Development of Isotope Dilution-Liquid Chromatography/Tandem Mass Spectrometry as a Candidate Reference Method for the Determination of Acrylamide in Potato Chips

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An isotope dilution-liquid chromatography/tandem mass spectrometric method was developed as a candidate reference method for the accurate determination of acrylamide in potato chips, starch-rich foodstuff cooked at high temperature. Sample was spiked with $^{13}C_3$-acrylamide and then extracted with water. The extract was further cleaned up with an Oasis HLB solid-phase extraction (SPE) cartridge and an Oasis mixed-phase cation exchange (MCX) SPE cartridge. The extract was analyzed by using LC/ESI/Tandem MS in positive ion mode. LC with a medium reversed-phase (C4) column was optimized to obtain adequate chromatographic retention and separation of acrylamide. MS was operated to selectively monitor $[M+H]^+$ ions of the analyte and its isotope analogue at m/z 72 and m/z 75, respectively. Sample was also analyzed by the LC/MS with selectively monitoring the collisionally induced dissociation channels of m/z 72 → m/z 55 and m/z 75 → 58. Compared to the LC/MS chromatograms, the LC/MS/MS chromatograms showed substantially reduced background chemical noises coming from solvent clusters formed during ESI spray processes and interferences from sample matrix. Repeatability and reproducibility studies showed that the LC/MS/MS method is a reliable and reproducible method which can provide a typical method precision of 1.0% while the LC/MS results are influenced by chemical interferences.

Key Words : Acrylamide, Potato chips, ID-LC/MS, Method validation, Reference method

Introduction

In early 2002, a Swedish group reported that starch-rich foodstuffs processed or cooked at high temperature contain relatively high levels of acrylamide.[1] Mottram *et al.* later reported that acrylamide is formed by Maillard reaction of reducing sugars with asparagines at temperatures above 120 °C.[2-5] The Swedish findings attracted considerable public and scientific attention worldwide as acrylamide has been known as a potential genetic and reproductive toxin with mutagenic and carcinogenic properties.[6-9] Since the breakout of the acrylamide issues, several government bodies and international organizations initiated special actions to carry out survey and research on acrylamide in foodstuffs.[10-13] To date, no national governments have taken any regulatory action on the matter of acrylamide in food yet. All are waiting until further scientific data are gathered and evaluated.

Only a few analytical methods had been available for the determination of acrylamide at the time when the Swedish report was published. Analytical methods based on high performance liquid chromatography or gas chromatography was typical methods at that time.[14-20] Due to the high polarity of acrylamide, a cumbersome step of derivatization of acrylamide by bromination is required in GC methods to increase selectivity and sensitivity,[15,21-23] while the use of LC methods is limited as acrylamide has poor retention with conventional reversed-phase LC column[23] and is lack of a chromophor for UV detection.[24,25] In most of recent studies, mass spectrometry (MS) has been coupled with LC[24,26-37] or GC[22,23,27,34,38,39]-based online separation techniques for better identification of acrylamide from food samples. Especially, isotope dilution techniques[22,29,33-35] have been introduced to the MS methods to improve reliability of measurement results.

In addition to the development of a new analytical method, harmonization of acrylamide assay results among laboratories worldwide began to lead a considerable concern in scientific community as any scientific decision on risk assessment studies and subsequent setup of regulatory actions should be based on reliable analytical results.

It is well known that certified reference materials (CRMs) with their certified values traceable to the International System of Units (SI) play a key role for the harmonization of assay results among laboratories both nationwide and worldwide as they can be used for the validation of analytical methods used in individual laboratories and also for testing proficiencies of laboratories that carry out acrylamide measurements. However, to our knowledge, no matrix CRM with its certified value for acrylamide is available yet. Our laboratory is currently developing CRMs for the determination of acrylamide in potato chips, which are one of common starch-rich foodstuffs cooked at high temperature. While we are developing methodologies for preparing homogenized candidate reference materials, we carried out a study to develop a reference method for the accurate assignment of acrylamide levels in the CRMs which is traceable to SI. Isotope dilution mass spectrometry coupled with a reliable chromatographic separation technique is

usually a method of choice for this purpose due to its accuracy without systematic bias through the accurate correction of the recovery of the target analyte along the sample clean up processes. In this study, we established an isotope dilution LC/tandem MS method and carried out an evaluation of this method to determine whether this method has accuracy, repeatability, and reproducibility reliable enough to be used as a candidate reference method in our laboratory, which is the designated national metrology laboratory in Korea.

Experimental

Materials. Acrylamide (99 + %) was purchase from Aldrich (Milwaukee, WI, USA) and was used as a primary reference material without further purification. Purity assay of acrylamide is out of scope of the current study and thus was not carried out in our laboratory. Instead we adopted the purity provided by the manufacturer. According to the manufacturer's certificate analysis, the purity has a rectangular distribution from 99% to 100%. Therefore, 99.5% was assigned as its purity with a standard uncertainty of 0.3%. $^{13}C_3$-Acrylamide stock solution (99% isotopic purity, 1 mg/mL in methanol) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). A $^{13}C_3$-acrylamide working standard solution of a 5 mg/kg level was prepared by diluting the stock solution with water. HPLC grade organic solvents (methanol and acetonitrile) were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid was obtained from Aldrich (Milwaukee, WI, USA). Pure water was prepared by using a membrane-filtering system and further purified by passing through a Millipore Corp Milli-Q RG purification system. Oasis MCX SPE Cartridges (6 mL, 150 mg) and Oasis HLB SPE Cartridges (6 mL, 200 mg) were purchased from Waters (Milford, MA, USA). Potato chips from several manufacturers were purchased from local stores and were used as samples.

Calibration Standard Solutions. The standard solution used in this study was prepared and verified according to a procedure maintained in our laboratory. The brief description of the procedure is as following. We gravimetrically prepared at least two independent acrylamide standard solutions of 5 mg/kg level in water. For each of the acrylamide standard solutions, at least two calibration standard mixtures with 1:1 isotope ratio were prepared by mixing the weighed aliquots of the standard solution and the $^{13}C_3$-acrylamide working standard solution. We tested the self-consistency within and among the standard solutions by inter-comparing the calibration standard mixtures by the LC/MS. Based on the inter-comparison test results, repeatabilities and reproducibilies of preparing standard solutions and calibration standard mixtures were evaluated, and one calibration standard mixture was selected and used for further sample analysis as a single-point (exact matching) isotope ratio calibrant. In this study a single point isotope ratio calibration method was used as LC/MS measurement in the range of 0.7:1 to 1.3:1 ratio showed a good linearity.[40,41] For the sample measurement at each different time period, a new set of the standard solutions and the calibration standard mixtures were prepared and verified.

Sample Preparation and Clean-up. Potato chips were ground and homogenized using a laboratory mill prior to subsampling. A subsample of 5 g was taken into a 50 mL conical tube. An appropriate amount of the $^{13}C_3$-acrylamide working standard solution was spiked to the subsample so that the ratio of acrylamide to $^{13}C_3$-acrylamide was close to 1.0. The exact amounts of the sample and the $^{13}C_3$-acrylamide working standard solution taken into the tubes were determined by weighing the tube before and after addition of each of them into the tube. Then, 40 mL of water was added into the tube. The tube was caped tightly, and was shaken for complete mixing of the contents. Thereafter, the tube was left alone for 20 minutes at room temperature for the equilibration of spiked $^{13}C_3$-acrylamide with acrylamide in the sample. The tube was then shaken for 20 min for acrylamide extraction. The tube was centrifuged at 3900 g for 20 min. The tube contains oily layer, aqueous layer, and solid layer from the top to the bottom. The aqueous layer was transferred into a 50 mL conical tube. 5 mL of acetonitrile was added into the aqueous layer. The tube was centrifuged at 3900 g for 15 min to spin down precipitated proteins. The whole aqueous layer was concentrated to 1 mL by evaporation of liquid by placing the tube in a 100 ^{o}C water bath. The concentrated aqueous layer was then subjected to two stages of solid-phase extraction (SPE). The sample extract was first loaded onto an Oasis HLB SPE cartridge (200 mg/6 mL from Waters), which was preconditioned by eluting 5 mL of methanol and 5 mL of water. The eluent obtained during the sample loading was discarded. The cartridge was then eluted with 1 mL of 5 % methanol in water and the eluent was collected for MCX SPE clean up. The collected eluent was loaded to a Oasis MCX SPE cartridge (200 mg/6 mL from Waters), which was preconditioned with 5 mL of methanol and 5 mL of water. The MCX cartridge was eluted with 1 mL of water. The eluent obtained during sample loading and elution was collected after discarding the first 0.2 mL portion and then were bottled into an HPLC vial.

LC/MS Analysis. The LC/tandem MS used in this study was an API 2000 mass spectrometer from Applied Biosystems (Forster City, CA, USA) combined with an 1100 Series LC system from Agilent Technologies (Palo Alto, CA, USA) through its electrospray ionization interface. Sample extracts and calibration standard mixtures were loaded in 5 μL units to a Symmetry 300^{TM} C4 column (150 mm length, 4.6 mm i.d., 5 μm particle size) from Waters Corp (Milford, MA, USA). The analytical separation was performed using an isocratic mixture of 10% methanol in water containing 0.01% formic acid at a flow rate of 0.2 mL/min. The mass spectrometer was operated in the positive ion mode with optimized spray conditions. Two different operational modes were tested for the detection of acrylamide and its isotopic analogue by the mass spectrometer. For the LC/MS analysis, MS was operated on a selected ion monitoring

(SIM) mode for monitoring the $[M+H]^+$ ions of acrylamide and $^{13}C_3$-acrylamide at m/z 72 and 75, respectively. For the LC/MS/MS analysis, MS was operated on a selected reaction monitoring (SRM) mode for monitoring the collisionally induced dissociation (CID) channels of m/z 72 → m/z 55 and m/z 75 → m/z 58, which are the ammonia loss from the $[M+H]^+$ ions of acrylamide and $^{13}C_3$-acrylamide, respectively. For the SRM mode, the collision cell, the second quadrupole of the mass spectrometer, was filled with nitrogen gas at a pressure of ~0.2 Pa (~2.0×10^{-3} mbar) and the collision energy was 21 eV.

Measurement Protocol. For sample measurements, a single LC/MS run in the SIM mode was carried out in the order of the calibration standard mixture and a series of sample extracts. The single run cycle was repeated for 4 times. The LC/MS/MS measurement in the SRM mode was then carried out in the same order as the LC/MS measurement in the SIM mode.

Results and Discussion

ESI/Tandem MS Spectra. Figure 1(a) and (b) show ESI MS spectra of acrylamide and $^{13}C_3$-acrylamide dissolved in a mixture of 10% methanol and 90% water with 0.01% formic acid which was selected as an isocratic mobile phase for the analytical separation (see below). In the spectrum of acrylamide, $[M+H]^+$ ion peak at m/z 72 is the most intense peak. The spectrum of $^{13}C_3$-acrylamide also shows the most intense peak of $[M+H]^+$ ion at m/z 75. A weak peak due to the post source decay of the $[M+H]^+$ ions to the corresponding $[M+H-NH_3]^+$ ions is observed on both spectra. In this study, the $[M+H]^+$ ions were selected for the LC detection of acrylamide and $^{13}C_3$-acrylamide in the SIM mode. Figure 1(c) shows the background noise spectra obtained by spraying the mobile phase only. The spectrum shows that background noise peaks due to the solvent ions from the ESI source are not negligible at low mass range below m/z 100. The influence of these solvent ions on the acrylamide analysis will be discussed later. Figure 2(a) and (b) show the CID spectra of $[M+H]^+$ ions of acrylamide and $^{13}C_3$-acrylamide, respectively. On both spectra, $[M+H-NH_3]^+$ peaks at m/z 55 and 58, respectively, are dominant, and other fragment peaks are relatively weak. Therefore, $[M+H]^+ \rightarrow [M+H-NH_3]^+$ were selected as CID channels for the detection of both acrylamide and $^{13}C_3$-acrylamide in the SRM mode.

Sample Cleanup and Liquid Chromatographic Separation. Previous studies have reported several liquid chromatographic methods to obtain retention and separation of highly polar acrylamide.[42] As most of researchers have used, we decided to use reversed-phase chromatography as it is convenient to use and provides a good repeatability in retention time. Among several column assayed in the initial stage of method developments for the acrylamide analysis, a C4 phase column (Symmetry 300™ C4, 150 mm length, 4.6 mm i.d., 5 μm particle size; Waters Corp) was chosen as this stationary phase provided adequate retention of acrylamide and stable chromatographic performances when a proper mobile phase was used. Isocratic mixtures of water and methanol containing 0.01% formic acid have been tested as a mobile phase. In the initial stage of optimizing LC separation condition, the extract of potato chips fortified with acrylamide at the level of 10 mg/kg without any further

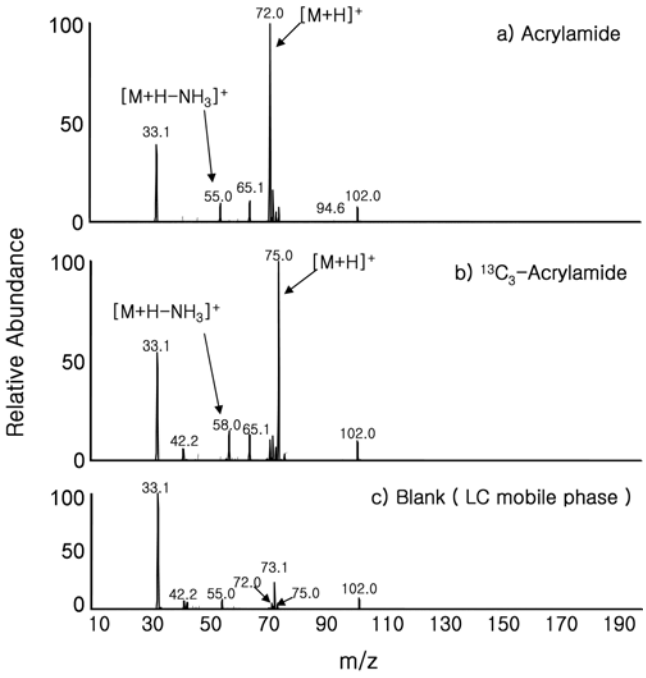

Figure 1. MS spectra of (a) acrylamide and (b) $^{13}C_3$-acrylamide in a mixture of 10% methanol and 90% water with 0.01% formic acid by positive electrospray ionization. (c) MS spectra obtained by spraying the mobile phase only, representing background chemical noises from solvent ion clusters formed at ESI processes; m/z 33 and 65 peaks are protonated methanol monomer and dimmer, m/z 102 peak is protonated triethylamine, carry-over residue in the source.

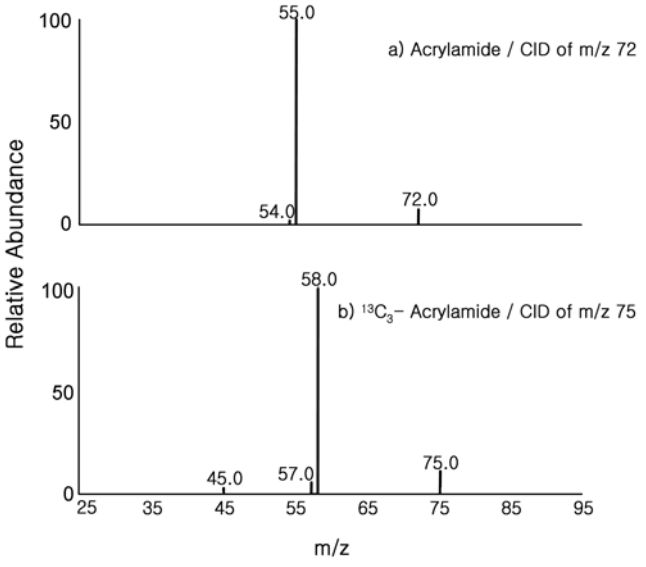

Figure 2. Collisionally induced dissociation spectra of $[M+H]^+$ ions of (a) acrylamide and (b) $^{13}C_3$-acrylamide, respectively.

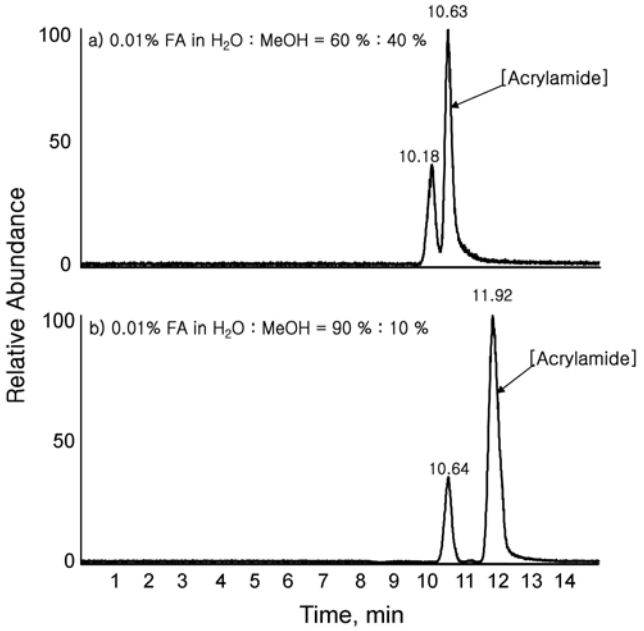

Figure 3. SRM chromatograms of acrylamide in the extract of potato chips fortified with acrylamide at the level of 10 mg/kg without further clean up. The collisionally induced dissociation channel of m/z 72 → m/z 55, the ammonia loss of the $[M+H]^+$ ions of acrylamide, was selectively monitored. Symmetry 300^{TM} C4 column (150 mm length, 4.6 mm i.d., 5 μm particle size). Mobile phase: (a) water 60% + methanol 40% + formic acid 0.01%, (b) water 90% + methanol 10% + formic acid 0.01%.

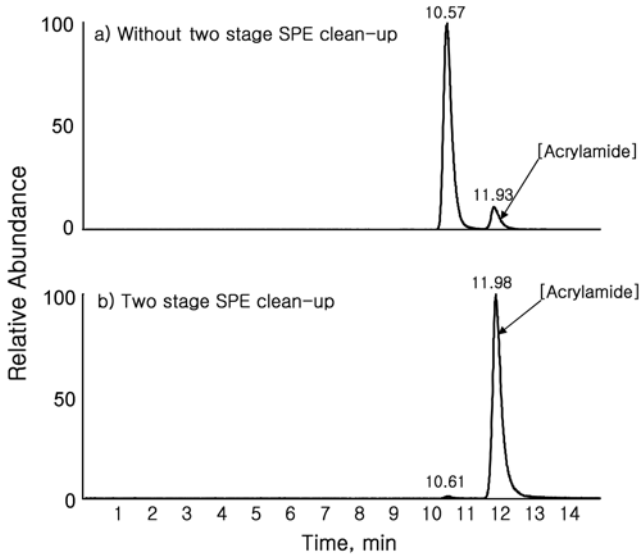

Figure 4. SRM chromatograms of acrylamide in the extract of potato chips fortified with acrylamide at the level of 2 mg/kg (a) without further clean up after extraction and (b) after two stages of SPE clean up with a HLB SPE cartridge and a MCX SPE cartridge.

cleanup after extraction was used as test sample. It was noticed that the C4 LC column could not retain acrylamide when mobile phase contains more than 40% of methanol. Figure 3 shows the chromatograms of the potato chips obtained by mass spectrometer in the SRM mode. The chromatogram using an isocratic mixture of 40% methanol and 60% water at a flow rate of 0.2 mL/min show that acrylamide begins to be retained and separated from an intense peak due to co-extracted matrix interferences. As shown in Figure 3(b), retention time of acrylamide increases and acrylamide is separated out from the interferences when methanol content is further decreased. Based on these results, the analytical separation was performed with the C4 column using the isocratic mixture of 10% methanol and 90% water with 0.01% formic acid at a flow rate of 0.2 mL/min.

Ground and homogenized potato chips are usually in the form of thick paste as substantial amount of cooking oil in pores of the chips is mixed with pulverized potato chip powder. Therefore, it is relatively easy to prepare homogenized sample. This grinding method was used to prepare homogenized potato chip sample in this study.

Proper correction for the recovery of acrylamide from sample and possible losses occurring during the whole sample preparation processes are a critical fact which can influence reliability of measurement results. Though we used $^{13}C_3$-acrylamide as an internal standard, complete equilibrium of native acrylamide with the isotopic analogue should be ensured for the proper correction of the recovery from the sample extraction. In this study, sample was spiked with $^{13}C_3$-acrylamide and mixed with water, then seated for 20 minutes, and shaken for another 20 min for equilibration of acrylamide and its isotopic analogues. Giving further time for equilibrium time did not change the analysis results, indicating that 20 minutes is enough for the equilibration of the native acrylamide with the spiked isotopic analogue.

Figure 4(a) shows the SRM chromatogram of acrylamide obtained by injecting the extract of potato chips fortified with acrylamide at a level of around 2 mg/kg just after protein precipitation without the two stage SPE clean-up. In addition to the acrylamide peaks at 11.9 min, an intense peak appears at 10.6 min due to co-extracted matrix interferences. Figure 4(b) shows the SRM chromatogram of acrylamide in the same sample extract obtained after the two stages of SPE clean-up. The intensity of the major interference peak is substantially reduced compared with that of the acrylamide peak.

ESI/Tandem MS Performance as LC Detector. Figure 5 shows SIM and SRM chromatograms of acrylamide and $^{13}C_3$-acrylamide in potato chips from a local market. The potato chip sample (with acrylamide level of 0.7 mg/kg) was spiked with an appropriate amount of $^{13}C_3$-acrylamide and went through sample preparation processes described in the experimental section and LC was operated with the analytical separation conditions described above. The SIM chromatogram of acrylamide is dominated by the acrylamide peak, but also shows a few weak peaks from co-extracted matrix interferences in addition to the major interference peak shown in Figure 3. A tiny peak is slightly overlapped with acrylamide peak at its head. The acrylamide peak is seated over a broad unstructured background level, which can be attributed to co-extracted matrix interferences. The influence of the background peak on the accurate

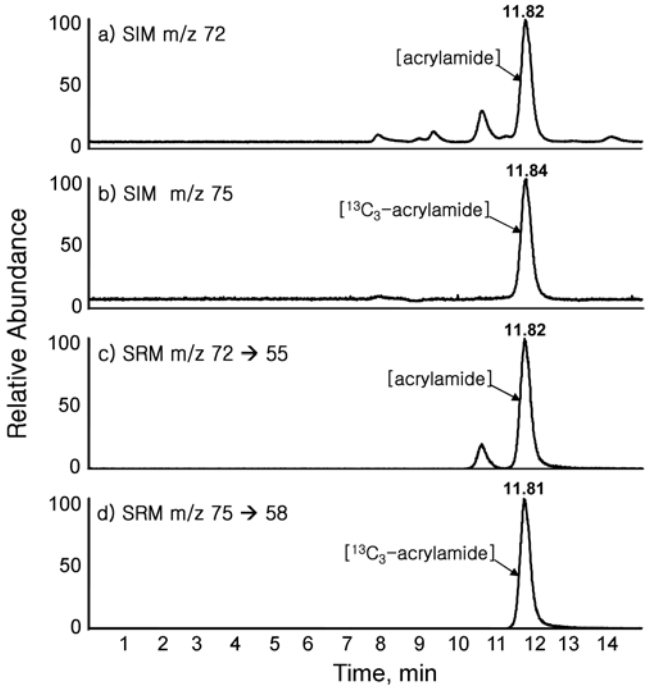

Figure 5. SIM and SRM chromatograms of acrylamide and $^{13}C_3$-acrylamide in potato chips with acrylamide in a natural level of 0.7 mg/kg.

determination of acrylamide level will be discussed later. In the SRM chromatogram of acrylamide, most of the co-extracted matrix interference peaks including the peak at the head of acrylamide peak are not observable any more. However, the major interference peak at 10.6 min is still observed at a substantially reduced intensity, but this peak is well separated from the acrylamide peak. It is noticeable that the baselines of the SIM chromatograms of acrylamide and $^{13}C_3$-acrylamide are at consistently elevated levels throughout the whole retention time range, which show a stark contrast to those of the corresponding SRM chromatograms. SIM chromatograms of calibration standard mixtures also showed similar levels of elevated baselines when compared to the corresponding SRM chromatograms. The elevated baselines of SIM chromatograms are attributed to the solvent ions from the ESI source as shown in Figure 1(c).

To test the LC/MS performance of the acrylamide analysis, the standard deviation of the area ratio of acrylamide and $^{13}C_3$-acrylamide was evaluated from multiple runs of sample extracts and calibration standard mixtures. The relative standard deviation of the area ratio from the LC/MS runs in the SIM mode was typically around 0.8% for both sample extracts and calibration standard mixtures, while it was reduced to 0.4% when the LC/MS was operated in the SRM mode. We note that sample extracts and calibration standard mixtures showed similar levels of repeatability though SIM chromatograms of acrylamide from sample extracts have interference peaks as described above. Integration of the acrylamide peak with a consistent manner seems to reduce possible variation even with the interfering peaks in the SIM chromatograms. The relatively poorer repeatability with the SIM mode can be attributed to the inaccuracy in integrating peak areas of acrylamide and $^{13}C_3$-acrylamide because of the elevated background noise coming from the solvent ions formed at the ESI source. The above results indicate that the SRM mode can provide the higher metrological quality in measurements as well as its capability in further reducing possible bias that can arise from co-extracted matrix interferences. Therefore, we have chosen LC/MS/MS in the SRM mode as a candidate reference method for the analysis of acrylamide in potato chips. However, LC/MS in the SIM mode is also further evaluated in parallel with LC/MS/MS in the SRM mode for the comparison purpose.

Method Validation and Analytical Quality Check: We carefully evaluated if ID-LC/MS/MS in the SRM mode has an adequate quality as a reference method that can be used in national metrology institutes. ID-LC/MS in the SIM mode was also evaluated in parallel to test whether it is compatible with ID-LC/MS/MS in the SRM mode. As no matrix CRM with a certified value for acrylamide is available yet to our knowledge, "validation by using well-characterized standard or published methods" is not applicable. In this case, the careful evaluation of performance parameters of the candidate reference method including repeatability, reproducibility, and sources of uncertainty is required. The followings are experimental results obtained for the validation of the method.

Recovery of Acrylamide in Sample Preparation. The overall recoveries of acrylamide and $^{13}C_3$-acrylamide from potato chip sample ranged from 40% to 60%. The recoveries of acrylamide and $^{13}C_3$-acrylamide from a blank sample (extracting solvent spiked with the two analytes) was similar to those from real samples, indicating that the recoveries were mostly affected by the recoveries of the analytes at the sample clean-up processes. The eluent during the sample loading contains most of co-extracted polar matrix interferences. Therefore, this portion of eluent was discarded though it also contains the front head of acrylamide elution. Further action was not taken to improve the recovery as it is not a critical factor in IDMS methods.

Detection Limit. As acrylamide-free potato chips are not available, the detection limit of the method was only estimated based on the signal to noise ratio of the SRM chromatograms of potato chip samples. The detection limit of the LC/MS/MS method in the SRM, with the signal to noise ratios of 3, was estimated to be 0.02 mg/kg. Meanwhile, the detection limit of the LC/MS method in the SIM mode was estimated to be 0.05 mg/kg, which is higher than that of the LC/MS/MS method in SRM mode due to elevated background noise level and the chemical interference from samples matrix.

Repeatability and Reproducibility. To test the repeatability and the reproducibility of the two LC/MS methods, an adequate amount of a homogenized sample is required. According to our screening test, the acrylamide levels of potato chips purchased from market range from 0.3 to 1.5 mg/kg. More than 200 g of potato chips with medium level of acrylamide was ground and homogenized using a laboratory mill. The pulverized sample in the form of thick

Table 1. Results of acrylamide in homogenized potato chip sample by ID-LC/MS in both SIM mode and SRM mode in three different time periods within 3 months

	Subsample No.	Measurement Results (mg/kg)	
		LC/MS/MS in SRM mode	LC/MS in SIM mode
Period 1	#1	0.673	0.641
	#2	0.669	0.646
	#3	0.680	0.656
	#4	0.671	0.672
	#5	0.677	0.670
	#6	0.673	0.646
	Average	0.674	0.655
	Standard deviation	0.004 (0.59%)	0.013 (1.99%)
	Expanded uncertainty[a]	0.007 (1.01%)	0.015 (2.31%)
Period 2	#1	0.670	0.659
	#2	0.677	0.651
	#3	0.688	0.668
	#4	0.689	0.667
	Average	0.681	0.661
	Standard deviation	0.009 (1.35%)	0.008 (1.22%)
	Expanded uncertainty[a]	0.014 (2.04%)	0.012 (1.86%)
Period 3	#1	0.674	0.650
	#2	0.669	0.646
	#3	0.671	0.650
	#4	0.684	0.668
	Average	0.675	0.654
	Standard deviation	0.007 (0.98%)	0.009 (1.45%)
	Expanded uncertainty[a]	0.010 (1.47%)	0.015 (2.26%)
Average		0.676	0.657
Standard deviation among period		0.004 (0.58%)	0.004 (0.62%)

[a]The expanded uncertainties are with a level of confidence of 95%.

paste was bottled into an amber jar. The jar was purged with argon, tightly sealed with a Teflon lined cap, and stored at 4 ^{o}C. Repeatability and reproducibility test was done by using this sample.

To test the repeatability of the candidate reference method described above, multiple subsamples were taken, and were subjected to the sample preparation processes, and the extracts were analyzed by the LC/MS both in the SIM mode and in the SRM mode. To test reproducibility of the methods, the same repeatability test on the same potato chip paste was carried out after a reasonable time interval of weeks to months. In each time period, a new set of multiple standard solutions were prepared and used after verification by the self-consistency test described in the experimental section. Table 1 lists the measurement results obtained at three different time periods.

The relative standard deviation of the results obtained from the LC/MS/MS method in the SRM mode within a time period ranges between 0.59% and 1.35% of the corresponding mean values, indicating that the method has an excellent repeatability. The relative standard deviation of results obtained from the LC/MS method in the SIM mode ranges from 1.2% to 2.0%, which is larger than that obtained from the LC/MS/MS method in the SRM mode. It is presumed that the poorer repeatability of the SIM mode compared to the SRM mode can be attributed to the variation of the amount of matrix interferences among subsamples due to slight variation in sample clean up conditions for each of subsamples. Further investigation to explain the difference between the two modes was not carried out.

The relative standard deviation of the means of the three different time periods was 0.58% for the SRM mode and 0.62% for the SIM mode, which is smaller than the repeatability of within periods, indicating that the LC/MS methods with both the SRM mode and the SIM mode have the high degree of reproducibility. The measurement results from different time periods agreed to each other within their expanded relative uncertainties (see below).

Comparison of Results of LC/MS in SIM Mode and in SRM Mode. In Table 1, the mean value of the measurement results from the LC/MS method in the SIM mode is 3% lower than the value from LC/MS/MS method in the SRM mode at all three time periods. The difference between the values from the two methods is larger than their repeatability, indicating that either of the methods has inherent bias sources. The difference of the measurement results between the two methods attributed to the bias in the SIM mode due to matrix interferences as the LC/MS/MS method in the SRM mode was proved to provide higher chromatographic quality and its additional specificity have capability to further reduce possible bias due to co-extracted matrix interferences. Therefore, the LC/MS/MS method in the SRM mode is selected as a candidate for a reference method, and the method using the LC/MS method in the SIM mode is not further considered as a reference method.

Uncertainty Sources. For a reference method to be fit for its purpose, the uncertainty of the measurement value must be evaluated and must be confirmed that the uncertainty is at least many fold smaller than that of the method generally used in field laboratories. Uncertainty sources of the results from the ID-LC/MS/MS method in SRM mode are listed in Table 2. As a full discussion of measurement uncertainty is beyond the scope of this article, only a brief description is given here. Note that the standard deviation of the measurement results of multiple subsamples within a time period in this case represents the random uncertainty due to the uncertainties in weighing sample taken for analysis, weighing the amount of the acrylamide-$^{13}C_3$ solution spiked to the sample, and the LC/MS measurements of the calibration standard mixture and sample extracts.[43,44] The uncertainties in the standard solution and the calibration standard mixture give systematic effects on the results and are not included in the repeatability estimated in this way. In addition to the random uncertainty sources of the repeatability test in a single period, the reproducibility also includes the uncertainties in the gravimetric preparation of the standard solution and the calibration standard mixtures. As a single pure acrylamide material was used throughout this study, the

Table 2. Uncertainty sources in the ID-LC/MS/MS in SRM mode for the determination of acrylamide in potato chips

Uncertainty Components	Sources (Evaluation Methods)	Typical value (Relative %)	Relation with Repeatability and Reproducibility[a]	
Acrylamide standard solution	Purity of the reference material (from the certificate)	0.3%	Systematic uncertainty	
	Gravimetric preparation (from cross-check of independent sets of calibration solutions)	0.2%	Systematic effects to multiple measurements within a time period	Included in reproducibility
Calibration standard mixture	Gravimetric mixing (from cross-check of independent sets of calibration standard mixtures)	0.3%		
Weight of sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01%	Included in repeatability within a time period	
Weight of $^{13}C_3$-acrylamide solution spiked into sample taken for analysis	Readability and linearity of the balance used (from the certificate of the balance)	< 0.01%		
Peak area ratio of acrylamide and $^{13}C_3$-acrylamide from LC/MS measurements of calibration standard mixtures	Standard deviation of multiple measurements	0.2%		
Peak area ratio of acrylamide and $^{13}C_3$-acrylamide from LC/MS measurements of sample extract	Standard deviation of multiple measurements	0.2%		

[a]Relationships of each uncertainty sources with the repeatability and the reproducibility are based on the measurement protocol used in this study.

uncertainty in the purity gives a systematic effect on all the results. Therefore, all the uncertainty sources but the uncertainty of the purity of the acrylamide reference material is included in the reproducibility as random effects. The uncertainty of the measurement results of the ID-LC/MS/MS method in SRM mode at each time period is evaluated and listed in Table 2. The expanded uncertainty in 95% confidence level is less than 2%, indicating that indicating that the method has a high metrological quality as a reference method.

Conclusions

An LC/tandem MS-based isotope dilution mass spectrometric method has been established and evaluated as a candidate reference method for the analysis of acrylamide in potato chips. For the LC detection of acrylamide and its isotopic analogues by the mass spectrometer, the selected reaction monitoring mode of the collisionally induced dissociation channels of their $[M+H]^+$ ions to the $[M+H-NH_3]^+$ ions was chosen rather than the selective ion monitoring mode of their $[M+H]^+$ ions as the SIM mode detection provide poorer chromatographic quality and contains bias sources due to the co-extracted matrix interferences. The repeatability and reproducibility test results proved that the LC/MS/MS method in the SRM mode has a high metrological quality as a reference method to be used in national metrology institutes. The candidate reference method will be used later in our laboratory for the certification of acrylamide in potato chip reference materials.

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