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Analysis of acrylamide in coffee and cocoa by isotope dilution liquid chromatography–tandem mass spectrometry

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Abstract An accurate and precise method for the quantification of acrylamide using stable isotope dilution liquid chromatography–tandem mass spectrometry was developed and used to measure acrylamide in coffee and cocoa samples. The sample preparation involved extraction of the analyte and its internal standard, $^{13}\text{C}_3$ -acrylamide, into water and subsequent defatting of the aqueous extract with dichloromethane. An aliquot of the resulting aqueous extract was then azeotropically dried under reduced pressure and subsequently purified using an aminopropyl-bonded silica cartridge. The purified extracts were then chromatographed on a 5- μm 2.1 \times 150 mm Hypercarb column, the effluent of which was monitored for the analyte and its internal standard using positive-ion APCI-selected reaction monitoring. The intra-laboratory reproducibility of the method, expressed as a relative coefficient of variation (%), ($n=5$), was determined at four levels of concentration (12.3, 42.3, 139.3 and 464.8 $\mu\text{g kg}^{-1}$) and was found to vary between 0.6–2.5%. The accuracy of the method was assessed using a reference sample of coffee. The average result obtained using our method differed from the assigned value of the reference material by less than 1%. An analysis of a cocoa sample revealed that the method is capable of precisely estimating acrylamide in challenging matrices down to a level of at least 12.3 $\mu\text{g kg}^{-1}$.

Keywords Acrylamide · Tandem mass spectrometry · Isotope dilution · Coffee · Cocoa · Atmospheric pressure chemical ionisation

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Introduction

In April 2002, the Swedish National Food Administration and the University of Stockholm [1] held a press conference to announce that acrylamide—a probable human carcinogen—was found in a select sample of heated foods. Within months of the announcement, analysts from the Central Sciences Laboratory [2] in the United Kingdom provided the first independent peer-reviewed confirmation of this phenomenon to, arguably, a sceptical scientific community.

Using a previously developed gas chromatographic–mass spectrometric (GC-MS) method [3] to measure acrylamide in hydroponically grown fruit, Ahn et al. [2] verified the Swedish discovery by quantifying the amount of acrylamide in a limited sample of cooked carbohydrate-rich foods. In the process of providing verification these analysts also demonstrated the validity of an expedient liquid chromatographic–tandem mass spectrometric (LC-MS/MS) method. By concurrently analysing samples they showed that there was good agreement between the results obtained using the two alternative mass spectrometric methods.

In contrast to the GC-MS method [3], which involves conversion of acrylamide to 2,3-dibromopropanamide and subsequent liquid-liquid extraction, the more expedient LC-MS/MS method [4] simply involves purification of aqueous food extracts using mixed mode (both cation and anion) non-polar disposable cartridges. Indeed, Ahn et al. [2] noted that the majority of the foods they surveyed for acrylamide could be analysed without any sample preparation other than extraction of the analyte into water. However, these authors mentioned that they routinely perform solid-phase extraction (SPE) using multi-mode cartridges prior to analysis of the aqueous food extracts by LC-MS/MS to reduce ion suppression [5] or so-called matrix effects.

Others [6, 7] have since shown that sample preparation using multi-mode non-polar SPE cartridges is an adequate and reliable means of reducing the matrix effects seen in the analysis of cooked carbohydrate-rich foods. However,

their application in preventing or reducing matrix effects in chemically more complex extracts appears to be less successful. For instance, Riediker and Stadler [8] noted that acrylamide could not be reliably measured in either coffee or cocoa using a method that employed two non-polar multi-mode SPE cartridges to prepare such samples for analysis by LC-MS/MS. Severe ion suppression has also been reported [9] to occur during the LC-MS/MS analysis of coffee extracts obtained using two non-polar SPE cartridges, one of which was of the multi-mode variety. Similarly, Granby and Fagt [10] found that ion suppression was an issue in the analysis of coffee extracts obtained using a multi-mode non-polar cartridge.

Whilst the use of co-eluting isotopically labelled internal standards is helpful in overcoming the quantitative issues associated with ion suppression, it is not a panacea for matrix effects. Selective sample preparation techniques that remove matrix components that co-elute with analytes are the preferred strategy for reducing these problematic effects. We report here a method that uses an aminopropyl SPE cartridge to process extracts prior to LC-MS/MS analysis to quantify acrylamide in coffee. It also appears that the method can be used to measure acrylamide in cocoa.

Experimental

Chemicals and consumables

Unless otherwise stated, all organic solvents were of HPLC grade. 1,2,3- $^{13}\text{C}_3$ -acrylamide was obtained as a solution in methanol (1 g L^{-1}) from Cambridge Isotope Laboratory (Andover, MA, USA). Acrylamide was purchased from Sigma (St Louis, MO, USA). Aminopropyl (500 mg, 4 mL) solid-phase extraction cartridges were purchased from Alltech Associates (Deerfield, IL, USA).

Food samples

The FAPAS reference coffee material (T3008, assigned value $174\text{ }\mu\text{g kg}^{-1}$, acceptable range $102\text{--}247\text{ }\mu\text{g kg}^{-1}$) was obtained from the Central Science Laboratory (York, UK). Coffee and cocoa samples were purchased at a supermarket in Sydney, Australia, on 26 July 2005. Coffee beans were ground using a domestic coffee grinder.

Analytical instruments

Tandem mass spectrometric analysis was performed using a TSQ Quantum (Thermo Finnigan, San Jose, CA, USA) triple-stage quadrupole mass spectrometer, which was operated in the positive ion mode. The mass spectrometer was equipped with an atmospheric pressure chemical ionisation source, and the inlet system consisted of a Surveyor (Thermo Finnigan) autosampler and Surveyor MS quaternary pump.

Sample extraction

The sample (2.0 g) was placed in a 50-mL polypropylene test tube and covered with water (15 mL). After addition of the internal standard ($^{13}\text{C}_3$ -acrylamide, $1.5\text{ }\mu\text{g}$), the sample was mixed and then allowed to stand at room temperature with occasional mixing. After 2 h, the sample was centrifuged at 3,500 rpm for 30 min, and the supernatant was transferred to a glass centrifuge tube. Dichloromethane (10 mL) was added, and the resulting mixture was vortexed. After centrifuging the sample (2,000 rpm, 15 min) to separate the phases, an aliquot (2 mL) of the aqueous layer was mixed with 1:3 ethyl acetate/acetonitrile (20 mL) in a 50-ml polypropylene test tube. The sample was briefly vortexed and then centrifuged at 3,000 rpm for 30 min. The supernatant was decanted from the pellet into a pear-shaped flask (50 mL) and, after the addition of iso-octane (1.0 mL), was concentrated to approximately 1 mL under vacuum (250 mm Hg) with mild heating ($55\text{ }^\circ\text{C}$). The resulting dark brown concentrate was diluted with ethyl acetate (3 mL), vortexed, and cyclohexane (2 mL) was then added. The mixture was vortexed again and then applied to an aminopropyl solid-phase extraction cartridge (500 mg) that had been conditioned with ethyl acetate (2 mL) and cyclohexane ($2\times 2\text{ mL}$). Pentane (2.5 mL) and then 1:2 acetone/pentane (1 mL) were successively passed through the cartridge to waste before elution of acrylamide and its internal standard in 1:2 acetone/pentane (5 mL). The solvent was carefully evaporated to dryness under a gentle stream of nitrogen and with the aid of mild heating ($40\text{ }^\circ\text{C}$). The resulting colourless residue was dissolved in HPLC-grade water (0.5 mL).

Liquid chromatograph tandem mass spectrometry

An aliquot (20 μL) of the above extract was chromatographed on a Hypercarb (Thermo, Bellefonte) analytical column ($2.1\times 150\text{ mm}$), which was kept at $30\text{ }^\circ\text{C}$ in an oven. The mobile phase, water, was pumped through the column at a rate of $350\text{ }\mu\text{L min}^{-1}$. Under these conditions, acrylamide and its internal standard eluted from the column in approximately 5 min. The column effluent was diverted to waste for the first 4 min and then switched to the detector for the next 2 min. Detection of acrylamide and its internal standard was achieved by monitoring the daughter ions arising from the collision-induced dissociation of the parent ions of acrylamide ($m/z=72$, $\text{M}+1$) and $^{13}\text{C}_3$ -acrylamide ($m/z=75$). Argon (1 mTorr) was used to fragment these ions, and in the case of acrylamide, two daughter ions ($m/z=55$) [$\text{CH}_2=\text{CHCO}^+$] and 44 [$\text{O}=\text{C}=\text{NH}_2^+$] were produced by applying collision energies of 10 and 26 V respectively to the parent ion. Similarly, applying the same collision energies to the parent ion of the internal standard produced the corresponding daughter ions with m/z values of 58 and 45. A scan width of 1 amu and a dwell time of 300 ms were used to monitor the daughter ions. The resolution of both quadrupoles was set to 0.7 amu FWHM. The parent ions of acrylamide and its internal standard,

$^{13}\text{C}_3$ -acrylamide, were produced under atmospheric pressure chemical ionisation conditions. The vaporizer temperature and discharge current were set to 250 °C and 7 μA respectively. The temperature of the ion capillary transfer tube was set to 275 °C, and nitrogen at a flow of 25 L h^{-1} was used as the sheath gas. Other parameters such as source CID and tube lens were optimised using a 0.3 $\mu\text{g mL}^{-1}$ solution of acrylamide.

Acrylamide calibration

A set of ten calibration standards was made with concentrations 0.098, 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 and 25.0 $\mu\text{g mL}^{-1}$ in water. Calibration standards (including a blank) for the LC-MS/MS calibration curve were prepared by adding 100 μL of each standard and 1.5 μg of internal standard (30 $\mu\text{g mL}^{-1}$ in water, 50 μL) to 20 mL of water. A calibration curve was constructed by plotting the amount, in nanograms, of acrylamide against the response (area) of the target ion, $m/z=55$, of the analyte relative to the response of the target ion, $m/z=58$, of the internal standard. The calibration curve was linear ($y=0.008x+0.0015$, $R^2=0.99998$) over the range of 0–2,500 ng.

Results and discussion

The adventitious [11] discovery of acrylamide in certain heated-treated foods has created a need to measure this compound in a wide variety of food commodities. In this respect, we were recently called upon to quantify the amount of acrylamide in coffee and cocoa samples. Our initial attempts at using non-polar multi-mode SPE cartridges to prepare the aqueous extracts of such samples for LC-MS/MS analysis were unsuccessful. In a sequence analysis of coffee extracts we noticed that in some samples there was a severe suppression, presumably because of matrix effects, of the mass spectrometric response for the internal standard. Interestingly, in addition to being the source of choice for flow rates above 0.1 mL min^{-1} and mobile phases with a high content of water [12], atmospheric pressure chemical ionisation is reported [13] to be less prone to ion suppression, which is thought [5] to be caused by co-eluting non-volatile solutes changing the colligative properties of a mobile phase and thereby preventing the transfer of analyte from solution into the gas phase. Unfortunately, the use of a polar graphite analytical column to retain very polar compounds that elute around the column void on a reverse-phase column and that can potentially co-elute with acrylamide did not remedy the

RT: 0.00 - 5.50 SM: 7B

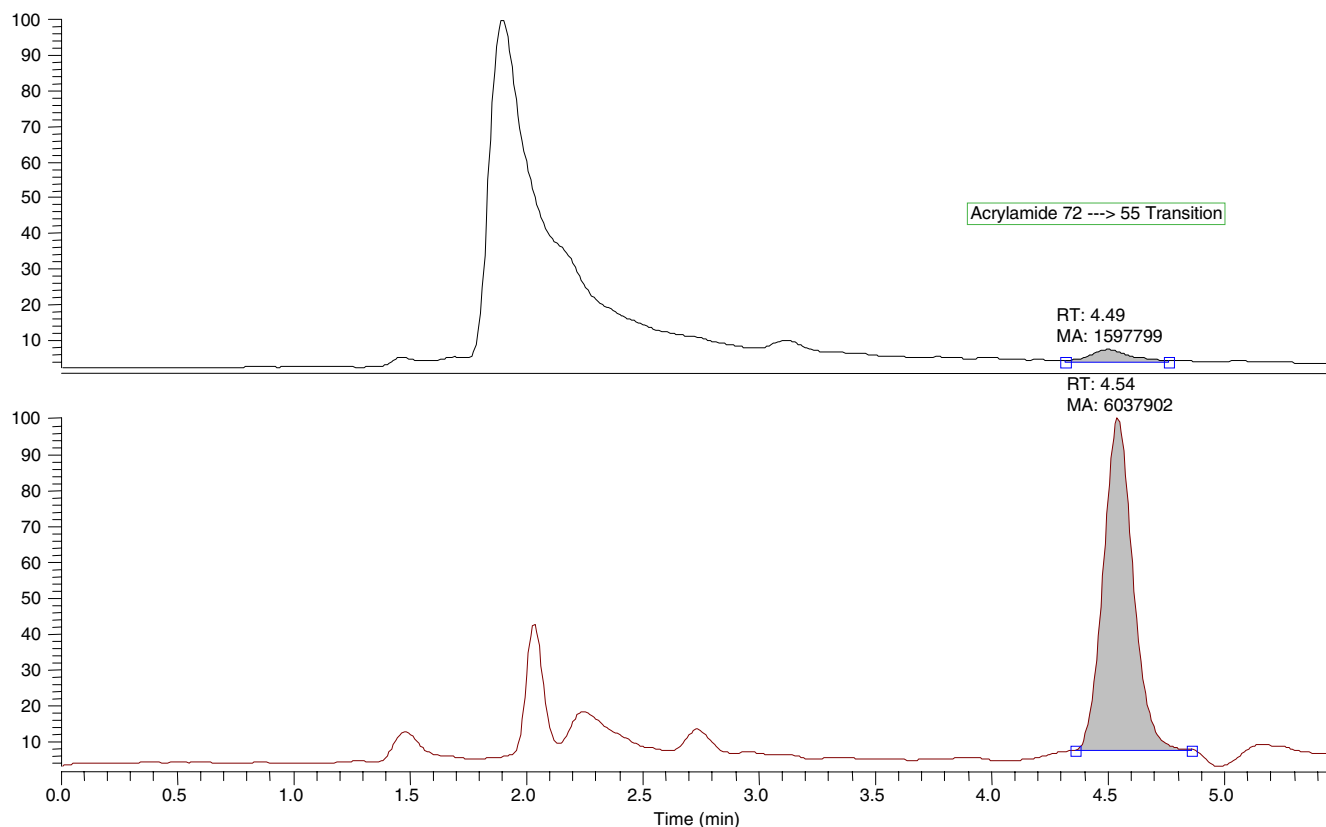


Fig. 1 Chromatograms obtained from the LC-MS/MS analysis of a coffee sample contaminated with 43 $\mu\text{g kg}^{-1}$ acrylamide using multi-mode non-polar (*top*) and aminopropyl (*bottom*) cartridges

problem. The combination of a polar graphite column and water as a mobile phase, however, gave excellent retention of acrylamide, which eluted as a Gaussian peak and with a capacity factor of about 2.5 when the flow through the column was $350 \mu\text{L min}^{-1}$. This is more than twice the retention of acrylamide on the C18 reverse-phase column (Phenomenex, Aqua 2.0×150 mm, 125A, $3 \mu\text{m}$, $275 \mu\text{L min}^{-1}$) we initially used in this work.

A review of the literature (*vide supra*) suggested to us that matrix effects and co-elution were significant issues when non-polar multi-mode SPE cartridges were used as the primary means of purifying coffee and cocoa extracts for the analysis of acrylamide. A solution to this problem was reported by Stadler and his co-workers [14]. The key feature of their sample clean up was the recovery of acrylamide from a salted aqueous matrix by repeated extraction with ethyl acetate. After the addition of water, the organic phase was evaporated and the aqueous phase, into which acrylamide had transferred, was purified by passage through a C18 multi-mode cartridge. Although this method provides a solution to the vexing problem of ion suppression, it is laborious because it requires multiple extractions and aspirations, four centrifugations, and two concentration steps, one of which is noted to take approximately 1 h.

It occurred to us that an alternative approach to the analysis of acrylamide in difficult matrices such as coffee and cocoa would be to use a normal phase sorbent to perform the sample clean up. This would provide an opportunity to selectively elute the amide from more polar impurities that would otherwise co-elute with acrylamide from non-polar sorbents such as C18. The aminopropyl sorbent is of the normal-phase variety, which also has weak anion exchange properties. Thus in addition to its absorption properties, it would also retain extraneous matrix components by an ionic mechanism.

This alternative approach, however, would require that the coffee extract be in the form of a relatively non-polar solution so that acrylamide is retained on the aminopropyl cartridge. We were aware from previous work that aqueous extracts can be expediently and efficiently dried by azeotropic distillation under reduced pressure with a mixture of acetonitrile and ethyl acetate. The other benefit of this solvent mixture is that it efficiently precipitates proteins. Recovery studies using analytical standards indicated that acrylamide did not co-distil with the solvent.

In the event, the addition of one part of an aqueous coffee extract to ten parts of the solvent mixture formed a precipitate, which was removed by centrifuging the mixture. Evaporation of the supernatant under reduced

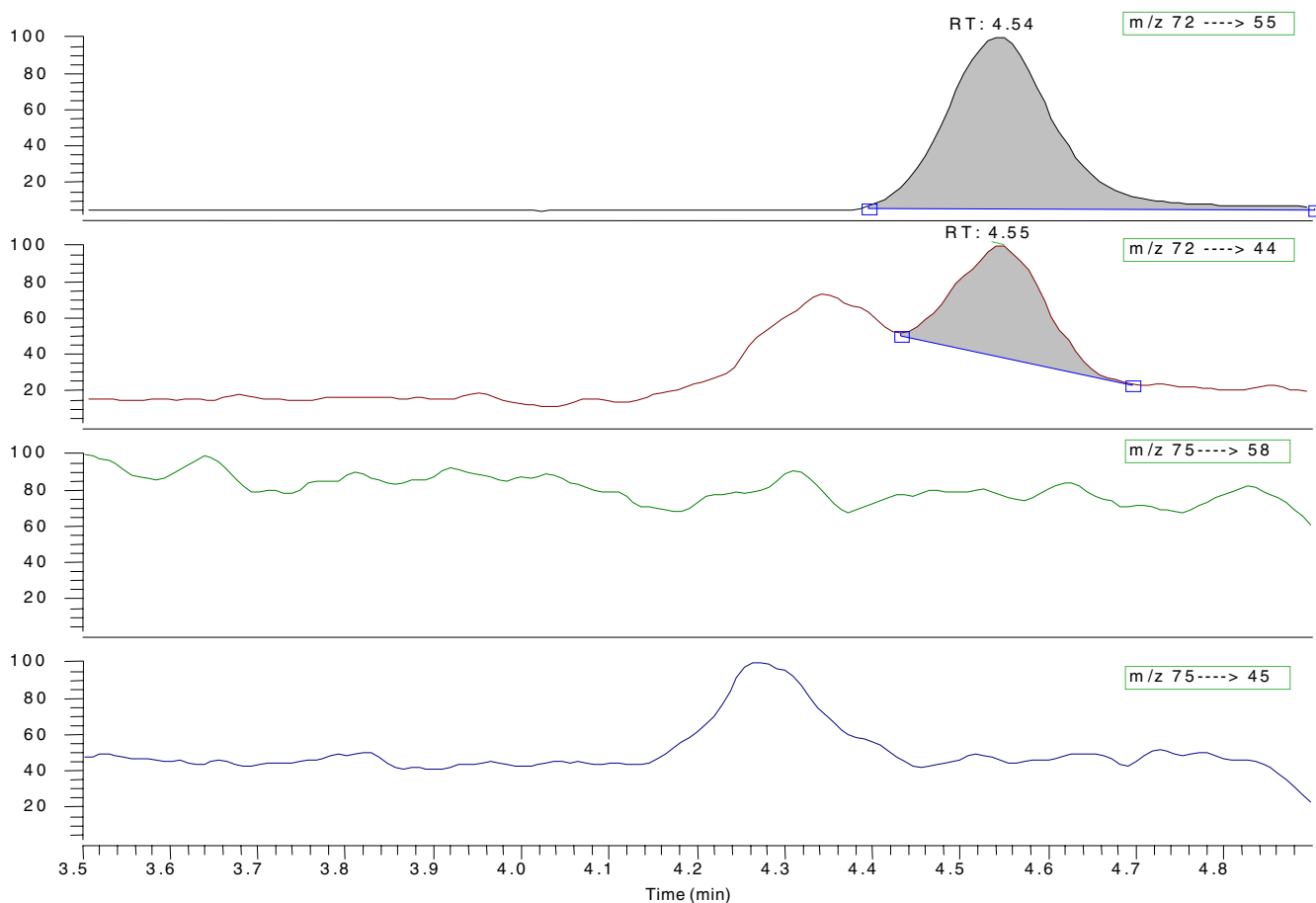


Fig. 2 Chromatograms of selected transitions used to monitor acrylamide and $^{13}\text{C}_3$ -acrylamide

Table 1 Results of the replicate ($n=5$) analysis of coffee samples and a cocoa sample

Average concentration ($\mu\text{g kg}^{-1}$)	Standard deviation	Coefficient of variation (%)
12.3 ^a	0.3	2.5
43.4	1.1	2.5
139.3	3.0	2.2
464.8	2.7	0.6

^aCocoa sample

pressure gave a dry tarry residue. Dissolution of this material in a solvent that retained acrylamide on the aminopropyl cartridge proved to be rather difficult. The addition of a small volume of isooctane to the supernatant before evaporation solved the problem by preventing the adhesion of the tarry residue to the wall of the pear-shaped flask. The concentrate obtained in this way readily dissolved in a solvent of an appropriate polarity to retain acrylamide on the aminopropyl cartridge.

With the analyte adsorbed on the cartridge, we experimented with various solvents to elute acrylamide from the cartridge. Not surprisingly, polar solvents such as acetone, diethyl ether and ethyl acetate quantitatively eluted the analyte from the cartridge. However, we found that elution with a 1:2 mixture of acetone/pentane gave a cleaner extract in that the solutions were colourless and, more importantly, the mass of the residue obtained after evaporation of the solvent was less than a third of that obtained using the polar solvents.

The chromatogram (Fig. 1) obtained from the LC-MS/MS analysis of the reconstituted residue revealed that the aminopropyl cartridge had, compared with a multi-mode non-polar, retained the extraneous polar matrix components in coffee, in that the early region of the chromatogram was not particularly remarkable. It was also evident from the response of the signal that this sample preparation technique provides a means of concentrating acrylamide. The bottom chromatogram shown in Fig. 1 was obtained from the analysis of a coffee sample that was contaminated with $43 \mu\text{g kg}^{-1}$ of acrylamide. The m/z $72 \rightarrow 44$ transition, which corresponds to the fragment $\text{O}=\text{C}=\text{NH}_2^+$ and has an abun-

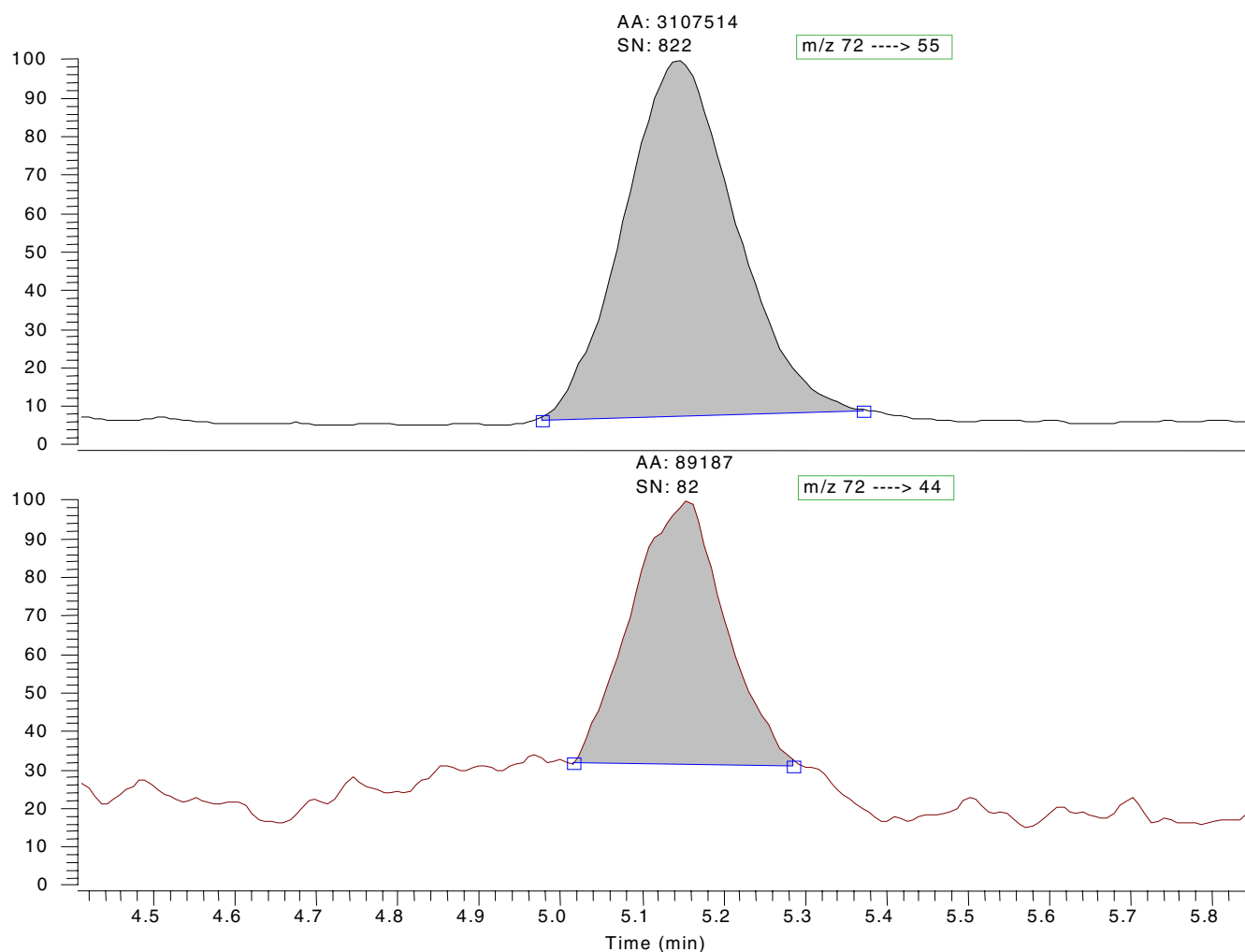
**Fig. 3** Chromatograms obtained from the analysis of a cocoa sample that was found to contain $12.3 \mu\text{g kg}^{-1}$ of acrylamide

Table 2 Summary of the reference material used to assess the accuracy of our method and the results of the analysis of this sample

Matrix	FAPAS code	Assigned value ($\mu\text{g kg}^{-1}$)	Acceptable range ($\mu\text{g kg}^{-1}$)	Found ($\mu\text{g/kg}$) [n=5,±SD]
Coffee	T3008	174	102–247	179±9

dance of 3% relative to the m/z 72→55 transition, was clearly discernible (Fig. 2). Although there was a partially co-eluting compound in some of the m/z 72→44 chromatograms (Fig. 2), the acrylamide response in this channel could still be used as a qualifier ion. Similarly, the closely eluting compound in some of the m/z 75→45 chromatograms did not prevent the $^{13}\text{C}_3$ -acrylamide signal in this channel from being used as a qualifier ion. The m/z 75→58 transition channel for the internal standard was free of interferences around the region where acrylamide elutes. In this respect, it is noteworthy that in all our survey work the relative abundances of the qualifier ions were within the 10% tolerance criteria we set as a quality control measure of selectivity.

Unfortunately, during our work with coffee we did not find a sample that was much below $45 \mu\text{g kg}^{-1}$ to test our expectation that the method could be used to measure low $\mu\text{g kg}^{-1}$ levels of acrylamide in coffee. To that end, we used a cocoa sample, which we knew from literature reports to contain low amounts of acrylamide, to demonstrate the precision of the method around its limit of quantitation. The results of the precision study are summarised in Table 1.

The analysis of the cocoa sample not only proved that the method could readily quantify acrylamide below a level of $12 \mu\text{g kg}^{-1}$, but also that it was capable of doing this with good precision. Furthermore, the response of the m/z 72→44 transition was clearly discernible in the chromatogram (Fig. 3). Also, its abundance, relative to that of the m/z 72→55 transition, was within our 10% tolerance quality control criteria. The signal-to-noise ratio of this qualifier ion (m/z 44) was 82:1 and that of the target ion (m/z 55) was 822:1. For comparative reasons, it is worth noting that Stadler and his co-workers [14] reported an “intermediate precision” (CV) of 22.8% for the analysis of a soluble chocolate powder that contained $12.7 \mu\text{g kg}^{-1}$ of acrylamide and a detection limit of $9.2 \mu\text{g kg}^{-1}$ for their method.

The need to assess the performance of analytical methods to measure acrylamide in foods has led to the

availability of commercial reference materials [15]. In this respect, the Central Sciences Laboratory, through its Food Analysis and Performance Assessment Scheme (FAPAS), provides several reference materials, including coffee, to assess the performance of analytical methods to measure acrylamide [16]. To that end, we obtained a FAPAS reference material (Table 2) to gauge the accuracy of our method. The average results of our analysis of these samples are shown in Table 2.

Conclusion

A selective and accurate method has been developed and partially validated for the analysis of coffee for acrylamide. The applicability of this method to the analysis of cocoa has also been demonstrated. The precision of the method and its ability to concentrate acrylamide are two particularly notable features of our method.

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