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Validation of an Improved LC/MS/MS Method for Acrylamide Analysis in Foods

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ABSTRACT

An improved LC/MS/MS method was set up and validated for analyzing acrylamide (AA) in foods including fried potato snack food, baked breakfast food, bread, coffee and tea drinks which are the most popular stimulant drinks in Taiwan. Two types of cartridge in tandem use, elution volume, and a long column (250 mm), were used to improve the separation of interferences and reduce detection limit. Reproducibility, repeatability, and recovery tests were conducted to assure the analytical method applicable in house validation testing. Participation in the FAPAS® inter-comparison study resulted in a satisfactory result. The limit of detection was 3 μg/kg; mean recoveries ranged from 95 to 113%; coefficients of variation ranged from 1.3 to 10.0% for repeatability test and 3.3 to 6.9% for reproducibility test. The good results showed the method has been successfully set up to serve as a routinely analytical method. We also found that brown sugar with high AA contents lead to high AA content in some foods including those not cooked at high temperature.

Key words: acrylamide, food analysis, LC/MS/MS, brown sugar

INTRODUCTION

First reported in April 2002 by the Swedish National Food Administration, acrylamide (AA) is known to be formed by high cooking temperature in various foods, particularly fried and baked starch foods[(1)]. Studies have shown that reducing sugar (such as glucose, fructose) and amino acid (asparagine) content are the major precursors to the AA formation through Maillard reaction[(2,3,4)]. AA is a synthetic monomer used as a precursor in the production of polyacrylamide, which has been used in water treatment, paper processing and electrophoretic separations in bioscience fields. Studies have shown that AA is metabolized to glycidamide, an neurotoxic eposide[(5)] and is a potential mutagen and reproductive toxicant. The International Agency for Research on Cancer (IARC, 1994) has labeled AA as a Group 2A, probable human carcinogen since 1994[(6)]. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO)[(7)] have warned that the unintentional contaminant AA in certain foods might be of public health concern since it has been shown to cause cancer in animals.

Gas chromatography mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) are two main methods for analyzing AA in foods[(1,8-11)]. LC/MS/MS is used in most laboratories for its high selectivity and simple procedure. It is necessary for us to evaluate a method including transition ions' sensitivity, columns length, cleanup procedure and validation test (repeatability, reproducibility, recovery and detection limit) before routine in-house operations. Transition ion's sensitivity depending upon instrument and optimized parameters needs to be evaluated for individual instrument. The separation between desirable compound and interferences is affected by column length; particularly the retention time is relatively close. Cleanup procedure is also helpful for reducing noise and enhancing sensitivity during analysis. Validation test is needed to assure the reliability of data before a method can be adapted in house analysis.

The presence of AA in foods had been surveyed in different countries, including the United States, the United Kingdom, Norway, Switzerland, German, Japan, Hong Kong, Austria, Turkey, and Taiwan[(12,13-17)], for evaluating the exposure dosage of AA in foods. Many tea drinks are popular diet drinks in Taiwan[(18)]. Researchers have reported that roasted tea could yield high AA (up to 520 μg/kg) in Japan[(19)] , but no such data is available in

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Taiwan. Thus, the contents of AA in tea and tea drinks were evaluated in this study.

The objectives of this study were to validate an improved LC/MS/MS method for routinely analyzing AA, determine AA contents in some traditional foods and elucidate the correlation of AA and constitutes in foods.

MATERIALS AND METHODS

I. *Internal Standard and Standard Solution Preparation*

$^{13}C_3$-acrylamide (1 mg/mL, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was used as an internal standard. It was transferred to a 100 mL volumetric flask and made up to the volume with methanol as an internal standard stock solution. Ten milligrams of AA (99.9%, J. T. Baker, Phillipsburg, NJ, USA) was dissolved in deionized water and diluted to 100 μg/mL of AA standard stock solution. Calibration curve was established using the standard solution with 100 ng/mL of $^{13}C_3$-acrylamide combined with the AA standard solutions at five concentrations between 5 and 1000 ng/mL.

II. *Sample Preparation*

Sample was prepared according to the methods reported by Andrzejewski *et al.*[10] and Roach *et al.*[20]. Some modifications including solid phase extraction cartridge usage, and eluent volume were examined to improve the detection limit. Two columns (Atlantics dC18 and AQASIL C18 column) with different lengths were evaluated for better chromatographic separations by eliminating possible interferences.

Sample was pulverized by a food processor and weighed into a 50 mL centrifuge tube (i.e. around 1 g for bread, cake, and potato chips samples; 0.1 g for instant coffee samples; 1 mL for drink samples). Test portions were spiked with 1 mL (1 μg/mL) of $^{13}C_3$-acrylamide as an internal standard. The test portions were then diluted with 9 mL (8 mL for drinks samples) of deionized water. The mixture was homogenized for 3 min, shaken at high speed on a horizontal shaker for 20 min. The homogenate was centrifuged (Sorvall® RC-5B, Du Pont Instruments Inc., Germany) at 10,000 × g for 20 min at 5°C. A glass pipette was used to transfer 5 mL of the clarified aqueous to be filtrated through a syringe filter (Nylon membrane, pore size: 0.45 μm, filter size: 25 mm). The filtrate (1.5 mL) was subjected to Oasis® HLB cartridge (Waters, Milford, MA, USA) gravitationally pass through and was followed with another 0.5 mL of deionized water. The eluent was discarded. The sample solution loaded Oasis® HLB cartridge was then connected in tandem to an Oasis® MCX cartridge (Waters, Milford, MA, USA). The tandem cartridges were further eluted with 3 mL of deionized water. The eluent was collected in a 10 mL test tube and reduced to about 0.5 mL by a gentle steam of nitrogen before transferred to another 2 mL glass autosampler vial for further LC/MS/MS analysis. Both Oasis® HLB and Oasis® MCX cartridges were conditioned with methanol (5 mL and 3 mL, respectively) and water (5 mL and 3 mL, respectively) before use.

III. *LC/MS/MS Analysis*

LC-MS/MS analysis was carried out using a Waters 2695 Separations Module HPLC (Waters Corp., Milford, MA, USA) coupled to a Micromass Quattro Premier (Waters Corp., Milford, MA, USA) triple-quadrupole mass sptectometer equipped with an electrospray source and Masslynx version 4.0 software for separation, detection and quantification. The analytical column was a AQUASIL C18 column (5μm, 2.1 mm × 250 mm) (Thermo Hypersil-Keystone, Thermo Electron Corp., Walthan, MA, USA) maintained at 30°C. The mobile phase was 10% methanol with 0.1% formic acid and the flow rate was maintained at 0.2 mL/min. The injection volume was 20 μL. The AA was analyzed using electrospray ionization in positive ion mode. Multiple reaction monitored mode (MRM) was acquired with the characteristic fragmentation transitions *m/z* 72 > 55 ($[M+H-NH_3]^+$), 72 > 54 ($[M+H-H_2O]^+$), 72 > 44 ($[M+H\text{-ethene}]^+$), 72 > 27 ($[M+H\text{-formamide}]^+$) for AA and *m/z* 75 > 58 for $^{13}C_3$-acrylamide (Figure 1). MS/MS conditions were as follows: capillary voltage, 2.5 kV; cone voltage, 20 V; source temperature, 125°C; desolvation gas temperature, 250°C; desolvation gas flow, 900 L/h; cone gas flow, 100 L/h nitrogen. The argon collision gas pressure was adjusted to 6.86×10^{-3} mbar for MS/MS. The collision energy was varied and optimized for each MRM transition. The transitions monitored for AA were *m/z* 72 > 72 at 5 V, 72 > 55 at 13V, 72 > 54 at 11V, 72 > 44 at 10V, and 72 > 27 at 25V. The transitions monitored for $^{13}C_3$-acrylamide were *m/z* 75 > 75 at 5 V, 75 > 58 at 13V, and 75 > 29 at 25V. Four identification points (IP = 4) were achieved for analysts identification in the criteria of European Commission Decision 2002/657/EC[21]; we selected one precursor ion (*m/z* 72) and two transition ions (*m/z* 72 > 55 and *m/z* 72 > 54) to monitor the analysis. The MRM transitions *m/z* 72 > 55 and *m/z* 75 > 58 were acquired for the quantification of AA concentration as shown in Figure 2. The ion ratio of *m/z* 54/55 and 55/72 were 8.3 and 11.8%, respectively. The dwell time for each transition was 0.3 s with 0.02 s of interchannel and interscan delay.

IV. *Quantification*

AA was quantified using a linear calibration curve with standard solutions of AA dissolved in water at concentrations ranging between 5 and 1000 ng/mL with 100 ng/mL $^{13}C_3$-acrylamide as an internal standard. AA in samples was determined from calibration curve constructed by plotting the peak area ratios (*m/z* 55 and *m/z* 58) against the concentrations of AA.

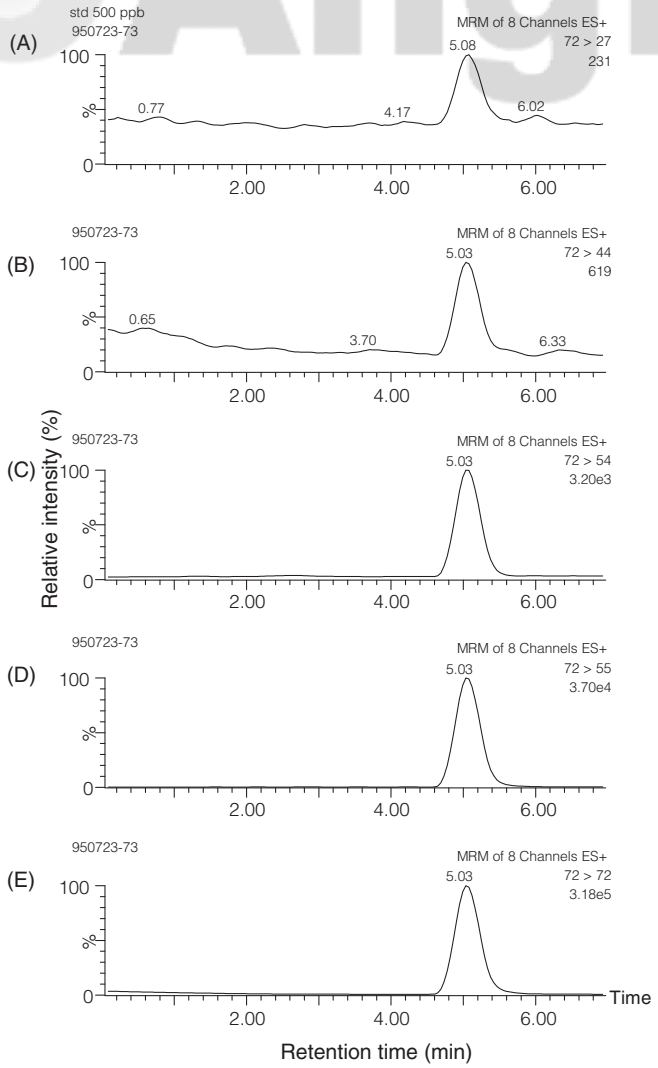

Figure 1. MRM chromatograms obtained from calibration standard of acrylamide (500 ng/mL) (A) *m/z* 72 > 27 (B) *m/z* 72 > 44 (C) *m/z* 72 > 54 (D) *m/z* 72 > 55 (E) *m/z* 72 >7 2.

RESULTS AND DISCUSSION

I. *Method Improvement*

Baked food consists of lipid which would be solidified at low temperature and be removed for better cleaning up. We found the procedure for extracting AA from foods by water at 5°C in high speed centrifuge could eliminate interference of lipid. Furthermore, the sample quantity for preparation was considered for avoiding serious interference during instrumental analysis. For baked food and tea, sample weight was around 1 g, but 0.1 g was enough for AA analysis of instant coffee powder. Too much coffee (1 g) resulted in the matrix effects and then masked the AA signal. Andrezjewski *et al*.[10] reduced the instant coffee sample portion to 0.5 g for better analysis.

Single cartridge or tandem cartridge combination were used for cleanup, such as Oasis® HLB (poly(divenylbenzene-co-*N*-vinylpyrrolidine)[15], Isolute Multi-Mode[1,22], Bond Elut-Accuat, Oasis® HLB and Bond Elut-Accuat[10], Isolute Multimode and Accubond II SCX[23], Oasis HLB® and Accuat SPE[20], Oasis HLB® and Bond Elut-Accuat[24]. Oasis® HLB and Oasis® MCX (sulphonic acid groups) cartridge were used in a previous study[12] and further compared with the Isolute® Multimode cartridge in this study. When only Isolute® Multimode cartridge was used, AA signal was interfered by overlapped noises (Figure 3). Isolute® Multimode cartridge has the multifunctional characteristics for being with non-polar C18, strong cation exchange ($-SO^{3-}$) and strong anion exchange ($-NR^{3+}$) group. Nevertheless, the capacity of the column to remove all interferences simultaneously appeared to be limited. In addition, it was necessary to increase the elution volume for obtaining all possible AA from Oasis® HLB cartridge. We found that at least 3.0 mL water was needed to elute the AA from Oasis® HLB cartridge sorbent as shown in Figure 4 other than 1.5 mL used in literatures[10, 20].

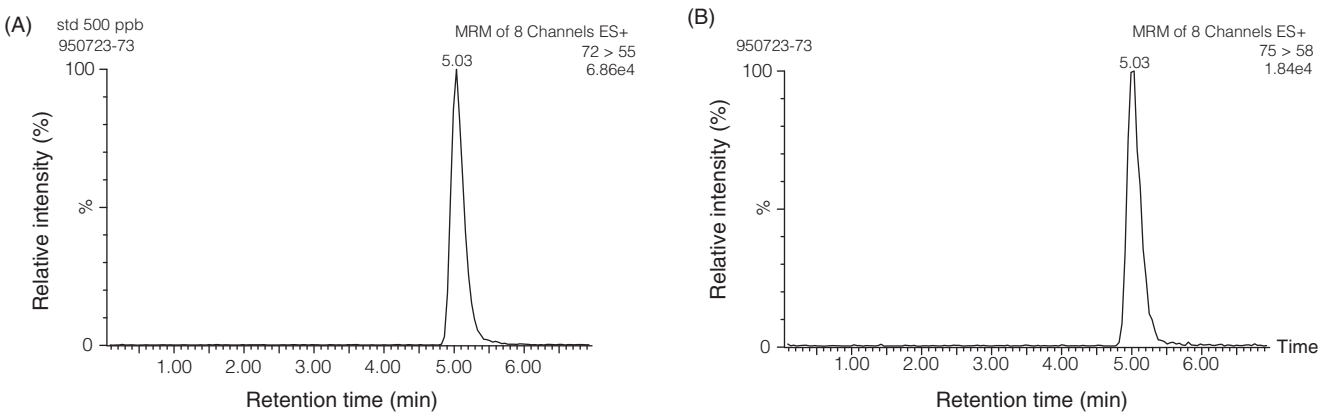

Figure 2. MRM chromatograms of (A) *m/z* 72 > 55 and (B) *m/z* 75 > 58 obtained from calibration standard of acrylamide and $^{13}C_3$-acrylamide used for quantification in LC/MS/MS analysis.

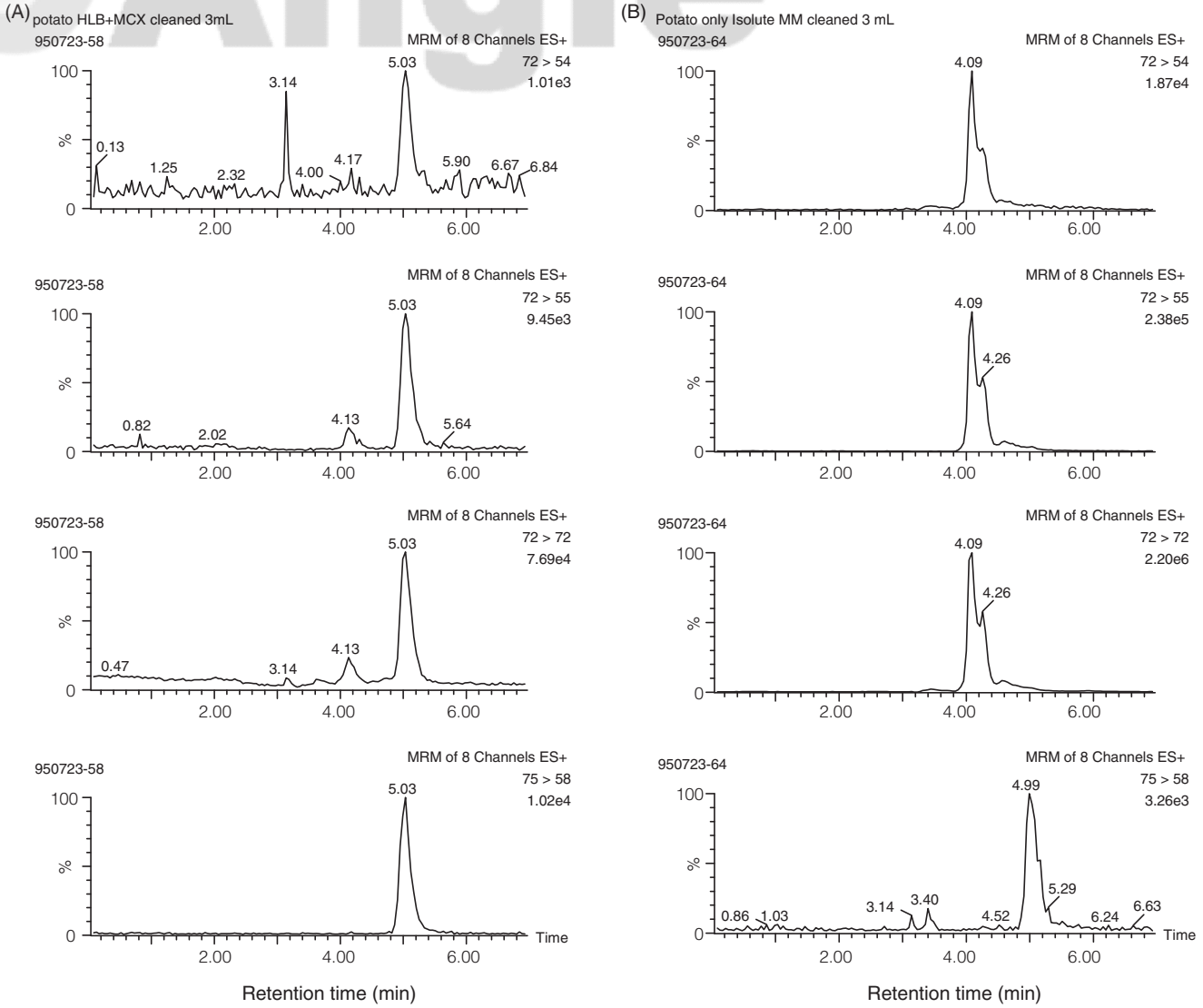

Figure 3. Comparison of MRM chromatograms for a potato chips sample cleaned up with different SPE cartridges (A) SPE with Oasis® HLB and Oasis® MCX (B) SPE with Isolute® Mulitmode.

Two columns, Atlantics dC18 (3 μm, 2.1 × 150 mm) and AQASIL C18 (5 μm, 2.1 × 250 mm), were used to examine the effect of column length on analytical results at retention time less than 6 min. The longer one, AQASIL C18, yielded better separation efficiency for obtaining AA from sample matrix and for higher resolution (Figure 5). The data showed that the modified procedures effectively resolved the interferences problems.

II. *Method Validation*

The standard curve was established based on the area ratios of the AA product ion at *m/z* 55 and the internal standard $^{13}C_3$-acrylamide product ion at *m/z* 58. The standard curve was linear in the range of 5-500 ng/mL with R^2 greater than 0.99 (data not shown). The reproducibility of standard curve was evaluated by analyzing the standard

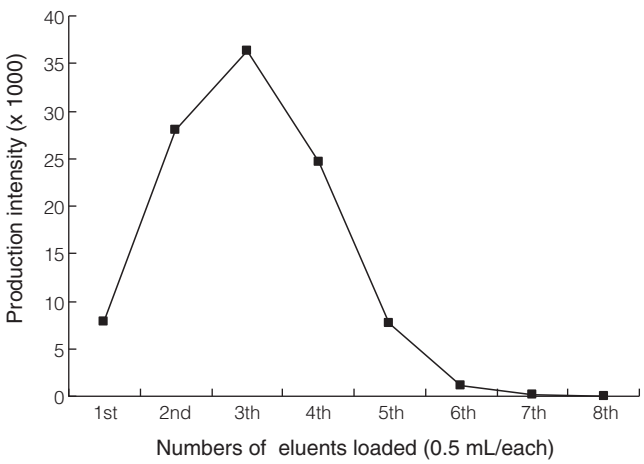

Figure 4. The product ion (*m/z* 72 > 55) intensity of AA in eluents from Oasis® HLB cartridge loaded with acrylamide (1 μg) after eluted with water (0.5 mL each).

Table 1. Recovery test of the LC/MS/MS method*

Sample	Acrylamide added (μg/kg)	Recovery (%)	CV (%)
Potato chips	100	104	5.1
	500	100	3.5
Sesame seed cake	100	95	2.4
	500	96	2.1
Tea	100	100	2.9
	500	98	4.2
Coffee	100	86	4.6
Bread (crumb)	10	113	8.0

* n=6 (duplication)

solution in 3 days and the studies showed good results with CV% ranged from 3.3 to 13.3% (data not shown).

The recovery studies were performed by spiking homogenized potato chips, sesame seed cake, tea and coffee with AA stock solution at concentrations of 100-500 μg/kg. Bread crumb was spiked with 10 μg/kg of AA stock solution to assure suitability of the method at low concentration. These spiked samples (n=6) were held 20 min in hood for AA absorption before analysis. The recoveries ranged from 86 to 113% with CV less than 10% (Table 1). The recovery for coffee was the least one among the samples tested. Bread yielded the greatest recovery of 113%.

The repeatability was examined by analyzing homogenized potato chips, sesame seed cake, tea and bread (n=6). Among four products, potato chip had the lowest CV of 1.3% and tea had 10% CV (Table 2). The data indicated that the repeatability of the method was acceptable. Homogenized sesame seed cake and potato

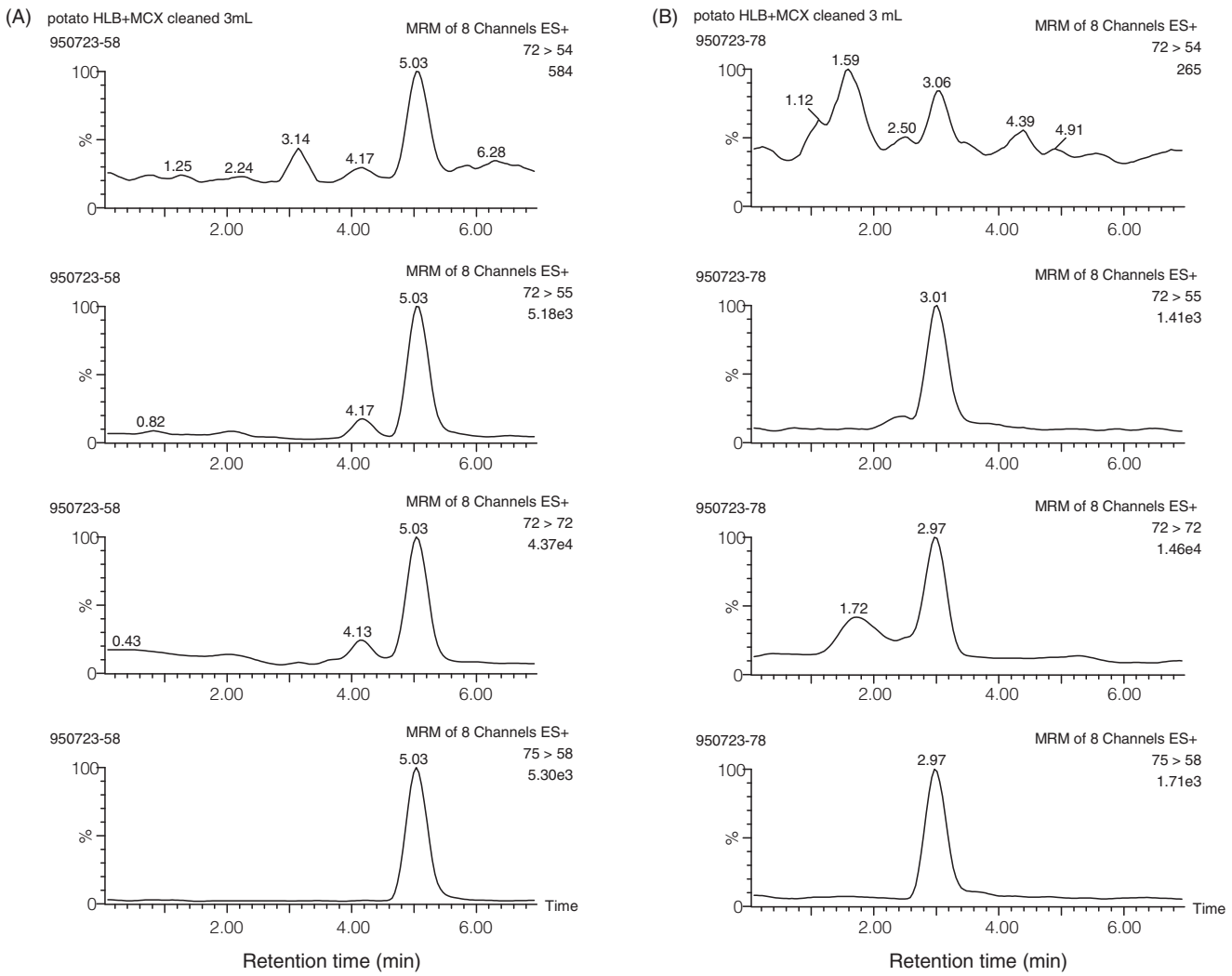

Figure 5. Comparison of MRM chromatograms for a potato chips sample separated with different column (A) AQUASIL C18 5 μm (2.1 × 250 mm) and (B) Atlantis dC18 3 μm (2.1 × 150 mm).

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Table 2. Repeatability test of the LC/MS/MS method

Test No.	Acrylamide (μg/kg)			
	Potato chips	Sesame seed cake	Tea	Bread (crumb)
1	1202	55	36	39
2	1221	58	41	35
3	1199	57	37	37
4	1180	58	31	37
5	1221	55	35	38
6	1201	59	33	39
Mean ± SD	1204 ± 13	57 ± 2	35 ± 4	37 ± 2
CV %	1.3	2.9	10.0	6.3

chips were further analyzed 12 times in 3 days to examine the reproducibility. The CV of the reproducibility ranged from 3.3% to 6.9%. Both the repeatability and reproducibility data met the criteria of European Commission Decision 2002/657/EC[21].

One gram of homogenized bread crumb was spiked with 10 ng AA and analyzed by the established method 7 times. The detection limit (MDL = $t_{(n-1,\ 1-\alpha=0.99)} \times SD$) was 3 μg/kg as calculated from standard deviation. The detection limits were reported in the range of 5-15 μg/kg for GC/MS method[1,12,26] and 3-20 μg/kg for LC/MS/MS method[1,10,20,22,24,25]. The sensitivity and validation test have demonstrated that the improved method is appropriate for analyzing AA content in food.

III. *Proficiency Test*

We participated in the proficiency test organized by the Food Analysis Performance Assessment Scheme (FAPAS) of the Central Science Laboratory York (UK) in March 2008[27]. Each participant received a biscuit test material to be analyzed for AA. The result reported by our laboratory for AA in dispatched test material with a Z-score (-0.8) less than ± 2 successfully met requirements of the organization. The result supported accuracy of the improved method for quantification of AA.

VI. *Acrylamide Contents in Foods*

The validated method was used to analyze AA contents in samples including baked sweet cake, bread, brown sugar, French fries, instant coffee, and various drinks as shown in Table 3. Specifically, the AA content in steamed bread no brown sugar added was lower than 3 μg/kg. Nevertheless, adding brown sugar resulted in higher AA content of 43 μg/kg. Both soft bread and hard bread yielded lower than 3 μg/kg of AA content. Again, AA content was high at 297 μg/kg for soft bread with brown sugar flavored. Low AA content in steamed bread was expected due to the processing temperature being no higher than 100°C since AA was formed at 120°C[28]. The AA content in brown sugar might attribute to the high AA content in steamed bread (brown sugar added) and soft bread (brown sugar flavored). Another analysis in 4 brown sugars showed the high AA contents ranged from 347-1107 μg/kg. Table 4 lists the AA content in different sugars including crystal sugar, granulated sugar (white color), granulated sugar (brown color) and brown sugar

Table 3. The acrylamide contents in foods using LC/MS/MS methods[a]

Sample	AA (μg/kg)
Baked sweet cake	
A, B, C, D, E, F and G[b]	< 3 - 12
Bread	
Steamed (no brown sugar added)	< 3
Steamed (brown sugar added)	43 ± 4[c]
Hard bread (A, B, C, D)	< 3
Soft bread (A, B, C, D, E)	< 3
Soft bread (brown sugar flavored)	297 ± 22
Brown sugar	
A	1107 ± 48
B	347 ± 25
C	405 ± 28
D	561 ± 22
French fries	
A	45 ± 0
B	76 ± 2
French fries cookie	308 ± 8
Coffee	
Instant coffee	98 ± 5
Coffee drink (A, B, C, D, E)	< 3
Tea drink and others	
Green tea drink	< 3
Oolong tea drink	< 3
Black tea drink	< 3
Asparagus juice drink	375 ± 3

[a] n=2 (repetition).
[b] A, B, C…represents different brands or products.
[c] Standard deviation.

Table 4. The compositions and acrylamide contents of different kinds of sugar

Compositions	Crystal sugar	Granulated sugar (white color)	Granulated sugar (brown color)	Brown sugar
Carbohydrate (%)	99.7	98.2	99.3	79.0
Protein (%)	0	0	0.2	1.9
Asparagine	N.D.	N.D.	N.D.	+
AA (μg/kg)	< 3	< 3	11	119

ND: not detected.

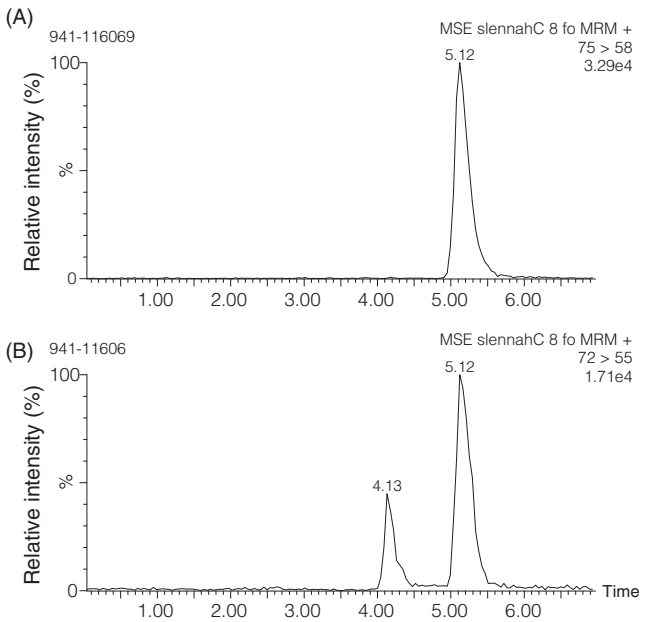

Figure 6. MRM chromatograms of (A) $^{13}C_3$-acrylamide and (B) acrylamide for brown sugar sample.

from one producer. The results showed that brown sugar consisted of high AA (119 μg/kg), which was ten times higher than granulated sugar (brown color). AA was not detected in crystal sugar and granulated sugar of white color. The chromatograms of AA in brown sugar sample are shown in Figure 6. Brown sugar is an unrefined product that consists of impurities, such as free amino acid, protein and minerals. Asparagine was detected positive in brown sugar, possibly explaining the high AA content in brown sugar. Since brown sugar is a popular food ingredient in Asian countries like Taiwan and Japan, it is necessary to further evaluate the risk from the intake of brown sugar-added foods in Taiwan.

The AA contents in French fries and French fries cookie ranged from 45 to 308 μg/kg. As for the stimulant drinks, the AA content in instant coffee was 98 μg/kg and negligible in coffee and tea drinks. The results were quite different from the results for roasted tea in Japan[19]. Roasted tea has been processed at temperature higher than 120°C, which might result in high AA content. It was unexpected to observe that asparagus juice consisted of 375 μg/kg of AA which was higher than tea and instant coffee, probably due to the high temperature during sterilization process. Stimulant drinks accounted for 10-16% dietary intake in Taiwan. To obtain an overall understanding on the intake of AA via drinks, it is necessary to run a complete survey on different drinks.

CONCLUSIONS

An improved LC/MS/MS method had been validated for analyzing AA contents in different foods. Combination of tandem cartridge and a long column are suggested to analyze AA for minimizing undesirable interferences and improving detection limit. In addition, brown sugar and asparagus juice were found to carry high AA content and thus increased AA contents in related products. More background data of AA contents in foods are needed to conduct the risk assessment of AA in diet.

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