Simultaneous GC-MS Determination of Azide, Cyanide and Thiocyanate *via* Phase-Transfer-Catalyzed Pentafluorobenzylation

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A convenient analytical procedure based on one-step, phase-transfer-catalyzed derivatization has been established for the simultaneous determination of azide, cyanide and thiocyanate in aqueous samples by gas chromatography – mass spectrometry (GC-MS). In order to obtain cleaner extracts, a polymer-bound phosphonium salt was employed as the catalyst, and pentafluorobenzyl bromide (PFB-Br) as a derivatization reagent. The extraction and derivatization of these analytes, as well as the concentration of the resultant derivatives into the organic phase were simultaneously achieved during a 30-min phase-transfer-catalyzed process at 38°C. Rapid and reliable GC-MS determination could be readily performed for these analytes, not only in various beverages but also in diluted, large-volume aqueous samples such as food washings. In addition to electron impact ionization MS (EI-MS), negative-ion chemical ionization MS (NICI-MS) was also employed to detect trace-level analytes, as well as to accomplish incontrovertible identification. The detection limits in various beverages were typically 0.1—0.5 μg/ml by EI-MS and 25—100 ng/ml by NICI-MS, both in the full-scan mode.

INTRODUCTION

Since the Wakayama curry-poisoning incident in July 1998, a chain of poisoning incidents has taken place throughout Japan. These incidents include a random homicide *via* cyanide in beverages in Nagano, as well as azide-poisoning cases, in which drinks were spiked with sodium azide, in Niigata and three other prefectures.

Numerous samples were submitted for toxicological analyses at forensic laboratories throughout Japan. In Osaka, for instance, 17 samples out of more than 600 samples submitted between August and December 1998 were found to be contaminated with poisons and/or potentially harmful substances. Therefore, the establishment of a systematic and reliable, yet rapid analytical method which covers all kinds of organic and inorganic poisons and harmful sub-

stances is the ultimate goal in toxicological circles. In order to be utilized as an effective tool in such analyses, we have established a one-step triphasal extractive derivatization method for the GC-MS of various anionic substances[1-3], including cyanide and its analogue/metabolite thiocyanate[4].

Various analytical methods have been developed for the detection of cyanide ; these include spectrophotometry[5], ion chromatography[6] and headspace GC with nitrogen-phosphorus detection (NPD) without derivatization[7], along with GC with flame-ionization detection[8] and electron-capture detection[9,10] after derivatization. Azide[11,12] and thiocyanate[13] are also detectable by ion chromatography, liquid chromatography or spectrophotometry without derivatization.

Although a variety of detection methods can be utilized, GC-MS after an appropriate derivatization serves as the preferred confirmation method owing to its specificity, particularly for forensic identification purposes. Conventional extraction methods, however, cannot provide an

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appropriate sample preparation for the GC-MS determination of inorganic anions in a complex sample matrix, especially at low concentration levels.

This study aims to achieve further improvements to our triphasal pentafluorobenzylation method[4] and to establish a convenient simultaneous GC-MS procedure for determining azide, cyanide and thiocyanate in beverages and food extracts, utilizing a polymer-bound phosphonium salt as a phase-transfer catalyst.

MATERIALS AND METHODS

Chemicals —— Potassium cyanide and potassium thiocyanate were obtained from Katayama Kagaku (Osaka, Japan), and sodium azide from Nacalai Tesque (Kyoto, Japan). Pentafluorobenzyl bromide (PFB-Br) (99＋%) and tetraline, used as an internal standard, were obtained from Aldrich (Milwaukee, WI, U.S.A.) and Katayama Kagaku, respectively. Polymer-bound tributylmethylphosphonium bromide [0.86 mmol Br^-/g; (TB-0.86)] was prepared from polymer-bound tributylmethylphosphonium chloride [0.9 mmol Cl^-/g; 200—400 mesh; polystyrene −1% divinylbenzene copolymer (Fluka, Buchs, Switzerland)] according to the previously described method[1].

A stock standard solution of cyanide (1.0 mg/ml aqueous solution) was prepared monthly by dissolving potassium cyanide in 0.1 M NaOH, and standardized by titration with 0.1 M silver nitrate. Stock standard solutions of thiocyanate and azide (1.0 mg/ml aqueous solutions) were prepared monthly by dissolving potassium thiocyanate and sodium azide in deoxidized water. These solutions were stored in the dark at 0—5℃. Spiked beverage and food washing samples used for the experiments were prepared by adding known amounts of these three analytes to blank samples at 0.01—10.0 μg/ml.

Triphasal Derivatization Procedure —— Before analysis, the pH of the samples was adjusted to approximately 7—8 with 0.1 M sodium hydroxide and/or acetic acid. A 30 mg amount of polymer-bound catalyst TB-0.86 was placed in a 2 ml micro test tube with safe lid lock (Eppendorf, Hamburg, Germany). An aqueous sample (0.1—1.0 ml), 100 μl of 0.1 M phosphate buffer (pH 7.5) and 400 μl of methylene chloride solution containing 1.5% v/v of PFB-Br and 4 μg of tetraline (internal standard) were successively added to the test tube. Five-milliliter test tubes were used for processing samples containing 1.0—4.0 ml.

The test tubes were stoppered and vigorously shaken (without evaporation) for 30 min at 38℃ using a test tube evaporator "Vapor-mix" (Yamato Rikagaku, Tokyo). After the reaction, the organic layer was withdrawn, and was dried with anhydrous sodium sulfate. Brief centrifugation (*ca.* 10 s) with a portable centrifuge facilitated layer separations. One-microliter aliquots of the extracts were injected onto GC-electron impact ionization (EI)-MS and GC-negative-ion chemical ionization (NICI)-MS instruments.

Instrumentation —— GC-EI-MS : A GCMS-QP5050 mass spectrometer (Shimadzu, Kyoto) was operated in the EI mode in combination with a DB-1 capillary column (30 m × 0.53-mm i.d., 1.5-μm film thickness ; J & W Scientific, Folsom, CA, U.S.A.). Helium was used as a carrier gas at a flow rate of 22 ml/min and a split ratio of 10. The column temperature was programmed from 70℃ to 250℃ at 10℃/min. The injection temperature was set at 250℃. The ionization energy and interface temperature were 70 eV and 250℃, respectively. GC-NICI-MS : A GCMS-QP5050 mass spectrometer (Shimadzu) was used in the NICI mode with isobutane as moderation gas at 0.2 kg/cm^2 in combination with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness ; J & W Scientific). Samples were injected in the split mode at the ratio of 30. Helium was used as the carrier gas at a flow rate of 1.86 ml/min. The column temperature was held at 50℃ for 1 min, then raised to 250℃ at 10℃/min. The electron energy, injection and interface temperatures were set at 70 eV, 250℃ and 230℃, respectively.

RESULTS AND DISCUSSION

Derivatization Optimization

Based on the results of our previous studies[1,14], the catalyst TB-0.86, namely polymer-bound tributylmethylphosphonium bromide (0.86 mmol Br^-/g), was chosen as the phase-transfer catalyst and methylene chloride as the organic solvent. Conditional optimization of phase-transfer-catalyzed derivatization was carried out employing a diluted standard solution containing azide, cyanide and thiocyanate ions at 10 μg/ml each.

Effect of pH on the Resultant Derivatives

With reactive alkyl halides, such as PFB-Br utilized in this study, cyanide displacement reac-
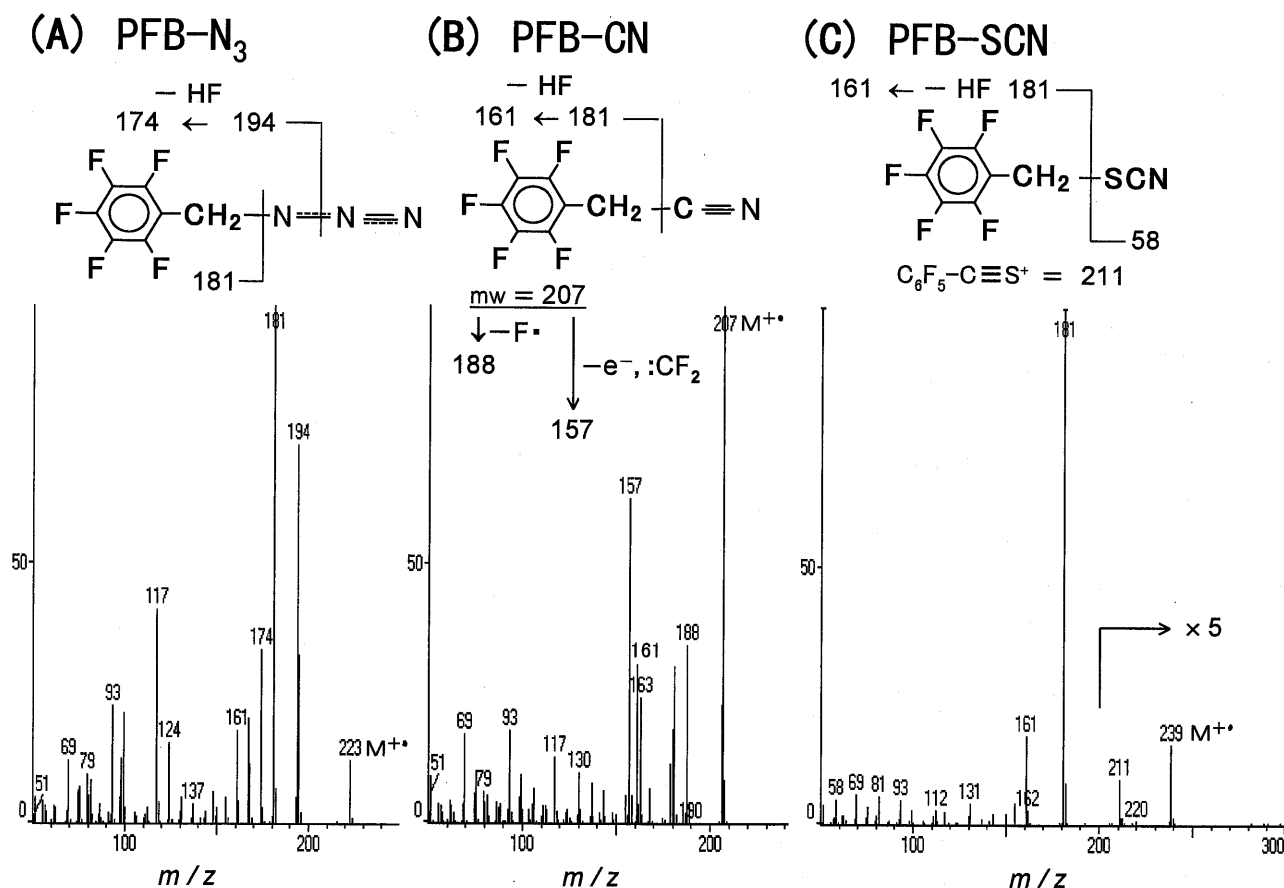
Fig. 1. EI Mass Spectra of PFB Derivatives of Cyanide, Thiocyanate and Azide

tions occur rapidly to produce the primary al-kylated nitriles (Ar-CH$_2$-CN), but these can lead to alkylated by-products [*i.e.*, Ar (Ar-CH$_2$) CH-CN and Ar (Ar-CH$_2$-)$_2$C-CN]. In fact, when the triphasal pentafluorobenzylation of cyanide was carried out at pH 9.2, the secondary alkylated nitrile Ar (Ar-CH$_2$-) CH-CN was the most abundant product. On the other hand, the primary alkylated derivative was predominantly generated from cyanide at pH 7.5. Thiocyanate and azide exclusively gave their primary alkylat-ed derivatives at a pH of 9.2 or lower. Since the formation of two different derivatives from a single analyte tends to complicate analyses, the derivatization was carried out at pH 7.5. Figure 1 shows the EI mass spectra of PFB derivatives of cyanide, thiocyanate and azide. Structures of some of the fragments are also proposed in Fig. 1.

Derivatization Time and Influence of Coexist-ing Chloride Ion

The time changes of the derivatization of azide, cyanide and thiocyanate in a diluted stan-dard solution at 10 μg/ml each are shown in Fig.

2-(A). Azide, the most nucleophilic, was quantita-tively converted within several minutes. The conversion of the least reactive, cyanide, reached a plateau after 20 min. Because almost no decreases in yields were observed for all the analytes, even after 60 min, the derivatization time was set at 30 min.

As mentioned in our previous paper[1], it was suggested that coexisting anions at high levels could depress the phase-transfer-catalyzed der-ivatization. In general, interference with the der-ivatization rate of an anionic analyte caused by coexisting anions depends on the differences in their nucleophilicities and affinities for the cata-lyst, as well as on relative activity as a leaving group in the nucleophilic displacement reac-tions[15,16]. The derivatization efficiencies of azide, cyanide and thiocyanate were examined in the presence of sodium chloride at concentrations of 0.5% (for all three analytes) and 0.1% (for the less nucleophilic cyanide only). Figure 2-(B) shows the time changes under these conditions. A sodium chloride concentration of 0.5% strongly interfer-ed with the derivatization of cyanide. Therefore,
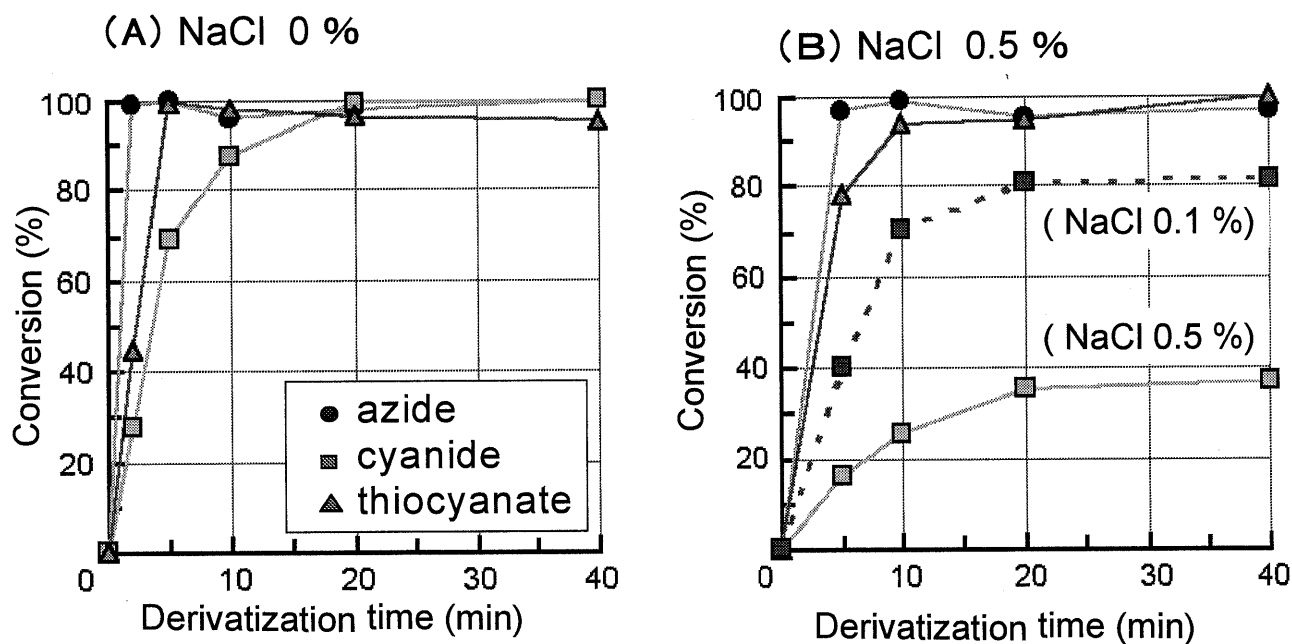
(A) NaCl 0 %

(B) NaCl 0.5 %

Fig. 2. Time Changes of the Derivatization of Azide, Cyanide and Thiocyanate —— The effects of NaCl concentration on the derivatization
(A) 0% NaCl; (B) 0.5% NaCl.

the quantitation of cyanide in samples with such a high-level sodium chloride should be performed after an appropriate sample dilution and/or by employing a standard addition method. No notable influences were observed for azide and thiocyanate, which both have highly nucleophilic properties[17].

Recoveries from Spiked Beverage and Food Samples

Table 1 lists the recoveries of azide, cyanide and thiocyanate from various spiked beverages and food washing samples at 10 μg/ml each. Sample dilutions were made for protein-rich and/

Table 1. Recoveries of Azide, Cyanide and Thiocyanate Spiked to Various Beverage and Food Washing Samples at 10 μg/ml

Beverage/food washing	Sample volume (μl)	Sample dilution	Recovery (%)		
			N_3^-	CN^-	$SCN^{-a)}$
Coffee with cream	200	5	72.8	46.7	66.7
Orange juice	200	5	63.8	20.0	59.3
Milk	200	5	85.7	33.3	73.9
Misoshiru soup	100	10	67.6	25.2	53.2
Green tea	400	1	92.1	65.5	74.7
Rice ball washing	1000	1	95.3	77.5	84.3

a) Measured by a standard addition method.

or high-ion samples in order to minimize protein binding as well as anionic interference. Since thiocyanate exists as a regular component of most biological materials, a standard addition method was employed to estimate its recovery. Highly nucleophilic properties of azide and thiocyanate ions, which lead to binding or even the reaction of these analytes to/with sample matrices, are most probably responsible for their incomplete recoveries. The evaporation of volatile cyanide in an acidic sample (such as orange juice) before analysis, protein binding and anionic interference probably accounts for the low recoveries of cyanide. In quantitating cyanide in a complex sample matrix, both bound and free cyanides have to be taken into account in the determination. Please note that headspace GC-NPD analysis[7] would be preferable for quantifying cyanide in a protein-rich sample like whole blood, since this method can quantitatively unbind the analyte from hemoglobin and detect it as hydrogen cyanide[7]. On the other hand, it should also be noted that such GC analysis could not serve as a confirmation method, as opposed to the present GC-MS procedure. Thus, the authors conclude that the present triphasal GC-MS procedure provides specific toxicological analysis of azide, cyanide and thiocyanate in various beverages and food samples when this method is per-
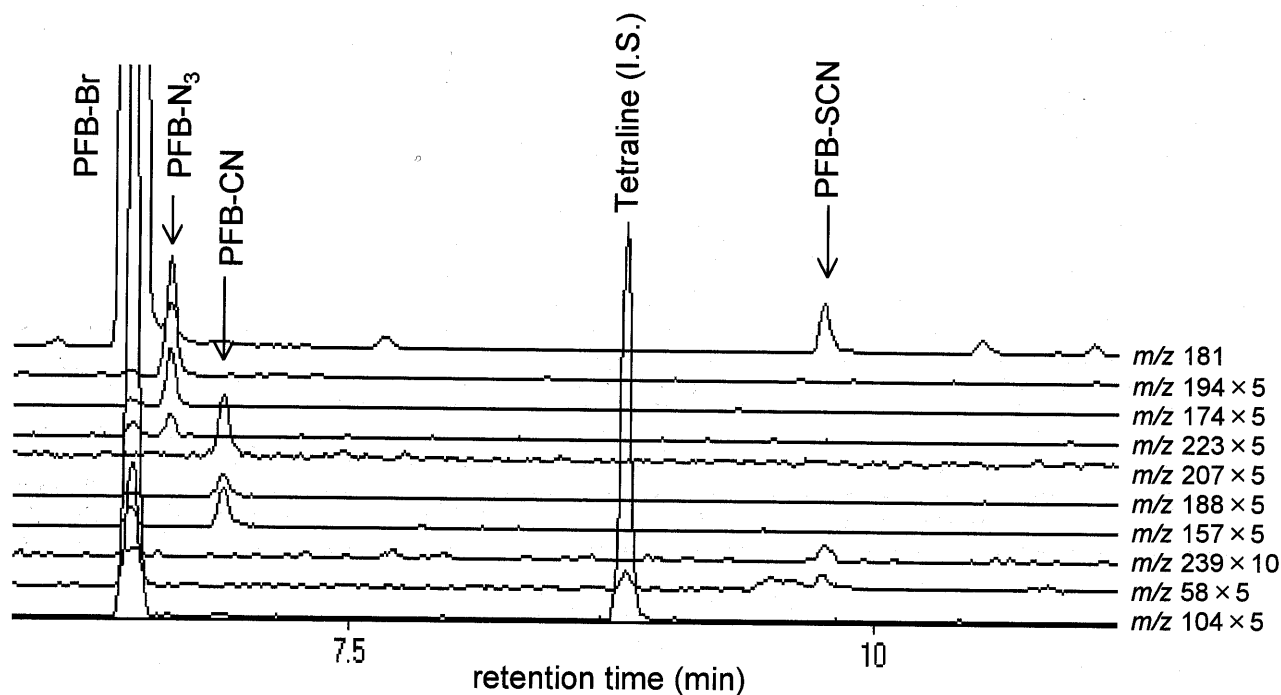
Fig. 3. GC-EI-MS Chromatograms Obtained from Spiked Misoshiru Soup at 10 μg/ml Each
A 100μl sample was diluted with water by 10-fold, then analyzed.

formed after an appropriate watering down dilution. Fig. 3 shows GC-EI-MS chromatograms obtained from spiked *misoshiru* soup, each at 10 μg/ml, taken in the full-scan mode.

Quantitative Accuracy

The calibration graphs for azide, thiocyanate and cyanide in spiked coffee with cream (sample volume: 200 μl; 5-fold water down dilution was made) were constructed by employing GC-EI-MS. The peak areas at m/z 194 for azide, m/z 207 for cyanide, and m/z 181 for thiocyanate against those at m/z 104 for the I.S. tetraline, were measured in the selected-ion monitoring (SIM) mode. The graphs gave good linearity over the range from 0.2 to 10.0 μg/ml; $y = -0.11 + 15.27\chi$ ($r^2 = 0.999$) for azide, $y = -0.34 + 14.77\chi$ ($r^2 = 0.997$) for cyanide, and $y = -0.16 + 5.86\chi$ ($r^2 = 0.998$) for thiocyanate. The relative standard deviation values during the whole procedure, evaluated at 1.0 μg/ml, were 3.3%, 5.7% and 4.2%, respectively. The limits of detection in the full-scan mode (the detection limit of three or more characteristic ion peaks on each GC-EI-MS chromatogram; $S/N = 3$) were 0.2 μg/ml for azide and 0.5 μg/ml for both thiocyanate and cyanide. Calibration graphs were also prepared for spiked green tea in the

same manner. Good linearity was again observed over the range from 0.2 to 10.0 μg/ml; $y = -0.14 + 5.00\chi$ ($r^2 = 0.998$) for azide, $y = -0.16 + 5.53\chi$ ($r^2 = 0.999$) for cyanide, and $y = -0.11 + 1.91\chi$ ($r^2 = 0.999$) for thiocyanate. Since recoveries are influenced by the sample matrix, the quantitation should be performed after an appropriate sample dilution, and by employing calibration curves that have been constructed for the individual sample matrix. Also, the employment of a standard addition method is necessary in analyzing unknown samples.

Application to Diluted, Large-Volume Aqueous Samples

As mentioned in our previous paper[1], the polymer-bound phosphonium salt catalyst has a function of capturing anions owing to its ion-exchange resin structure, and the derivatization takes place inside or on the surface of the resin. The most remarkable advantage of this triphasal derivatization is the fact that the extraction and derivatization of anionic analytes, and also the concentration of the resultant derivatives into a small-volume organic layer, are simultaneously achieved. Figure 4 presents the GC-EI-MS chromatograms obtained from a 4 ml volume of green tea sample spiked with three analytes at
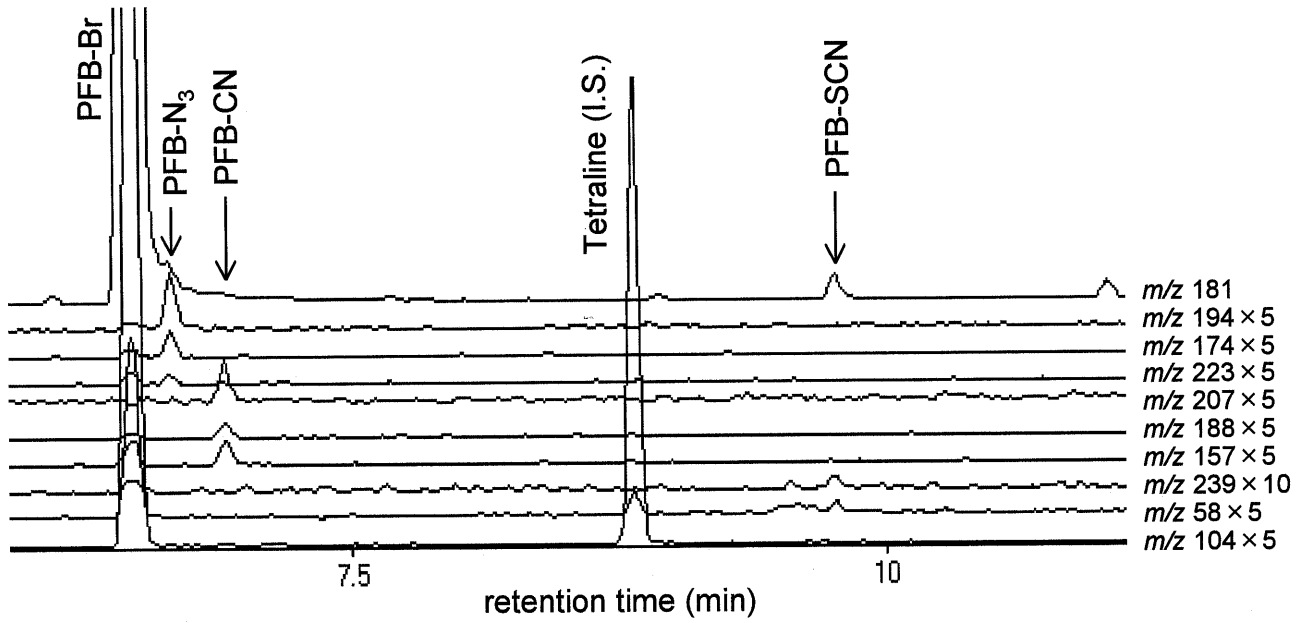
PFB-Br

PFB-N$_3$

PFB-CN

Tetraline (I.S.)

PFB-SCN

m/z 181
m/z 194 × 5
m/z 174 × 5
m/z 223 × 5
m/z 207 × 5
m/z 188 × 5
m/z 157 × 5
m/z 239 × 10
m/z 58 × 5
m/z 104 × 5

7.5

10

retention time (min)

Fig. 4. GC-EI-MS Chromatograms Obtained from a 4-ml Volume of Spiked Green Tea at 250 ng/ml Each

(A) PFB-N$_3$

167

195

42

42

50

195

167

m / z

(B) PFB-CN

$CH_2-C \equiv N$

mw = 207

↓ − HF

187

187

50

207

m / z

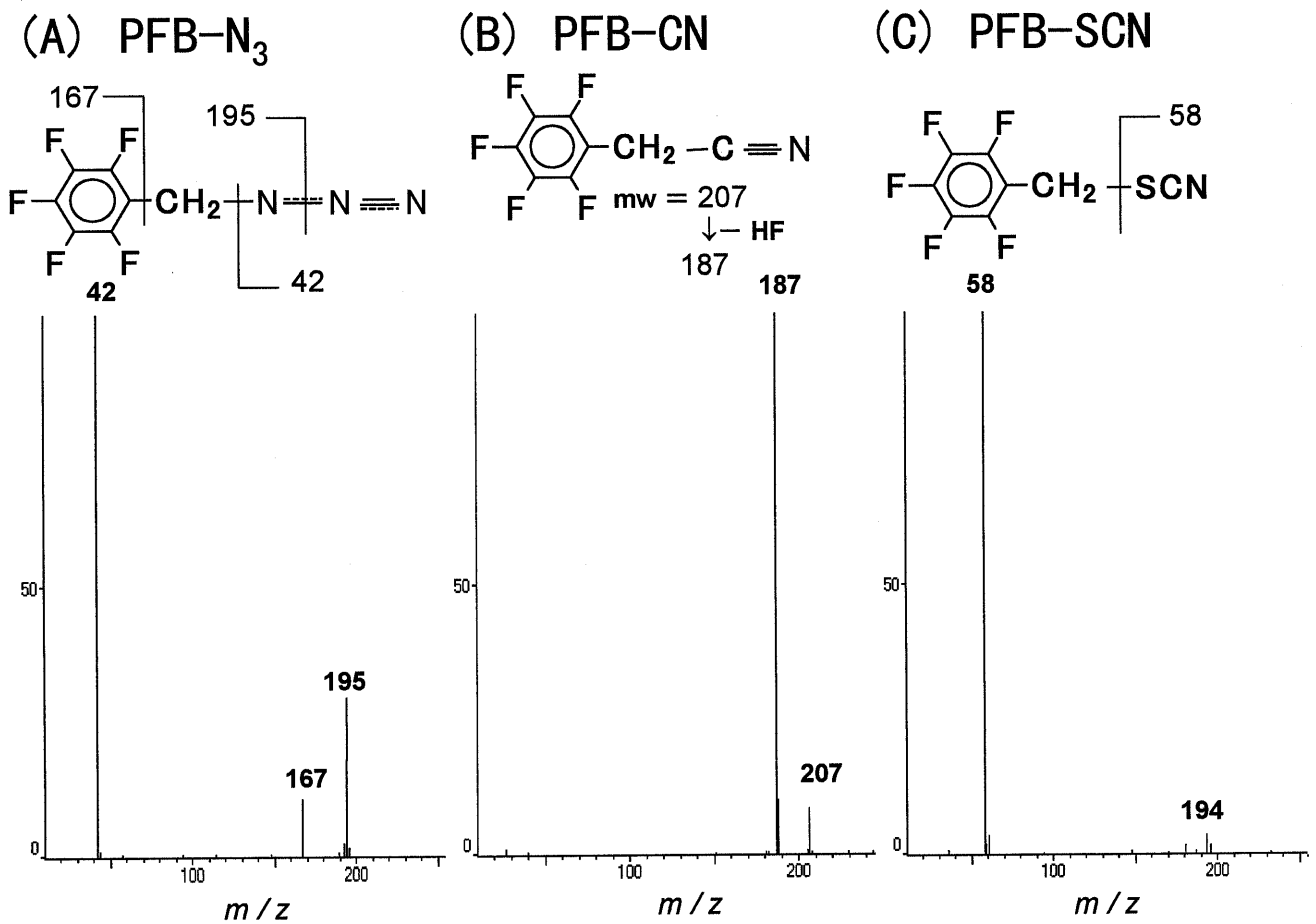## (C) PFB-SCN

58

CH_2 SCN

58

50

194

m / z

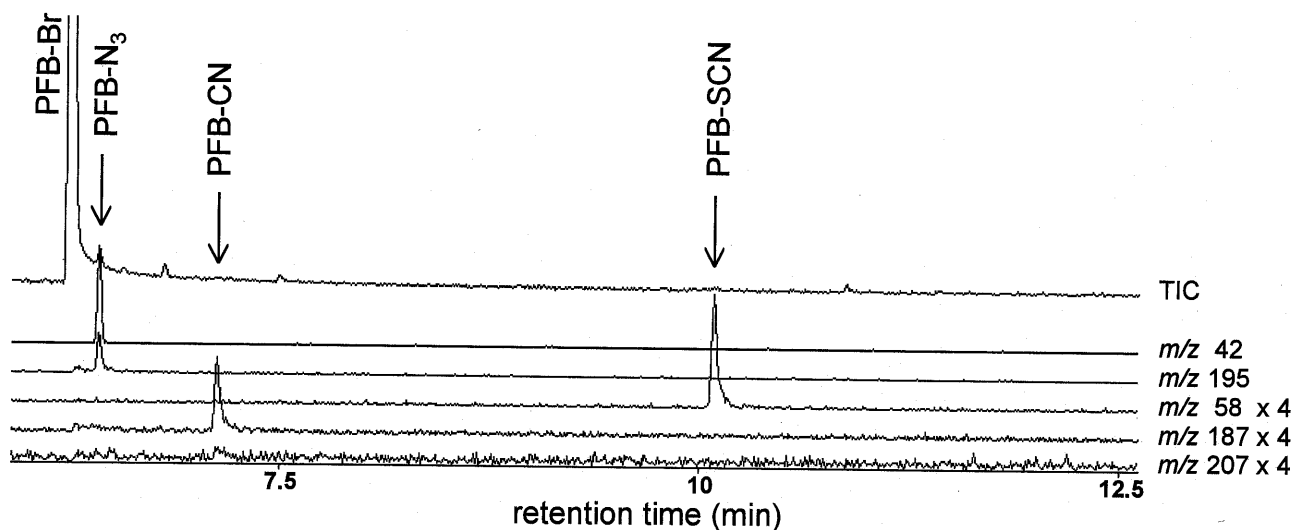**Fig. 5.** NICI Mass Spectra of the PFB Derivatives of Azide, Cyanide and Thiocyanate

Fig. 6. GC-NICI-MS Chromatograms Obtained from a 4-ml Volume of Spiked Green Tea at 100 ng/ml Each

250 ng/ml each, in the full-scan mode. The detection limits in this sample were 50 ng/ml for azide and thiocyanate, and 100 ng/ml for cyanide. It was thus demonstrated that this novel analytical technique is effective in analyzing diluted, large-volume aqueous samples such as tea and food washing samples.

GC-NICI-MS

It is well established that NICI-MS serves as an effective tool for the detection of electronegative compounds like PFB derivatives at trace levels. In addition, NICI-MS provides extensive information which enhances the reliability of identification. Although the PFB derivatives of azide and cyanide gave characteristic EI-MS spectra, as shown in Fig. 1, that of the thiocyanate derivative provides insufficient identity; only small peaks of characteristic ions (*i.e.*, m/z 239 and m/z 211) were given. Therefore, a combination of EI-MS and NICI-MS analyses was performed in order to obtain extensive information as well as higher sensitivity. Figure 5 shows the NICI-MS spectra of the PFB derivatives of azide, cyanide and thiocyanate. The limits of detection in various beverage samples in the full-scan mode (*i.e.*, the detection limits of the peaks of m/z 42 and 195 for azide, m/z 187 for cyanide, and m/z 58 for thiocyanate on each GC-NICI-MS chromatogram; $S/N = 3$) ranged between 25 and 100 ng/ml. Figure 6 presents the GC-NICI-MS chromatograms obtained from a 4ml volume of green tea spiked with these analytes at 100 ng/ml each. Lower background

levels in NICI-MS led to higher S/N ratios, and thus resulted in better sensitivity. The detection limit of azide in this sample was 10 ng/ml.

In the forensic field, GC-MS serves as one of the most frequently used confirmatory methods owing to its specificity and convenience. Employing a polymer-bound phase-transfer catalyst, a novel GC-MS procedure has been established for the simultaneous determination of azide, cyanide and thiocyanate. The catalyst allowed these anions in aqueous samples to be simultaneously extracted, derivatized and subsequently concentrated in a small-volume organic layer that was to be injected to GC-MS. Also, the resultant pentafluorobenzyl derivatives were suitable for both EI-MS and NICI-MS. Thus, this triphasal technique has bridged the gap between GC-MS and inorganic anions whose extraction has been problematic in conventional methods. The present procedure provides a rapid and specific analytical tool for these anions, not only in various high-ion and/or protein-rich beverage and food samples, but also in diluted, large-volume aqueous samples like food washings.

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