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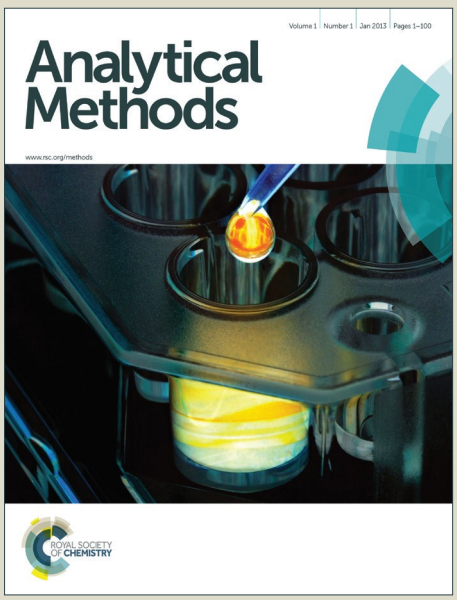

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COMMUNICATION

HT column GC/MS method for determination of anthraquinone and its toxic impurities in paper products

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High-temperature (HT) column gas chromatography – mass spectrometry (GC/MS) method was developed and validated for the measurment of anthraquinone (AQ) and its toxic impurities in various paper food packaging products. The present method will be basic framework for the risk assement of toxic chemicals in paper products.

1 Introduction

Anthraquinone (AQ) has been widely used as an additive in chemical alkaline pulp processes in the paper and pulp industry for the delignification [1,2]. However, impurities can be produced from reaction intermediate and/or side products during the manufacture of AQ [3,4], as shown in Fig. 1. Some of the impurities in AQ, such as 9-nitroanthracene (9-NA), 1-hydroxy- and 2-hydroxy-anthraquinones (1-OH-AQ and 2-OH-AQ) can cause serious negative effects to human and can be even carcinogenic and mutagenic [4-6]. Especially, AQ together with hydroxy-anthraquiones and 9-NA were classified into "possibly carcinogenic to humans" as Group 2B and Group 3, respectively, by the International Agency for Research on Cancer (IARC) [1,5]. Because of AQ toxicity, the use of AQ is regulated and banned in paper industry. With regard to toxicity of impurities, the monitoring and measuring of these chemicals are of crucial important for ensuring the safety of commercial paper products. In addition, these hazardous chemicals can be transferred from paper packaging products to foodstuffs when foods were packed with paper packages [7]. Thus, the determination of AQ and its toxic impurities is important of the food safety and management of hazardous

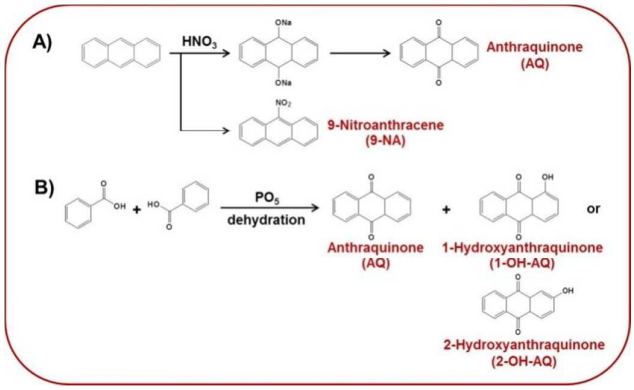

Fig. 1. Formation pathways of toxic impurities during the in manufacture of anthraquinone

substances in paper food packaging products. For the determination of AQ and poly-aromatic compounds various sample matrices, gas chromatography-mass spectrometry (GC-MS) [7-13] have been popularly used due to its high sensitivity and specificity. In some extend, high performance liquid chromatography (HPLC) methods using spectrophotometric detection [14-17], or MS [18] have been used for the determination of non-volatile aromatic compounds. Some of these methods focusing on the quantification of single or small set of aromatic compounds are sensitive.

Although conventional GC/MS method has favourable advantages in high specificity, sensitivity and peak capacity, some difficulties still remain. First, AQ and its impurities (9-NA and OH-AQ isomers) with higher boiling points (above 310 ^{o}C) could be adsorbed in capillary column GC systems and then produced irreproducible quantification in repeated analysis. The OH-AQ-isomers could be shown poor GC chromatographic properties due to their incompatible properties to GC systems. Thus, chemical derivatization method requires to reduce their polarity and to enhance GC chromatographic properties. Third,

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the detection of trace amounts of AQ and its impurities in paper products can be easily masked by components of the sample. Highly sensitive and selective detection method requires for the precise quantification of AQ and its impurities. Due to reasons, the determination of these toxic chemicals has not been detail investigated.

In this study, we developed rapid analytical method using a high temperature (HT) column GC/MS combined with trimethylsilyl (TMS) derivatization for the simultaneous determination of trace AQ and its toxic impurities in paper packaging products. In addition, analytical performance of HT column GC/MS method was compared with capillary column GC/MS and HPLC methods. Validation of HT column GC/MS method was conducted in terms of linearity, LOQ, precision, and accuracy. Established method was successfully applied for the determination of AQ and its hazardous impurities in various paper packaging products

2 Experimental

2.1 Materials and reagents

Anthraquinone (AQ, purity: 97%), 9-nitroanthracene (9-NA, purity: 93%), and phenanthrene-d10 used as internal standard (IS, purity: 98%) were purchased from Sigma Aldrich (St. Louis, USA). Individual 1-hydroxy- and 2-hydroxy-anthraquinones (OH-AQ, each purity: 95%) were purchased from TCI (Tokyo, Japan). The chemical structures of these chemicals are depicted in Fig. 1. HPLC grade of methanol and ethyl acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA). The silylation agent, N-methyl-N'-trimethylsilyltrifluoroacetamide (MSTFA), was purchased from Machery-Nagel (Duren, Germany).

2.2 Preparation of standard solutions

Each AQ, 9-NA, 1-OH-AQ, and 2-OH-AQ and internal standard (phenanthrene-d_{10}) stock solution containing 1 mg/mL was prepared in ethyl acetate. Working standard solutions containing 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL were prepared by suitable dilution of the stock solution with ethyl acetate. The working solution of internal standard was prepared by diluting of stock solution with ethyl acetate (5 µg/mL for HPLC-DAD and 0.1 µg/mL for GC-MS).

2.3 Sample preparation

A total of 100 paper food packaging samples were collected from local market: 40 teabags, 30 pizza and fried chicken delivery boxes, and 30 muffin paper cups from different manufacture company. Approximately 1.0 g of paper packaging sample was cut into 0.5 cm x cm and then spiked internal standard to sample [5 µg/mL for HPLC-DAD analysis and 0.1 µg/mL for GC/MS analysis]. Prepared paper sample was put in 30 mL methanol (or 100 mL methanol for teabag), and then analytes were extracted by ultrasonication for 20 min. The extract was filtered through a 0.2 µm membrane filter. Filtrated extract was dried by rotary evaporator and redissolved with 1.0 mL methanol for HPLC-DAD analysis or 1.0 mL ethyl acetate for GC-MS analysis. For HPLC-DAD analysis, 5 uL was injected into the HPLC system.

For TMS derivatization, 80 µL of extract was transferred into a 1 mL of Supelco reacti-vial and treated with 20 µL of MSTFA to make 0.1 mL sample volume. The resulting solution was heated at 80°C for 20 min. After cooling down to room temperature, 1 uL of resulting solution was injected into the GC/MS system.

2.4 Gas chromatography-mass spectrometry conditions

GC-MS analysis was performed with an Agilent 6890N gas chromatography instrument combined with an Agilent-5975 mass spectrometer equipped with electron ionization (EI) and a quadrupole analyzer. AQ and its impurities were separated using a DB-5MS capillary column (20 m x 0.25 mm i.d., film thickness 0.25 µm, J&W Scientific, USA) and a ZB-5HT column (15 m x 0.25 mm i.d., film thickness 0.25 µm, Phenomenex, USA). The temperatures of injector and interface were set at 290°C for DB-5MS column and at 400°C for ZB-5HT column. Sample was injected in split mode (10:1). The oven temperature was programmed to hold at 150°C for 2 min and then increase to 300°C at a rate of 5°C/min. The temperature of the ion source was 230°C and the electron energy (EI) was set at 70eV.

For the quantification and confirmation analysis by the selected ion monitoring (SIM) mode, m/z 180 and 152 for AQ, m/z 223 and 176 for 9-NA, m/z 263 and 251 for 1-OH-AQ-TMS, m/z 296 and 253 for 2-OH-AQ-TMS, and m/z 188 for phenanthrene-d_{10} were selected. The dwell time of each ion was set at 50 ms.

2.5 Method validation

The established method was validated by determining the linearity, limits of detection (LODs) and limits of quantitation (LOQs), precision, and accuracy. The linearity of the calibration curves was evaluated by spiking samples at seven concentration levels in five replicates. The LODs and LOQs of four target analytes were evaluated at signal-to-noise (S/N) ratios of 3 and 10, respectively.

The precision of the developed methods was evaluated using intra- and inter-day variations. The relative standard deviation (RSD) was taken as a measure of precision. Intra- and inter-day repeatability was determined on triplicates performed within one day and over three consecutive days, respectively. To evaluate the accuracy of the analytical methods, the recovery study was measured by spiking known amounts of standards in blank samples in triplicate according to US-FDA guideline [19]. Also matrix effect was determined at 100 ppb level and expressed as percentage ratio between analyte response in triplicate spiked paper samples and spiked solvent. Approximate 100 % and lower value means absence of matrix effect and signal suppression, respectively.

3 Results and discussion

3.1 Sample preparation and analysis

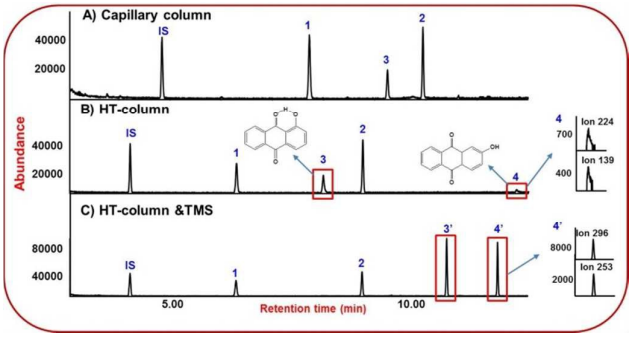

Fig. 2. TICs and EICs of standard mixture (each 10 µg/mL) and internal standard (3 µg/mL) A) without derivatization obtained by capillary column-GC/MS scan mode, B) without derivatization obtained by HT column-GC/MS, and C) with TMS derivatization obtained by GC/MS scan mode. Peak identities as follow: 1. AQ, 2. 9-NA, 3. 1-OH-AQ, 3'. 1-OH-AQ-TMS, 4.2-OH-AQ, 4'.2-OH-AQ-TMS, and IS. phenanthrene-d_{10}

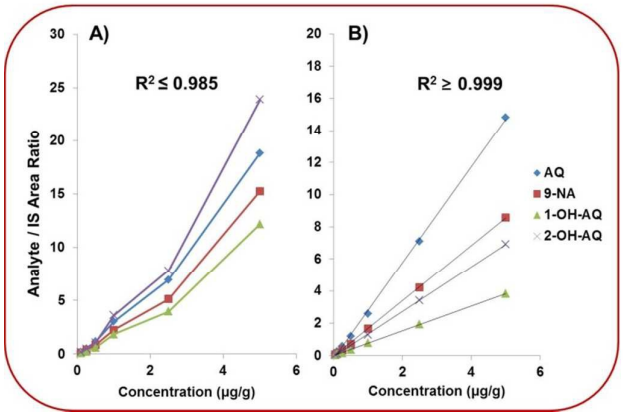

Fig. 3. Calibration curves of AQ, 9-NA, 1-OH-AQ, and 2-OH-AQ in paper packaging products obtained by A) capillary column GC/MS and B) HT-column GC/MS

GC/MS method for the analysis of AQ and its impurities in paper packaging products required simple sample preparation including extraction and TMS derivatization. To extract AQ and its impurities from paper packaging products, ultrasonication, reflux, and immersion were compared and various extraction solvents [methanol, hexane, and chloroform] were tested according to previous studies on polyaromatic compounds [7, 15]. Generally, AQ and its impurities with hydroxyl or nitro group are well soluble in most organic solvents. In view point of matrix effect and convenience, ultrasonication method using methanol was more suitable than other methods, showing the reasonable overall recovery of AQ and impurities from paper sample. For spiking samples at 2.5 µg/glevel in triplicate analysis, the extraction recoveries of AQ, 9-NA, 1-OH-AQ, and 2-OH-AQ (mean ± % standard deviation) were shown to be 92.1 ± 7.98, 82.1 ± 7.89, 87.5 ± 5.20, and 83.4 ± 8.42 %, respectively. Also, TMS derivatization of OH-AQ isomers was optimized according to temperature (40, 60, 80, and 100 °C) and time (10, 20, 30, and 40 min). As results, TMS derivatization temperature and time were set at 80 °C for 20 min.

In this study, capillary column GC/MS was preferentially applied for the determination of AQ and its impurities in paper packaging products based on previous study [7]. As shown in Fig. 2-A, the peaks of AQ, 9NA, and 1-OH-AQ were clearly observed in total ion chromatogram (TIC) of standard mixture containing 10 µg/mL of each analyte and 3 µg/mL of phenanthrene-d_{10}. However, 2-OH-AQ did not detected at 10 µg/mL level in scan mode. Moreover, the response on calibration solution of targets was shown less linear in both GC/MS-scan and selected ion monitoring (SIM) modes. The linearity of target compounds was shown exponential curves (linearity coefficient $r^2 \leq 0.9845$) when preparing calibration curves (Fig. 3-A). These phenomena can be explained that AQ and its impurities might be adsorbed in GC/MS system due to their high boiling points. Thus, precise quantification of AQ and its impurities in paper products could not be obtained by capillary column GC/MS In this study, to minimize their adsorption in GC/MS system, high temperature (HT)-column GC/MS method was attempted. Three target analytes, except for 2-OH-AQ, were observed with reasonable sensitivity in TIC obtained by HT-GC/MS (Fig. 2-B). It is interesting observation that the GC retention behaviour and detection sensitivity for 1- and 2-OH-AQ isomers are greatly different. It can be explained that free 1-OH-AQ experiences considerably more intramolecular hydrogen bonding than free 2-OH-AQ (inset of Fig. 2-B), resulting in increasing the volatility and relatively higher detection response. In order to improve detection sensitivity of 1-OH- and 2-OH-AQ isomers, trimethylsilylation (TMS) was applied. After TMS derivatization, all targets were detected with significant sensitivity and the retention time of 2-OH-AQ-TMS was significantly shorter than that of free 2-OH-AQ due to improving the volatility (Fig. 2-C).The detection responses of OH-AQ-TMS derivatives in GC/MS scan mode were greatly improved by approximately 3.9 times for 1-OH-AQ and 22.4 times for 2-OH-AQ. Furthermore, the linearity of calibration curves for all targets was greatly improved by HT-column GC/MS, as shown in Fig. 3-B.

For the sensitive detection of AQ and its impurities by GC/MS-SIM mode, two abundant ions for each compound were selected for quantification and confirmation. The base peak for each compound was typically used for quantification in SIM mode. For AQ and 9-NA, molecular ions and abundant $[M\text{-}CO]^+$ at m/z 180 and $[M\text{-}HNO_2]^+$ at m/z 176, respectively, were selected. For OH-AQ-TMS derivatives, $[M\text{-}15]^+$ ion at m/z 281 was shown as base peak. However, the ion chromatogram of the base peak at m/z 281 for OH-AQ-TMS derivatives exhibited higher baseline due to column bleeding. To eliminate any possible interferences, appropriate abundant ions (m/z 251 for 1-OH-AQ-TMS and m/z 296 for 2-OH-AQ-TMS), instead of the base peak at m/z 281, were selected for precise quantitation and positive peak confirmation by SIM mode.

3.2 Method validation

Table 1.The calibration curves, correlation coefficients, LODs, and LOQs of AQ and its impurities obtained by HT-column GC/MS SIM mode.

Compound	Linear range (ng/g)	Slope	Intercept	Correlation coefficient	LOD (ng/g)	LOQ (ng/g)
AQ	50-5000	2.9825	-0.1994	0.9992	6.1	20.3
9-NA	50-5000	1.7196	-0.0523	0.9998	8.0	26.8
1-OH-AQ	50-5000	0.7686	-0.0024	0.9999	14.3	47.5
2-OH-AQ	50-5000	1.3932	-0.0561	0.9998	12.2	40.8

To evaluate the linearity of the developed method, calibration spiked solutions for all target analytes were prepared by spiking the stock solutions to blank paper packaging sample. Calibration curves were constructed by linear regression analysis of the peak ratios of each analyte to the internal standard, *versus* concentration. The calibration equations, linear correlation coefficients, LOD and LOQ of target compounds are summarized in Table 2. The correlation coefficients were greater than 0.999, indicating good linearity within concentration range as indicated in Fig. 3-B.The LODs and LOQs of the target analytes were in the ranges of 6.1-14.3 ng/g and 20.3-47.5 ng/g, respectively. To test the accuracy and precision of the analytical method, known amounts of the target compounds were spiked to paper packaging products according to US-FDA guide line [19]. The intra- and inter-day variations for the target compounds in the paper packaging products were determined as described in the Experimental section, and the results are summarized in Table 2. The precision of the method for simultaneously determining the four targets was acceptable because the RSD did not exceed 11% at any given

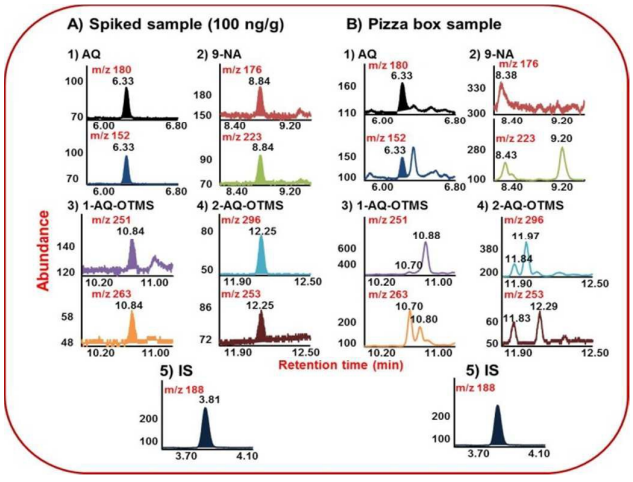

Fig. 4. Extracted ion chromatograms of A) spiked sample (each 100 ng/g) and B) pizza box extract obtained by HT-column GC/MS-SIM

Table 2. Inter- and intra-day precision (%CV) and accuracy (% bias) for AQ and its impurities obtained by HT-column GC/MS SIM mode.

Compound	Concentration (μg/g)	Intra-day (n=3)		Inter-day (n=3)	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
AQ	0.05	87.4	4.5	89.7	6.9
	0.25	100.4	2.8	104.5	3.4
	0.5	96.3	9.8	93.7	8.9
9-NA	0.05	91.4	10.8	95.3	5.6
	0.25	108.2	9.7	103.8	3.8
	0.5	103.6	11.2	101.2	9.4
1-OH-AQ	0.1	98.2	8.6	102.4	6.7
	0.25	102.7	6.3	99.3	2.5
	0.5	94.5	9.2	104.7	9.0
2-OH-AQ	0.1	112.9	11.4	88.1	10.3
	0.25	103.5	7.5	98.0	6.8
	0.5	97.8	8.2	113.1	9.5

concentration. At the same concentrations, the intra-day accuracy ranged from 87.4 to 112.9%, while the inter-day accuracy ranged from 88.1 to 113.1%, providing reasonable accuracy. These inter- and intra-day data demonstrated that the developed method was highly reproducible and precise for four target analytes.

3.3 Method application

The established HT-GC/MS method was applied to the analysis of AQ and its impurities in 100 food paper packaging samples purchased from Korean markets. Typical extracted ion chromatograms of spiked sample (each 100 ng/g) and AQ extracted from pizza box by GC/MS-SIM method are shown in Fig. 4-A and B, respectively. As indicated in Fig. 4-A, four target analytes were clearly detected without any significant interferences. Though not shown here, no significant peaks at retention times of the target analytes were observed in blank sample. As shown in Fig. 4-B, AQ extracted from pizza cartoon was clearly detected and quantified in the extracted ion chromatograms (EICs) of m/z 180 and 152. For confirmation of AQ, the peak area ratio of m/z 180 to m/z 152 was examined whether the value is within criteria range or not. To quantify AQ, peak area ratio of m/z 180 against m/z 188 (internal standard) in EIC was calculated and applied to calibration equation obtained by least squares method. However, any other toxic impurities were not detected.

In this study, HT-column GC/MS and HPLC-DAD methods were compared in terms of the interference effect, runtime, and sensitivity. Typical HPLC chromatograms of standard mixture and AQ extracted from pizza box extract are shown Fig. 5-A and B. As indicated in Fig. 5-A, four standard targets and internal standard were successfully separated within 20 min on C18 column and sensitively detected at UV wavelength 254 nm referred by previous study on anthraquinone and polyaromatic hydrocarbons analysis [14]. Based on optimized LC conditions, pizza paper extract was analyzed. As shown in Fig. 5-B, AQ was detected at retention time 9.25 min but UV spectrum of its peak might be overlapped with other matrix interference. In some cases, HPLC method could lead to false positive or overestimation when sample was not properly

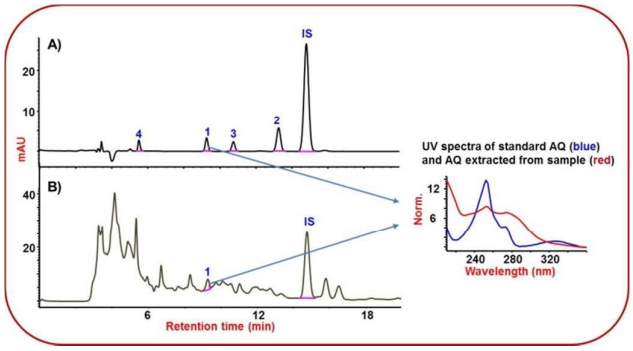

Fig. 5. HPLC-DAD chromatograms of A) standard mixture (each 0.5μg/mL) and B) pizza box extract. Peak identities as follow: 1. AQ, 2. 9-NA, 3. 1-OH-AQ, 4. 2-OH-AQ, and IS. phenanthrene-d_{10}

purified. Moreover, the detection sensitivity of AQ and its impurities obtained by HPLC method was significantly lower than that by HT-column GC/MS method. The LOQs for AQ obtained by HT-column GC/MS and HPLC-DAD were about 20.3 ng/g and 115.3 ng/g, respectively. Thus, HT-column GC/MS-SIM method could be suitable for determination of AQ and its impurities in paper products with high precision and accuracy.

As results on the analysis of 100 food paper packages by established method, AQ was observed in the range of 0.17-0.23 μg/g in5 samples among 30 pizza and chicken delivery boxes and 0.15-0.18 μg/g in two samples among 40 paper teabags whileas no other toxic impurities were observed in all paper samples. Also, AQ and its impurities were not observed in 30 muffin samples.

To investigate the migration percentage of AQ into foodstuff, experiment was performed by the official method proposed by Ministry of Food and Drug Safety, Korea [20]. Standard AQ was spiked into teabag and pizza delivery box at 250 ng/g level, respectively. As results, the migration of AQ into hot water and pizza crust was shown to be 2.16 ± 1.2% and 4.23 ± 3.5% (mean ± standard deviation, n=3), respectively. In comparison with previous literature [7], the migration percentage of AQ in pizza delivery box was almost similar to our result. In this study, the migration percentage of AQ in teabag was for the first time reported and could provide basic framework on the risk assessment of AQ.

Conclusions

Rapid HT-column GC/MS method has been developed for simultaneous determination of AQ and its toxic impurities in paper packaging products. Compared to HPLC-UV and capillary column GC/MS, HT-column GC/MS-SIM combined with simple sample preparation offers significantly improved sensitivity, selectivity, and linearity for AQ and its impurities. Thus, the established method can be used for the safety evaluation of paper food packaging products through the measurement of AQ and its toxic impurities.

Acknowledgments

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