gums were then sealed in glass bottles and refrigerated at 4°C.

3. Standard solutions for the standard addition method of HS-GC

Using water, 0.8 g each of methanol and 2-propanol were made up to 100 ml, 1 ml of each resultant solution was made up to 100 ml in water as standard A (Std A: 80 μg/ml), of which 10 and 5 ml were diluted to 20 ml in water as standard B (Std B: 40 μg/ml) and C (Std C: 20 μg/ml), respectively. In the case of gellan gum, the amount of 2-propanol present was so high that 10 times the concentration of the standard solutions had to be used.

4. Sample preparation for HS-GC

Exactly 0.04 g of the gum was weighed out in four 10 ml vials, and a magnetic stirring bar and 5 ml of either water, Std A, Std B or Std C were added to each vial. Then the vials were immediately sealed using an aluminium cap with a PTFE septum. The vials were left standing overnight at room temperature. After stirring the solution in the vials for 1 min, the vials were then placed in the headspace sampler.

5. Procedure for HS-GC

Helium served as the carrier gas at 250 kPa (1.5 ml/min). The oven in the GC was maintained at 40°C for 6 min, and then heated to 110°C at a rate of 4°C/min. Finally, the temperature was increased to 250°C at a rate of 25°C/min, and maintained for 10 min. The injection temperature was 200°C, and the detector temperature was 250°C.

The headspace in the vial was injected as follow: the vials were kept at 60°C for 40 min, then pressurised for 0.15 min with carrier gas, the sample loop temperature was adjusted to 110°C, and the transfer line temperature was 130°C. Headspace gas was introduced to the GC column in the split mode in a 10:1 or 90:1 ratio.

Quantification was performed by the standard addition method. The peak areas of methanol and 2-propanol were measured. The area of methanol or 2-propanol for each sample (one spiked with water and three spiked with different levels of standard solutions) was plotted on the y-axis and the concentration (μg/g) of solvent in the standard solution was plotted on the x-axis. A calibration curve was obtained by the simple linear least squares analysis method. The absolute x-intercept value at $y = 0$ represents the concentration of the solvent as C_{sa} (μg/g).

6. Sample preparation for distillation and GC chromatography

Tert-butanol (0.1 g) was dissolved in 100 ml of water as the internal standard solution. Methanol and 2-propanol (0.5 g) each were weighed out exactly and made up to 50 ml with water, of which 5 ml was diluted to 50 ml using water. 2 ml of the methanol and 2-propanol mixture solution, and 4 ml of internal standard solution, were made up to 100 ml with water as standard solution.

The gums were weighed out (2 g each) in 300 ml round flasks, and 200 ml of water, 5 ml of silicon oil and a few boiling stones were added and mixed. The flasks were then connected to a distillation apparatus. The flasks were heated using a heater, and when the water and gum started to boil, the temperature of the heater was reduced, to keep the mixture boiling without boiling over the connection to the distillation apparatus. The internal standard solution (4 ml) was added to a 100 ml volumetric flask. Distillate was added at a rate

of 2–3 ml/min to a 100 ml volumetric flask. The process of distillation was stopped when 90 ml of distillate was collected, and the distillate was made up to 100 ml by adding water.

GC was performed using a Shimadzu GC-14B equipped Aquatic-2 capillary column, with helium as a carrier gas at 250 kPa (1.0 ml/min). The oven in the GC was maintained at 40°C for 6 min, and then the temperature was increased to 110°C at a rate of 4°C/min. Finally, the temperature was increased to 250°C at a rate of 25°C/min, and maintained for 10 min. The injection temperature was 200°C, and the detector temperature was 250°C. The injection volume was 1 μl, and the injection was performed in split mode in a 100:1 ratio.

One microliter of the standard solution and distillate were injected into the GC with FID, and the areas under the peak for *tert*-butanol, methanol and 2-propanol were measured. The amount of 2-propanol and methanol was determined by the ratio of the area of methanol or 2-propanol to that of *tert*-butanol. The ratios of standard solution and distillate were denoted as Q_S and Q_T. Q_S and Q_T were calculated using the formula A_a/A_{TBA}, in which A_a is the area of each alcohol peak (a = methanol, 2-propanol), and A_{TBA} is the area of the *tert*-butanol.

The percentage of solvent was calculated as the weight of solvent (g)/weight of gum (g) × Q_T/Q_S × 0.4.

7. Comparison between packed column and capillary column with FID (GC 14B)

A standard solution of methanol, 2-propanol and *tert*-butanol for distillation was applied in a packed or capillary column with FID (GC 14B) and the peaks compared.

The packed column was equipped with GC 14B; the column temperature was 120°C; the injection port temperature was 200°C; the carrier was helium flowing at 250 kPa. The GC conditions, using the capillary column with GC 14 B, were the same as for the distillation method.

8. Recovery tests with spiked sample preparation by HS-GC

To evaluate the repeatability and the accuracy of the HS-GC, recovery tests were carried out by analyzing two different concentration levels of 2-propanol with 1 and 0.5% for carob bean gum and guar gum, or 0.075 and 0.0375% for gellan gum. Exactly 0.04 g of the gum was weighed out in four 10 ml vials, and a magnetic stirring bar and 5 ml of either water, Std A, Std B or Std C were added to each vial. In case of gellan gum Std A, Std B and Std C were diluted into 6, 3 and 1.5 μg/ml, respectively. Fifty microliter of standard solution containing 2-propanol with suitable concentration was added to all sample preparations before sealing the vials. The spiked samples were treated according to the sample preparation procedure for HS-GC.

Ⅲ Results and Discussion

1. Distillation-GC using capillary column

Figure 1 shows gas chromatograms of the standard solutions of methanol, 2-propanol and *tert*-butanol in water taken with GC-FID using packed and capillary columns.

In the chromatogram of the packed column (Fig. 1 a), methanol eluted at ca. 2.3 min, 2-propanol at 12.2 min and *tert*-butanol at 23.4 min. Although these peaks were clear, the methanol peak appeared near the injection artefact at around 2 min, and the peak of *tert*-butanol was broad. On the other hand, in the chromatogram of the capillary column (Fig. 1 b), methanol eluted at ca. 4.2 min, 2-propanol at 6.8 min and *tert*-butanol at 7.4 min. All the peaks were clear and the area of each peak was not spread out in this chromatogram. There was no degradation of the capillary column for repeated injections of the sample solutions. These results show that the method can accurately analyse distillate containing water from thickeners.

Carob bean gum, guar gum and gellan gum were distilled with water, and the distillate collected and analysed by GC-FID using a capillary column. Figure 2 shows the chromatogram of the distillate from guar gum, carob bean gum, and gellan gum analysed by GC-FID using a capillary column. No significant difference was observed in the area of the internal standard (*tert*-butanol), and the peak showed good resolution.

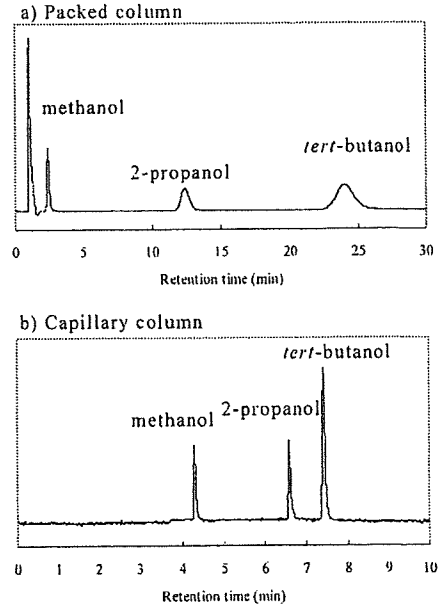

Fig. 1. GC-FID Chromatogram of methanol, 2-propanol and *tert*-butanol in water using a) a packed column, and b) a capillary column

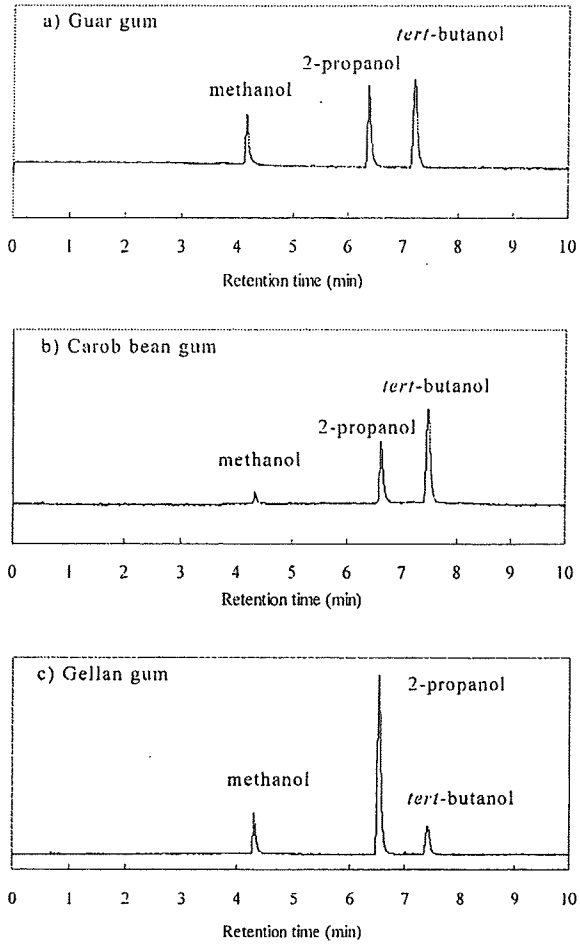

Fig. 2. GC-FID chromatogram of residual solvent in spiked a) guar gum, b) carob bean gum and c) gellan gum after distillation

2. Headspace Gas Chromatography (HS-GC)

The sample vials were kept for various pre-incubation times (5 min to overnight) at various temperatures (room temperature to 50°C), and HS-GC was performed (vial equilibration at 60°C for 40 min). The results all showed almost the same or less methanol and 2-propanol than obtained by distillation, but the amount of methanol varied for a short pre-incubation time or at a low equilibration temperature.

This variability was caused by incomplete swelling of the gums and equilibration in the vials. Therefore, we kept the sample vials overnight at room temperature. Furthermore, the peaks of methanol and 2-propanol widened, depending on the equilibration temperature for the headspace vials in the headspace sampler. To improve the precision of the analysis, we performed the analysis at an equilibration temperature of 70°C, but the residual solvent value was below that seen at 60°C.

The residual solvents absorbed in the thickener are released by swelling the thickener in water. Guar gum, carob bean gum and gellan gum were swollen in water or the standard solution in sealed vials overnight at room temperature. We used the standard addition method to avoid the affect of the sample matrix, because each thickener shows different viscous behaviour in water. Figure 3 shows the chromatogram of the HS-GC of the residual solvent in each sample. Methanol and 2-propanol eluted at ca. 5.4 min and ca. 8.1 min, respectively. These peaks were completely resolved from the other volatiles.

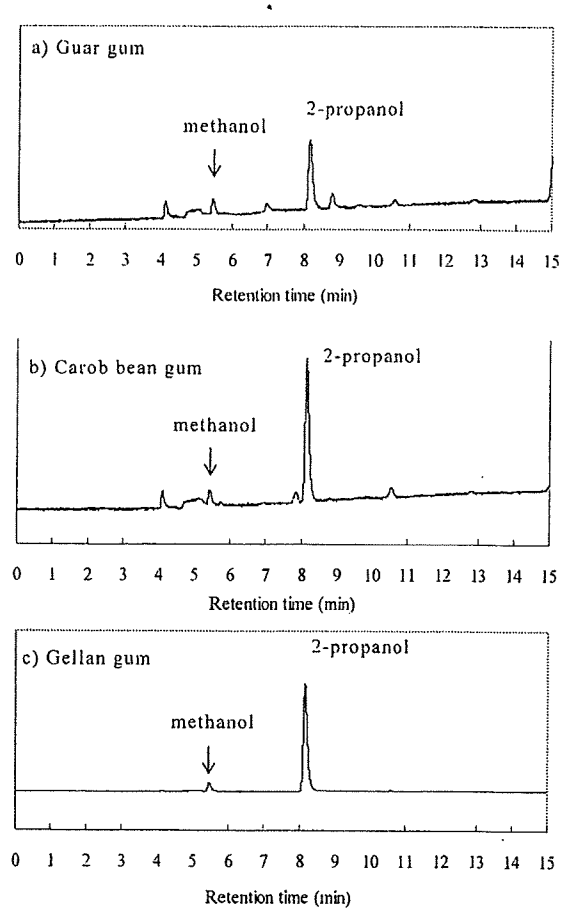

Fig. 3. HS-GC chromatogram of residual solvent in spiked a) guar gum, b) carob bean gum and c) gellan gum after keeping them overnight at room temperature HS oven temperature: 60°C, equilibration time: 40 min.

3. Quantification of methanol and 2-propanol by HS-GC and distillation-GC

Table 1 shows the quantitative values of methanol and 2-propanol in carob bean gum, guar gum and gellan gum analysed by HS-GC and distillation. The values obtained by HS-GC were similar to those obtained by distillation-GC for all the gums, however, the values of 2-propanol by distillation of carob bean gum and gellan gum were slightly higher than those by HS-GC. The values of 2-propanol in gellan gum were

spread out, probably due to overload of the capillary column. The differences in values were not statistically significant. From Table 1, both methods obtained similar quantitative values and the distillation-GC and HS-GC methods showed similar effectiveness in analysing the residual solvent in thickeners.

HS-GC is widely used for determining residual solvents in pharmaceutical substances, and is also used for some natural food additives and flavours[4-6], but the method has not yet been introduced in Japan's Specification and Standard for Food Additives. When HS-GC is used for quantitative determination of residual solvents, the sample must be well dissolved or suspended uniformly to volatilise the residual solvents. However, coexisting matrices in the sample affect the partial coefficient between the gaseous and sample phases in the HS vial because of their stickiness[3]. To avoid this affect of the matrix, we used the standard addition for the quantitative analysis of residual solvents in thickeners by HS-GC.

Table 1. Comparison of the quantitative values obtained by Distillation-GC and HS-GC methods

		Content (%)*	
		GC-FID	
		HS	
Sample		60°C, 40 min	Distillation
Guar gum (add)	Methanol	0.11 ± 0.02	0.13 ± 0.01
	2-propanol	0.21 ± 0.02	0.20 ± 0.01
Carob bean gum (add)	Methanol	0.02 ± 0.01	0.03 ± 0.00
	2-propanol	0.11 ± 0.02	0.13 ± 0.02
Gellan gum (add)	Methanol	0.33 ± 0.02	0.30 ± 0.02
	2-propanol	1.30 ± 0.14	1.31 ± 0.03

* Each value is mean ± S.D. of 3 trials

4. Recovery tests with spiked samples by HS-GC

The repeatability and accuracy of HS-GC were evaluated by a recovery test with each gum. Table 2 shows the recoveries of spiked 2-propanol from carob bean gum, guar gum and gellan gum. The recoveries were 83.1~107.5%, and the relative standard deviations (RSD) were 2.9~9.6%. RSD(%) at a spiked level of 1% were improved by modification of the GC injector split ratio from 10:1 to 90:1

The thickeners used in this study were insoluble in organic solvents, but soluble in water. However, adding a small amount of water made them into a hard gel, which made HS-GC analysis difficult. Because adding even 5 ml of water or Std solution to 0.04 g of thickeners is not enough to eliminate specific viscous behaviours, we tried to avoid the matrix effect by using the standard addition method in this study.

Table 2. Recovery tests of 2-propanol in sample preparations

Sample	Spiked level (%)	Recovery (%)[a]	RSD (%)
Carob bean gum	1	106.9	5.8
	0.5	83.1	9.3
Guar gum	1	103.7	9.6
	0.5	104.9	2.9
Gellan gum	0.075	107.5	7.2
	0.0375	106.9	6.2

[a] Mean of 3 replicates

Ⅳ Conclusions

Residual solvents in thickeners were determined by HS-GC using a standard addition method and the distillation-GC method. The results of both methods were very similar, and both methods were equally effective. The standard addition method is effective for determining the amount of residual solvent in viscous material. Capillary columns can be used to analyse the residual solvent containing water in thickeners in both HS-GC and distillation-GC methods, and there was no problem in separating the peaks of methanol and 2-propanol with good resolution.

Ⅴ Acknowledgement

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論　　文

標準添加法を用いたヘッドスペースガスクロマトグラフィー法による増粘安定剤中の残留溶媒の分析

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キーワード：残留溶媒、食品添加物、ヘッドスペースガスクロマトグラフィー（HS-GC)、標準添加法

概　要

ヘッドスペースガスクロマトグラフィー（HS-GC法）は薬品、食品添加物および食品中の残留溶媒の分析法として頻繁に用いられている方法である。本研究では、増粘安定剤（カロブビーンガム、グアーガム、ジェランガム）中のメタノールおよび2-プロパノールを分析対象とし、標準添加法を用いたヘッドスペースガスクロマトグラフィー法（HS-GC法）および水で蒸留後、留液をガスクロマトグラフィーで分析する方法（蒸留-GC法）により分析し、両方法で得られる溶媒量の比較検討を行った。その結果、HS-GC法と蒸留-GC法ではほぼ同程度の溶媒量が検出された。以上の結果から、増粘安定剤のような粘性の高い試料に対して、標準添加法によるHS-GC法は蒸留-GC法などと同等有用な方法であると考えられた。また、いずれの分析法においてもキャピラリーカラム（Aquatic-2 ジーエルサイエンス（株））を用いて良好な分離が得られた。

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Negative and Positive Ion Mode LC/MS/MS for Simple, Sensitive Analysis of Sorbic Acid

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Sorbic acid (SA: CH_3–CH=CH–CH=CH–COOH) is one of the widely used food preservatives, although there have been some reports of its toxic activity, for example, on DNA and skin cells. In order to examine the effects of SA on mammalian tissues, we have developed a highly sensitive analytical method using LC/MS/MS with positive and negative ion mode electrospray ionization (ESI). In a previous study, we found that a nonacidic eluent offers better ionization efficiency than acids or their ammoniun salts. However, optimal results could not be obtained because the anion form of SA is poorly retained on a conventional reversed phase column. To resolve this problem, we chose a new type of column and used high-resolution mass spectrometry and positive ion mode analysis. There have only been a few reports using these methods in the positive mode, for example derivatized SA, because acid compounds such as SA are usually used in the negative ion mode. However, a new type of low-carbon-content and polar-endcapped C18 phase column was developed for better separation of SA from the matrix. High-resolution selected reaction monitoring (SRM) gave the best signal to noise ratio in normal-resolution SRM. In the positive ion mode, the CH_3OH–0.05% HCOOH/0.1% CH_3COOH eluent system yielded the best ionization efficiency. We propose a highly sensitive and simple analysis using a two-ion-mode ESI SRM method. Such systems should allow quantification of the amount of SA in or around the cells, without the need for pretreatment such as solid phase extraction.

Key words sorbic acid; LC/MS/MS; electrospray ionization; selected reaction monitoring

Sorbic acid (SA) and its salts are commonly used as food additives because of their antibacterial and growth inhibitory activities against yeast and fungi. They are also used in cosmetics, pharmaceuticals and tobacco products.[1] Their usage for food preservation is usually considered to be safe for human consumption. However, some studies have shown that SA and its salts exhibit a weak genotoxic potential,[2—4] including causing damage to DNA[5] and having an alkylating activity on nucleophilic 4-(*p*-nitrobenzyl) pyridine.[6] Also, Soschin and Leyden reported that SA induced erythema and edema in human skin,[7] although the mechanism was not clarified.

There is a need to understand the actions of SA and its salts in terms of possible cytostatic or cytotoxic effects in mammalian tissues and cells. Some work has been done to examine the effects of SA on cultured mammalian cells.[8,9]

Mass spectrometry, which offers high sensitivity and selectivity, should be ideally suited to the detection of SA. However, there are few reports on the use of LC/MS for SA analysis. Negri *et al.* reported that SA in urine could be measured by selected ion monitoring (SIM) of *m/z* 111.13 with electrospray ionization (ESI)-LC/MS in the negative ion mode with the detection limit of 4 μmol/l,[10] and Cartwright *et al.* reported that SA derivative at less than 4 fmol was detected by using ESI-LC/MS/MS selected reaction monitoring (SRM) in the positive ion mode.[11] Generally, the SRM mode is more selective and sensitive than the SIM mode, and the technique for detecting the SA derivative requires extra time for analysis because of unwanted side products.

In a previous paper, we reported a simple and sensitive method for the determination of SA in or around cultured mammalian cells by using neutral solvents (CH_3OH/CH_3CN–H_2O) for HPLC (eluents) under negative ion mode ESI-LC/MS/MS.[12] Acids and their ammonium acetate solvents are usually used as eluents. However, this sacrifices the ionization efficiency of SA under negative ion mode ESI for retention on a conventional reversed phase column. On the other hand, the anion form of SA has high ionization efficiency under the negative ion mode ESI but poorly retains SA on a conventional reversed phase column, thus not giving the best separation of SA from the cytosol fraction.

To solve the problem, we developed simpler, more sensitive and better separation techniques for the determination of SA in the cells by using LC/MS/MS in the positive and negative ion modes. With the negative ion mode, we used two experimental approaches. First, HPLC columns were examined for their ability to retain and separate the anion form of SA from a matrix such as cytosol. The columns used were endcapped reversed phase columns (ODS), carbon-column and temperature-responsive silica columns filled with cross-linked poly (*N*-isopropylacylamide) hydrogel-modified amino silica beads.[13—15] Second, a high resolution mass spectrometer was used in an SRM mode for mass separation of SA from the matrix using the exact mass value. We also tried positive ion mode ESI-LC/MS/MS. To find the most sensitive eluent system under the positive ion mode, we compared the use of acid solvents with the use of ammonium acetate.

To evaluate the matrix effect for the analysis of SA in the positive ion mode, we measured the recovery of SA from the cytosol of mastocytoma P-815 cells, which are used as a proper model of growing mammalian cells because P-815 cells are favorable for examining growth and differentiation, and also for the evaluation of various compounds with re-

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spect to cytotoxicity, phototoxicity and immunotoxicity.[16—22]

Experimental

Materials SA, guaranteed grade, and LC/MS-grade of H_2O, CH_3OH and CH_3CN were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Guaranteed grades of HCOOH, CH_3COOH, $HCOONH_4$ and CH_3COONH_4 were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Argon gas (99.99%) used as the collision gas of the SRM mode was obtained from Neriki Gas Co., Ltd. (Hyogo, Japan).

Preparation of Cytosol Cytosol from the mastocytoma cells, preloaded with 2.5 mmol/l SA for 0.5 h or without SA, was prepared as described in a previous paper.[12] These extractions were analyzed by LC-MS/MS.

Columns and Conditions ODS columns, carbon column and cation column were used for the analysis of SA under negative ion mode ESI. ODS columns (4.6 mm i.d.×150 mm); Handy ODS (particle size 5 μm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), TSKgel ODS-100V (particle size 3 μm) and TSKgel ODS-100Z (particle size 5 μm) were from TOSOH Co. (Tokyo, Japan), Hypersil GOLD aQ (particle size 3 μm) and Thermo Hypersil-Keystone BetaMax Neutral (4.6 mm i.d.×150 mm and 2.1 mm i.d.×150 mm; particle size 5 μm) were from Thermo Fisher Scientific K. K. (Yokohama, Japan). Carbon column Hypercarb (4.6 mm i.d.×150 mm; particle size 5 μm) was obtained from Thermo Fisher Scientific K. K. (Yokohama, Japan). The temperature response cation silica column, which used cross linked poly (*N*-isopropylacylamide) hydrogel-modified amino silica beads, Aqua Way Cation (4.6 mm i.d.×150 mm; particle size 5 μm) was obtained from CellSeed Inc. (Tokyo, Japan). The retention time of SA under negative ion mode of these columns and these eluents are summarized in Table 1. A Thermo Hypersil-Keystone BetaMax Neutral (2.1 mm i.d.×150 mm; particle size 5 μm) was used for the separation column, under negative ion mode ESI high resolution SRM. A binary mobile phase consisting of H_2O (solvent A) and CH_3CN (solvent B) was used in the following program: 0 min, 10% B; 2.0 min, 70% B; 8.0 min, 70% B; 8.1 min, 10% B, 15 min, 10% B. The flow rate was 0.2 ml/min, and the injection volume was 5 μl. Under the positive ion mode ESI-SRM, a TSK gel ODS 100 V was used as a separation column. The eluents used and ionization efficiencies are summarized in Table 2.

Triple-Quadrupole Mass Spectrometer Conditions A Quattro premier triple-quadrupole LC-MS (Micromass, Manchester, U.K.), equipped with an ESI source was used for the negative ion and positive ion mode MS/MS analyses coupled to the Alliance HT Waters 2795 separation module (Waters Co., Milford, MA, U.S.A.). The instrumental parameters of the negative ion mode were used as described in our previous paper.[12] Under the positive ion mode, the parameters of the ionization efficiency were optimized by evaluating the sensitivity based on flow injection analysis. SA, 1 mmol/ml, was injected at 5 μl/min by syringe and connected with the line of the mobile phase of 0.05% HCOOH–CH_3OH (40 : 60), flow rate 0.4 ml/min, *via* a T-joint. The parameters of the analyzer were optimized under the same conditions, temperature (source and desolvation) and the nitrogen gas flow rate (cone and desolvation), of the negative ion mode. The product ion spectrum was obtained by scanning Q3 over the mass range of *m/z* 40—120. The flow rate of the argon collision gas for fragmentation in the SRM mode was 0.3 ml/min (3.37—3.39×10^{-3} mbar) by which the collisional energy was optimized for the fragment ion of SA. The optimized value of the cone was set at 19 V and the collisional energy at 10 eV. During the Q3 scan, the Low Mass (LM) and High Mass (HM) resolution values for both Q1 and Q3 quadrupoles were 15; while during SRM analysis, they were 10.

The high resolution SRM analysis under negative ion mode ESI was done using a TSQ Quantum Ultra (Thermo Fisher Scientific K.K., Yokohama, Japan) equipped with Prominence (Shimadzu Corp., Kyoto, Japan) HPLC system. The resolutions of Q1 and Q3 were set at 0.1 and 0.7 for high resolution SRM, respectively. The optimized parameters were: spray voltage 2000 V; vaporizer temperature, 350 °C; ion transfer tube temperature, 330 °C; sheath gas, 30 arbitrary units; auxiliary gas, 20 arbitrary units; skimmer collision-induced dissociation, 5 eV; collision gas, Ar; collision gas pressure, 0.6 m Torr.

Table 1. Retention Time of SA Using Various Columns

Column	Eluent	Carbon content (%)	Retention time (min)
Handy ODS	H_2O–CH_3CN (60 : 40)[a]	16	5.1
TSKgel ODS-100V	H_2O–CH_3CN (60 : 40)[a]	15	6.2
TSKgel ODS-100Z	H_2O–CH_3CN (60 : 40)[a]	20	2.8
Hypersil GOLD aQ	H_2O–CH_3CN (60 : 40)[a]	12	7.0
Hypersil-Keystone BetaMax Neutral	H_2O–CH_3CN (60 : 40)[a]	29	3.2
Hypercarb	0.02% HCOOH–CH_3CN (10 : 90)[a]	—	8.2
	5 mM $HCOONH_4$–CH_3CN (10 : 90)[a]		8.6
	H_2O–CH_3CN (5 : 95)[a]		N.D.
Aqua Way Cation	H_2O–CH_3CN (10 : 90)[b]	—	8.2

a) Flow rate: 0.4 ml/min, column temperature: 30 °C. *b*) Flow rate 1.0 ml/min, column temperature: 40 °C. N.D.: not detected.

Table 2. Effect of Eluent on Ionization Efficiency of Positive Ion Mode ESI

Eluent	pmol	Peak area	Peak area ratio
0.05% HCOOH/2 mM $HCOONH_4$–CH_3CN	2.5	112	1
0.05% HCOOH/2 mM $HCOONH_4$–CH_3OH	2.5	1297	11.6
0.05% HCOOH–CH_3CN	2.5	8211	73.3
0.05% HCOOH–CH_3OH	2.5	15842	141.4
0.1% CH_3COOH–CH_3CN	2.5	11308	101.0
0.1% CH_3COOH–CH_3OH	2.5	20515	183.2

The volumes of CH_3CN and CH_3OH were 50% and 60%, respectively. Flow rate: 0.4 ml/min.

Results and Discussion

Negative Ion Mode ESI Analysis Under the negative ion mode, we examined two strategies to solve the problem of poorly retained SA on ODS columns. First, we compared the retention time of SA among the five ODS columns and other types of column under isocratic eluent conditions (Table 1). In the case of ODS columns, SA is more strongly retained on Handy ODS, TSKgel ODS-100V and Hypersil GOLD aQ columns (Fig. 2A), than TSKgel ODS 100Z and Hypersil-Keystone BetaMax Neutral columns. The carbon content levels of these columns (%) were 16%, 15%, 12%, 20% and 29%, respectively and their accessible silanol groups were endcapped with various reagents, *i.e.* TSKgel ODS-100V with difunctional dialkylsilane reagents and Hypersil GOLD aQ with polar functional group(s). Hypercarb, one of the carbon columns, did not retain SA with neutral eluent. The Aqua Way Cation column, which responds to temperature and other external stimuli, showed good separation of SA from the cytosol fraction (Fig. 2B), but the sensi-

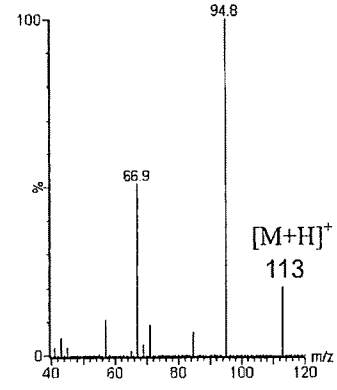

Fig. 1. Positive Ion Mode ESI Product Ion Spectra of $[M+H]^+$ *m/z* 113 with Optimized Collisional Energy

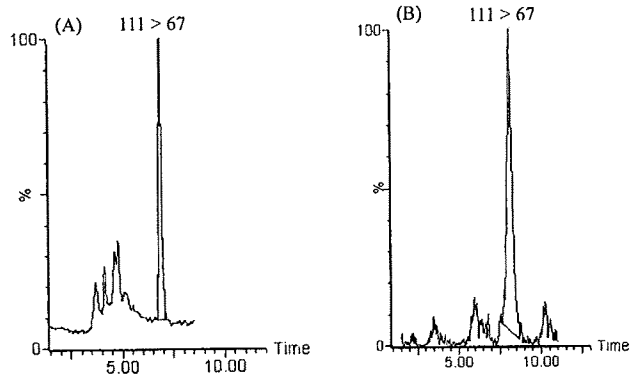

Fig. 2. Negative Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

(A) An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 20 μl was injected to LC/MS . Eluent: CH_3CN–H_2O (40 : 60), flow rate: 0.4 ml/min, column: Hypersil GOLD aQ. (B) An equal volume of SA 1.0 μmol/l was added to filtered cytosol, and 20 μl was injected to LC/MS. Eluent: CH_3CN–H_2O (90 : 10), flow rate: 1 ml/min, column: Aqua Way Cation.

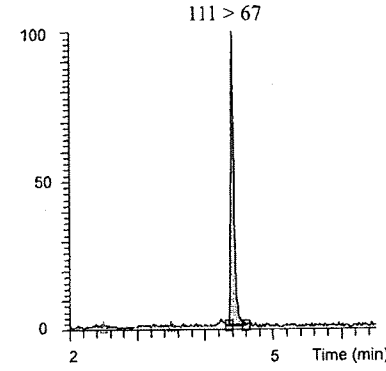

Fig. 3. Negative Ion Mode ESI High Resolution SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 μl was injected to LC/MS (SA 256 fmol, S/N 81). Eluent: CH_3CN–H_2O (10—70 : 90—30), flow rate: 0.2 ml/min, column: Thermo Hypersil-Keystone BetaMax Neutral.

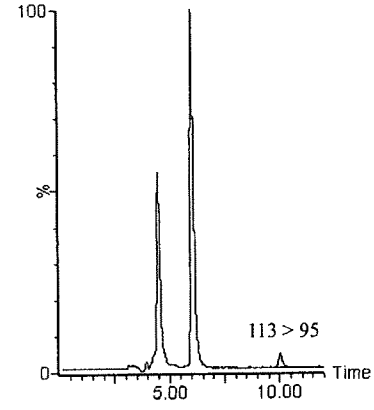

Fig. 4. Positive Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 μl was injected to LC/MS (SA 256 fmol, S/N 24). Eluent: 0.05%HCOOH–CH_3OH (40 : 60), flow rate: 0.4 ml/min, column: TSKgel ODS-100V.

tivity was poor, owing to the character that SA was tightly connected with the cation function on silica beads. These results indicated that low carbon % and polar endcapped C18 phase columns would be useful as separation columns for SA under neutral conditions. These polar endcapped columns were suitable for polar compounds, providing evidence for the reasonableness of these results. The carbon column (Hypercarb) did not provide good results for the anion form of SA. The cation column (Aqua Way Cation) utilizes temperature-responsive polymer as the stationary phase. The retention mechanism of SA on this column was very different from the case of cytosol fraction. SA flows out faster with 10% CH_3OH than 20% CH_3OH, but cytosol fraction came out faster with 10% CH_3OH than 5% CH_3OH. Using a 10% CH_3OH–H_2O eluent system, we achieved excellent separation between the two compounds, but poor recovery efficiency of SA. Second, for another approach to the analysis of the anion form of SA, we used a high resolution mass spectrometer. This mass spectrometer is capable of separating SA from cytosol matrices based on mass accuracy. Thus, the S/N of the SRM chromatogram was improved (Fig. 3), and a good quantitation limit was obtained. The linearity was good up to 5000 fmol (r^2=0.9991), and the detection limit was 5 fmol (S/N 3). The high resolution mass spectrometer was useful for identification as well as quantification of the analytes.

Positive Ion Mode ESI Analysis The negative ion mode ESI is usually used for SA analysis in trying to find the most sensitive eluent under the negative ion mode, we discovered that the neutral condition was suitable for highly sensitive detection of SA because it took the anion form at pH 7. However, the anion form of SA showed poor retention on ODS columns. This is the dilemma of negative ion mode analysis of SA. We therefore decided to try using a positive ion mode analysis for SA.

Optimization of MS and MS/MS Conditions MS conditions were optimized according to the previous paper. The capillary voltage was 3.5 kV and cone voltage was 19 V. The temperature of the source and desolvation were 120 °C and 350 °C, respectively. The gas flow rate (l/h) of the cone and desolvation were 100 and 1000, respectively. The product ion mass spectrum of the protonated molecular ion $[M+H]^+$ *m/z* 113 is shown in Fig. 1; *m/z* 95 appeared at the production mass spectrum when the collision energy was set at 10 eV. The mass transition pattern $[M+H]^+$ *m/z* 113→95 was selected to monitor SA in the positive ion mode. To obtain the most sensitive ionization conditions for analysis of SA under the positive ion mode, we compared the peak area of *m/z* 113→95 in SRM method using various eluents. For positive ion analysis, 0.05% HCOOH–CH_3OH/CH_3CN, 0.1% CH_3COOH–CH_3OH/CH_3CN, 0.05% HCOOH–2 mM $CHOONH_4$–CH_3OH/CH_3CN was used as the eluent. The peak area of 2.5 pmol SA (500 nM of SA was injected at 5 μl) was compared for the different eluent systems, and the results are summarized in Table 2. The results indicated that CH_3OH was better than CH_3CN (1.9 times under 0.05% HCOOH, 1.8 times under 0.1% CH_3COOH) and 0.1% CH_3COOH was better than 0.05% HCOOH (1.3 times under CH_3OH, 1.4 times under CH_3CN). The acetate buffer containing eluent did not yield good results (low peak area).

Sensitivity and Matrix Effect Using these optimized eluent conditions, 0.05% HCOOH and 0.1% CH_3COOH/CH_3OH, calibration was performed. The calibration graph for

SA was generated from the peak areas of the mass transition pattern, $[M+H]^+$ *m/z* 113→95 in SRM method (SRM chromatogram is shown in Fig. 4). A calibration curve was constructed using the least-squares method of quantities *versus* peak area. The linearity was good up to 25 pmol (r^2=0.9989 for 0.05% HCOOH, r^2=0.9996 for 0.1% CH_3COOH), and the detection limit (S/N 3) was 35 fmol for 0.05% HCOOH, 25 fmol for 0.1% CH_3COOH. Next, the matrix effect was evaluated by comparing the peak areas of the cytosol blank spiked with SA to those prepared in the mobile phase at the corresponding concentration. A linear calibration curve was constructed using the same method mentioned above. Good linearity was obtained up to 1250 fmol (r^2=0.9978 for 0.05% HCOOH, r^2=0.9991 for 0.1% CH_3COOH) and the detection limit (S/N 3) was 35 fmol for 0.05% HCOOH, 25 fmol for 0.1% CH_3COOH. In a previous study, we detected SA at 160 fmol/5×10^6 cells (n=3) content in the cytosol of P-815 cells treated with SA (2.5 mmol/l) for 0.5 h under the negative ion mode.[12] The positive ion mode ESI analysis was shown to be useful for quantification and separation of this volume of SA under the matrix, in mammalian cells.

Conclusions

In this study, we established a highly sensitive analysis method for SA in the cytosol fraction. In the negative ion mode ESI SRM method, a low carbon content and polar end-capped C18 phase ODS columns showed better retention time for the ion form of SA than other ODS columns. A column with a novel separation mechanism (Aqua Way Cation column) facilitated analysis of the ion form of SA from the cytosol fraction, but its ability to maintain the ion form of SA was too high resulting in a low rate of collection of the ion form of SA. Therefore, this column could not be used for highly sensitive quantification of the ion form of SA, but could be used for analysis to determine the amount of SA as an additive. The SRM chromatogram obtained using a high resolution mass spectrometer led to a higher S/N than the usual SRM chromatogram for analysis of SA in the cytosol fraction. Under the positive ion mode ESI SRM method, using the 0.1% CH_3COOH and 0.05% HCOOH–CH_3OH system as an eluent of HPLC gave a higher peak area of SA than 0.1% CH_3COOH, 0.05% HCOOH–CH_3CN and 0.05% HCOOH/2 mM $HCOONH_4$–CH_3OH/CH_3CN. Addition of the acid to the eluent system is effective for enhancement of $[M+H]^+$ under the positive ion mode, because H^+ adheres to the molecular form of SA in the acid eluent. This is the first demonstration of use of the positive ion mode ESI SRM method for highly sensitive analysis of the acidic compounds such as SA.

In positive ion mode, normal resolution SRM showed a better detection limit than in the negative ion mode, and it was better than separating SA using conventional ODS columns. Mass separation analysis of an analyte from a matrix using a high resolution mass spectrometer is effective for obtaining a clear S/N SRM chromatogram and a good detection limit.

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