Improvement of Measurement Repeatability for High Performance Liquid Chromatography/Atmospheric Pressure Chemical Ionization-Mass Spectrometry by Sample Introducing Method Using Auto Switching Valve

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In order to obtain good measurement repeatability for the high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS), the peak portion of an analyte eluted from LC was introduced into the interface by using an auto switching valve. This considerably reduced stains stuck to the interface, so that LC/APCI-MS was able to operate stably, and the repeatability was improved. This sample introducing method was applied to the selected ion monitoring (SIM) determination for 3-*tert*-butyl-4-hydroxyanisole (BHA) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) contained in dried sardines. The measurement repeatability for BHA and BHT were 2–3% as relative standard deviations. The results were compared with those obtained by the capillary gas chromatography (CGC) of the same sample solution, showing that the quantitative analysis can be performed by LC/APCI-MS with a high repeatability comparable to the CGC.

1. Introduction

Recently, selected ion monitoring (SIM) determinations by a high performance liquid chromatography/atmospheric chemical ionization-mass spectrometry (LC/APCI-MS) shows promise as an analytical means for analytes in the mixture samples. LC/APCI-MS apparatus for practical use are commercially available, and are used to determine a wide variety of compounds of low to high polarity.^{1)–14)} However, when the same sample solution is repeatedly determined by the SIM, the variation of peak area is larger than that obtained by using other LC detector or GC measurements. Therefore, it has been desired to develop LC/APCI-MS which can operate stably and can achieve SIM determination in good repeatability.

The authors¹⁵⁾ reported that differences were recognized in the measurement variations, caused by the different materials for corona discharge needles.

Further effort was carried out to disclose the causes of the variations in the SIM measurement of LC/APCI-MS for the same sample solution. As a result, it has been revealed that substances eluted from the LC analytical column stick to the APCI interface as stains, making the SIM determination unstable.

At present, the use of 3-*tert*-butyl-4-hydroxyanisole (BHA) and 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) as antioxidants in dried sardines in Japan is regulated based on the total amount of BHA and BHT. There are many reports on analytical methods

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for simultaneous determination of BHA and BHT in foods and in biological samples by using a high performance liquid chromatography (LC)^{16)~19)} or a gas chromatography (GC).^{20)~24)} However, these methods were impossible to separate BHA and BHT from coexisting substances in the dried sardines completely. Further, the use of LC for simultaneous determination of antioxidants has been increased. For these reason, LC method that can determine BHA and BHT without interruptions of coexisting substances and can confirm the eluted substances is highly needed.

In this paper, using BHA and BHT added to dried sardines as analytes, we report the causes of the variation in the SIM measurement of LC/APCI-MS. Only the peak portions of BHA, BHT, and internal standard separately eluted from the LC column were introduced into the APCI interface by using an auto switching valve to be determined by SIM. The stability of LC/APCI-MS was improved, and the measurement precision was comparable to those obtained by the capillary GC (CGC) determination.

2. Experiments

2.1 Reagents

BHA, BHT, and 3-methyl-4-isopropylphenol (Biosol) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo Japan). All the other chemicals were analytical reagent grade.

2.2.1 Internal standard (IS) solution Biosol was dissolved in ethyl acetate in such an amount as to give a concentration of 400 ppm.

2.2.2 Stock solution About 25 mg of BHA and 25 mg of BHT were separately weighed. The weighed BHA and BHT were transferred to the same 100 ml volumetric flask by ethyl acetate, and diluted to the volume with ethyl acetate and mixed.

2.3 Apparatus

2.3.1 Essential oil distilling apparatus Japanese pharmacopeia XII.

2.3.2 LC/APCI-MS The HPLC/APCI-quadrupole MS system was a model M-1200H (Hitachi Ltd., Tokyo Japan) which consisted of an L-7100 pump (pump A), an L-6200 pump (pump B), an L-4000 UV detector and an auto switching valve (SPV-N-4A (GL Science, Tokyo, Japan)).

2.3.3 Maintenance of APCI interface As mentioned above, as the corona discharge needle, an iron needle¹⁵⁾ was used. The needle was exchanged for a new one each time it was used, and at the same time, the APCI interface was cleaned.

2.3.4 CGC system A model 14 A gas chromatograph and a data processor of CHROMATOPAC C-R4AX (Shimadzu (Kyoto, Japan)) were used.

2.3.5 Conditions for analyses The analytical conditions of LC/APCI-MS and CGC are summarized, respectively (Table 1).

2.4 Preparation of sample

Dried sardines not contained with BHA and BHT were made into powder with a pulverizer. A 10 ml volume of the stock solution was added into the pulverized powder of 50 g. The powder added both BHA and BHT was sufficiently mixed, air dried for 48 hours. This powder contains both BHA and BHT at concentration of 50 ppm, respectively.

Table 1. Analytical Conditions

【LC/APCI-MS】	
(LC)	
Column	; TSK-gel ODS 80TS 150 mm×6.0 mm i.d. (TOHSON, Japan)
Mobile phase	; A: 80% methanol, B: methanol, linear gradient from A 100% to B 100% over 7 min with 5 min hold at 100% B at 1 ml/min.
Detection	; UV 280 nm.
Inject volume	; 10 μ l.
Column temp.	; 23±1°C
(APCI-MS)	
Detection mode	; negative
Method of detection	; selected ion monitoring (SIM) at m/z 149 (Biosol), m/z 179 (BHA) and m/z 219 (BHT).
Corona discharge needle	; iron needle
Needle voltage	; -2700 V
Drift voltage	; -30 V
Multiplier voltage	; 1800 V
Nebulizer temp.	; 200°C
Desolvator temp.	; 400°C
First aperture temp.	; 120°C
【Capillary GC】	
Column	; DB-FFAP 30 m, 0.25 mm i. d., film thickness 0.25 μ m (J & W, USA).
Detection	; FID
Temp. programing	; initial temp. 170°C, initial time 2 min, rate 10°C/min, final temp. 250°C, final time 5 min, detection temp. 250°C
Injection	; injection temp. 250°C, inject volume 1 μ l, split 1 : 50
Carrier gas	; He, 1.8 kg/cm ²

2.5 Sample solution

A 5 g amount of sample was correctly weighted into a 500 ml volume distilling flask. To the flask, 50 g of sodium chloride, 0.2 g of pyrogallol and 200 ml of water were added. In addition, 1 ml of the IS solution and 4 ml of ethyl acetate were added, and the flask was lightly shaken to distribute the ethyl acetate evenly. Then, a portion of 5 ml of ethyl acetate was added as extractants in the reflux condenser and the reflux condenser was attached to the distilling flask. The solution was heated and distilled for 4 hours. After cooling, the extracted ethyl acetate layer was collected and dried by 2 g of sodium sulfate anhydrous. This solution was used as the sample solution.

2.5.1 CGC measurement The above described sample solution was used for CGC measurement. The CGC measurement was achieved by internal standard method. Based on the peak area ratio between BHA and IS, the BHA content was calculated from the calibration curve. As the case of BHA, the content of BHT was calculated.

2.5.2 LC/MS measurement A portion of 1 ml of sample solution was dried away in a stream of N₂ gas. To the residue, 1 ml of methanol was added and residual matters were dissolved. This solution was used for LC/MS measurement. The LC/MS measurement was also performed by the internal standard method in the same manner as CGC measurement.

3. Results and Discussion

3.1 Affects of stains in interface portion

Few reports have described that the interface of LC/APCI MS is readily stained, and the stains may affect the measurement repeatability. Proctor *et al.*²⁵⁾ reported that regular maintenance required for a corona discharge as sputtered material may, in some system, block the sampling orifice. Kato²⁶⁾ reported that matters eluted from the analytical column were deposited onto the corona discharge needle, which might interrupt the corona discharge in the deposited area. As reported by the authors,¹⁵⁾ the corona discharge using an iron needle was able to work stably, as compared with that using discharge needle made of other materials, and the amount of deposits on the iron needle decreased, giving an enhanced measurement repeatability.

The APCI interface of the LC/APCI-MS apparatus (M-1200H, Hitachi) is shown (Fig. 1). Stains deposit on the interface, making it difficult to enhance the repeatability of SIM determinations by LC/APCI-MS. Possible mechanisms for this fact will be presented below.

1) The top end of portion of the metal capillary (12; Fig. 1) is clogged with stains, causing the spread and direction of the spray to change with a lapse of time. This changes the quantity of ions emitted by the corona discharge.

2) Organic substances present in the mobile phase sprayed from the nebulizer deposit as stains on the corona discharge needle (18; Fig. 1), making the corona discharge unstable.

3) An analyte ionized is adsorbed to stains present in the respective electrode fine pores (21, 22, 23; Fig. 1). This changes the quantity of ions arriving at the mass spectrometer each time the measurement is carried out.

4) A part of the surface of the primary electrode having stains deposited thereto is electrically insulated, making the corona discharge unstable.

5) Stains having an indeterminated ionic strength and exhibiting a spectra as shown in Fig. 2, for example, are gradually released from the portions of interface and detected by MS, deteriorating the repeatability of the measurement.

3.2 Sample introduction method

When such sample introduction methods are adopted that only the peak portion I (Fig.3) of an analyte eluted from the column is introduced into the interface by using

Fig. 1. Schematic diagram of atmospheric pressure chemical ionization interface: 11, nebulizer; 12, metal capillary; 13, 17, heater; 14, nebulizer space; 15, vaporizer; 16, vaporizer space; 18, corona discharge needle; 19, high voltage supply; 20, ionization chamber; 21, primary electrode; 22, intermediate electrode; 23, secondary electrode.

Fig. 2. Mass spectra of a stain from the APCI interface.

Fig. 3. Loading method of the analytes to interface using an auto switching valve. I: Loading of the analyte eluted from the analytical column to the APCI interface. II: Drain of the effluent from the analytical column.

one LC pump, no flow state of the interface occurs at the time for the needless portion II eluting from the analytical column (Fig. 3). This makes it impossible to obtain a stable baseline for SIM measurement, that is, to calculate the peak area accurately. In addition, the fine pore portion of the metal capillary and the respective electrodes may be clogged. Accordingly, it is undesirable to interrupt the flow of the mobile phase through the interface. In order to waste the portion II eluted from the column, which might deposit as stains, and introduce only the peak portion I thereof, two LC pumps having identical performance were used while they were switched with an auto switching valve (Fig. 3).

3.3 APCI-MS spectra of BHA and BHT

BHA and BHT gave abundant quasi-molecular ion $[M-H]^-$ at m/z 179 and m/z 219, respectively, in the negative ionization mode (Fig. 4). In the positive mode, the sensitivity of BHA and BHT was about 1/5 of that measured at in the negative mode.

Fig. 4. LC/APCI mass spectra of BHA, BHT, and Biosol. a) BHA; b) BHT; c) Biosol (IS).

For the biosol used as IS, an abundant quasi-molecular ion $[M-H]^-$ at m/z 149 was also observed in the negative mode (Fig. 4).

3.4 LC, CGC, and LC/APCI-MS chromatogram

Dried sardines contain a variety of compounds such as n-pentadecane and the like.²²⁾ These compounds, having no UV absorption in the vicinity of a wavelength of 280 nm, are difficult to be detected with a UV detector (Figs. 5a, 5b), so the wavelength of 280 nm is used for detecting BHA and BHT in the dried sardines by LC measurements.¹⁷⁾ In the sample solution for determination, also a variety of substances were contained as shown in Fig. 5a. Under the CGC measurement conditions adopted by this study, BHA could not be separated from coexisting substances in the dried sardine (Fig. 6b). Thus, it has been desired to develop an analytical method having a high measurement precision and assuring the reliable identification.

According to the LC/APCI-MS-SIM method adopted by this study, BHA and BHT could be determined, not affected by substances coexisting in the dried sardines (Figs. 5c–5f).

3.5 Analytical method for BHA and BHT

Simultaneous analytical methods for BHA and BHT have been reported, by which the sample is pretreated by solvent extraction,^{16), 18)–19)} distillation extraction^{20)–23)} or solid-phase extraction,¹⁷⁾ and then analyzed by GC or LC. The LC method using fluorescence detector,²⁷⁾ having a sensitivity for BHT which is about 1/100 of that for BHA, is impractical for the simultaneous determination. The LC method^{16), 19)} of determining antioxidants was reported, by which an acetonitrile–water system is used as the

Improvement of Measurement Repeatability for HPLC/APCI-MS

Fig. 5. HPLC-UV chromatograms of extracted solution followed by the proposed method from dried sardine added BHA and BHT a)-b), APCI-MS total ion chromatogram (TIC) c), and APCI-MS selected ion monitoring (SIM) chromatograms d)-f). A: Biosol (IS) and BHA.

Fig. 6. Capillary-gas chromatographic profiles for the authentic standard a) and for the dried sardine extracts, which contained no BHA and no BHT, followed by the proposed method b).

mobile phase for the reversed-phase column. However, the sensitivities of APCI-MS using the acetonitrile–water system to BHA and BHT were low, that is, were about 1/20 of that using a methanol–water system. For this reason, the methanol–water system was used as the mobile phase of LC/APCI-MS in this study.

3.6 Internal standard

It was considered that the SIM measurement accuracy can be enhanced and the precision can be improved by using the same ionizing conditions for an analyte and IS. Many reports describe the LC/MS determination using isotopic substances of the analyte as IS.^{28), 29)} However, for the preparations of isotopic substances, considerable labors and time are needed. In order to improve the SIM measurement precision in this study, biosol having peaks completely overlapping those of BHA in the LC conditions adopted was used as IS.

3.7 Calibration curve and limit of determination for LC/MS

For the simultaneous determination of BHA and BHT based on internal standard method, the standard solutions with different concentration level were made by dissolving equally amounts of BHA and BHT in a 10 ml ethanol solution which contained 400 μg of IS. These standard solutions ($n=6$), contained BHA and BHT ranged from 10 to 400 μg were used to construct the calibration curve. Both calibration curves for BHA and BHT, respectively, were found to be linear using a computer based linear least squares program (correlation coefficient, $r>0.999$).

The determination limits of LC/APCI-MS for BHA and BHT under the analytical conditions adopted were about 0.4 ppm (signal to noise ratio is 5), respectively.

3.8 Repeatability of SIM measurement

The samples (No. 1–6) for the recovery experiment were added with BHA and BHT to have a concentration of 50 ppm (250 $\mu\text{g}/5\text{ g}$), respectively. The sample solution of No. 1 extracted by the method adopted for the recovery experiment was repeatedly measured seven times, and the contents of BHA and BHT were determined (Table 2–1). There was observed no statistical differences in variations in the measurements, caused by the

Table 2–1. Comparison of Repeatability of Measurements¹ between LC/APCI-MS and CGC for the Same Solution of Sample No. 1²

No. of measurement	LC/APCI-MS		CGC	
	BHA	BHT	BHA	BHT
1	200	231	191	219 ($\mu\text{g}/5\text{ g}$)
2	199	221	198	215
3	192	217	194	208
4	190	215	184	220
5	189	219	195	226
6	181	217	192	223
7	189	215	195	220
$\bar{X}$ ($\mu\text{g}/5\text{ g}$)	191	219	193	219
σ_{n-1}	6.50	5.58	4.46	5.82
RSD (%)	3.4	2.5	2.3	2.7

¹ Measurement was achieved successively for seven times.

² A 5 g of dried sardine, added together 250 μg of BHA and 250 μg of BHT, respectively, was determined by the proposed method.

Improvement of Measurement Repeatability for HPLC/APCI-MS

Table 2-2. Raw Peak Areas Obtained by the LC/APCI-MS Measurements of Table 2-1

	BHA	BHT	IS
1 ¹	93345	167571	109689
2	94221	162949	111373
3	97966	172769	120204
4	97742	172734	121268
5	97592	176469	121686
6	97359	182682	127101
7	95201	169081	118704
$\bar{X}$	96204	172036	118575
σ_{n-1}	1908	6393	6098
RSD (%)	2.0	3.7	5.1

¹ No. of measurement is the same as Table 2-1.

different types of the detectors. Good results were obtained. The relative standard deviation (RSD) of LC/APCI-MS was 2–3%, which is comparable to the measurement results by CGC. The raw peak areas listed in Table 2-1 obtained by the LC/MS measurements were shown in Table 2-2.

3.9 Recovery test

3.9.1 Extraction method If the essential oil determining device is used, only about 60% of BHT present in dried sardines can be recovered.^{20), 23)} Improved extraction methods²³⁾ have been reported, by which butyl acetate or acetone is added directly to the sample. By these methods, IS is added to a collecting liquid in the essential oil determining device. In this study, the butyl acetate method was further improved. That is, IS and the sample were added together to the distilling flask of the essential oil determining device. As a result, good recovery ratios of 88% were obtained for BHT (Table 3).

3.9.2 Comparison between the recovery test results obtained by LC/APCI-MS and CGC There were found no difference in the average recovery ratios and the RSD for BHA and BHT between the measuring devices of LC/APCI-MS and CGC (Table 3).

As understood in the above results, the SIM determination by LC/APCI-MS is accurate, and the precision is comparable to those by CGC.

Table 3. Results of Recovery Test for BHA and BHT Added together in Dried Sardine for 250 $\mu\text{g}/5\text{ g}$, Respectively

Sample No.	BHA		BHT	
	LC/APCI-MS	CGC	LC/APCI-MS	CGC
1	200	192	217	220 ($\mu\text{g}/5\text{ g}$)
2	202	192	214	215
3	207	203	210	204
4	186	194	215	219
5	216	209	222	226
6	218	220	241	239
$\bar{X}$ ($\mu\text{g}/5\text{ g}$)	205	202	220	221
RSD (%)	5.7	5.6	5.0	5.2
Recovery (%)	82.0	80.8	88.0	88.4

4. Conclusion

In order to improve the repeatability of the measurement, only the peak portion of the analyte was introduced into the interface by using an auto switching valve. This introducing system served as the stable operation of LC/APCI-MS. BHA and BHT contained in dried sardines were determined with a good repeatability of 2–3% by LC/APCI-MS. The results of the SIM determinations by LC/APCI-MS showed that the quantitative analyses can be performed in good repeatability comparable to CGC.

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