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Determination of acrylamide in coffee and chocolate by pressurised fluid extraction and liquid chromatography–tandem mass spectrometry

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Abstract
A selective and sensitive procedure has been developed and validated for the determination of acrylamide in difficult matrices, such as coffee and chocolate. The proposed method includes pressurised fluid extraction (PFE) with acetonitrile, florisil® clean-up purification inside the PFE extraction cell and detection by liquid chromatography (LC) coupled to atmospheric pressure ionisation in positive mode tandem mass spectrometry (APCI–MS–MS). Comparison of ionisation sources (atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionization (APPI) and the combined APCI/APPI) and clean-up procedures were carried out to improve the analytical signal. The main parameters affecting the performance of the different ionisation sources were previously optimised using statistical design of experiments (DOE). PFE parameters were also optimised by DOE. For quantitation, an isotope dilution approach was used. The limit of quantification (LOQ) of the method was 1 μg kg^{-1} for coffee and 0.6 μg kg^{-1} for chocolate. Recoveries ranged between 81–105% in coffee and 87–102% in chocolate. The accuracy was evaluated using a coffee reference test material FAPAS T3008. Using the optimised method, 20 coffee and 15 chocolate samples collected from Valencian (Spain) supermarkets, were investigated for acrylamide, yielding median levels of 146 μg kg^{-1} in coffee and 102 μg kg^{-1} in chocolate.

Keywords: Acrylamide, pressurised fluid extraction, PFE, LC–MS–MS, experimental design, optimisation, coffee, chocolate

Introduction

In April 2002, researchers at the Swedish National Food Administration announced that carbohydrate-rich foods, either are processed or cooked at high temperatures, contain relatively high levels of acrylamide (SNFA 2002). Subsequently, the risk assessment of acrylamide evaluated by the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of the European Union (EU) demonstrated that the exposure of humans to acrylamide should be kept as low as possible with regard to its inherent toxic properties (Friedman 2003). During the last few years, extensive data on levels of acrylamide have been collected by the European Commission (EC 2005) and by the US Food and Drug Administration (FDA 2003).

Several methods have been developed over the past decade to determine acrylamide in water, biological fluids and non-cooked foods, such as sugar, mushrooms and field crops (Tekel et al. 1989; Castle 1993; Bologna et al. 1999). The majority are classical methods based on HPLC or gas chromatographic (GC) techniques. Rosén and Hellenäs (2002) first reported on the confirmatory analysis of acrylamide in different heat-treated foods using isotope dilution liquid chromatography mass spectrometry (LC–MS). Recently, more selective analytical methods have been published, mainly based on MS as the determinative technique, coupled with a chromatographic step either by GC (Pittet et al. 2004) or LC (Riediker et al. 2003; Roach et al. 2003). It can be summarised from recent methodological studies that GC–MS and

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LC–MS–MS are recognised as the most useful and authoritative methods for acrylamide determination. Limits of quantification (LOQ) range from 30–50 $\mu g\,kg^{-1}$ for LC–MS–MS to 10–30 $\mu g\,kg^{-1}$ for GC–MS. There is no significant difference between results obtained by GC–MS with derivatization and LC–MS–MS (Klaffke et al. 2005; Owen et al. 2005), albeit the advantage of the LC–MS-based methods is that acrylamide can be analysed without prior derivatization, which significantly simplifies and accelerated the analysis. However, no uniform method applicable to all food matrices currently exists and methods must be adapted for the relevant matrices to achieve better limits of quantification and recoveries. Many of the methods do not perform well in difficult matrices, such as chocolate, coffee and high salt powders. This is mainly due to considerable loss of analyte throughout the sample preparation steps and to the multiple responses observed in each of the mass transitions at different retention times but close to that of acrylamide, which may cause interference (Delatour et al. 2004). Thus, the challenge is to develop sensitive, reliable and robust methods for these problematic matrices.

Among the ionisation sources, electrospray ionisation (ESI) is the technique commonly used in LC–MS owing to its wide range of applicability. Acrylamide is currently analysed by LC–MS using ESI in positive mode, taking advantage of the high response provided by the ionised amino groups (Riediker et al. 2003; Yusa et al. 2006). However, in some cases, APCI is the technique of choice since higher responses can be obtained for weak acid–base compounds by ionisation in the gas phase, as happens with acrylamide (Galceran 2005). Atmospheric pressure photoionization (APPI) interface was introduced a few years ago as an alternative for APCI in LC–MS (Marotta et al. 2003) but no reference to the use of this type of source for acrylamide has been found. Molecules showing an ionisation potential (IP) less than 10.6 eV, such as, acrylamide (IP 9.5 eV) are ionised and odd-electron positive ions $M^{\cdot+}$ are produced, while solvents, such as methanol (IP 10.85 eV), acetonitrile (IP 12.20 eV) and water (IP 12.62 eV), are not ionised by the photons. In particular, signal suppression by matrix compounds showing higher charge affinities than the target molecules is considered a major drawback of ESI and APCI. In general, signal-to-noise ratio (S/N) is improved using APPI with little ion suppression, allowing modest or no sample clean-up (Scherpenisse et al. 2004).

The extraction of acrylamide from foods products is usually performed by sonication (Hoenicke et al. 2004), dispersion (Riediker et al. 2003), shaking at high speed (Pittet et al. 2004), or in a rotating shaker (Roach et al. 2003) using water as an extractant. Pressurised fluid extraction is an automated technique that has been frequently used to extract lipophilic pollutants from environmental matrices and foods (Dean 1998). However, PFE has been rarely used for the extraction of hydrophilic compounds. The use of PFE for the extraction of acrylamide from food has been described by Cavalli et al. (2003) and, in a recent publication, Yusa et al. (2006) developed a sensitive PFE method for acrylamide analysis in cereal-based foods.

Most clean-up procedures consist of a combination of several solid-phase extractions (SPE) (Riediker et al. 2003; Roach et al. 2003). These procedures are laborious and time-consuming. Some researches have included a defatting step before or in combination with the extraction step (Ren et al. 2006). This involves extraction with hexane, petroleum ether or cyclohexane. Moreover, some protein-rich sample matrices need to have a deproteination step. This is carried out by adding methanol, acetonitrile or saline solution at a relatively high concentration. Hoenicke et al. (2004) and Senyuva and Gökmen (2005a) developed a protein precipitation step using Carrez I and Carrez II to obtain a clear solution without further clean-up. The use of water as extractant in Carrez-based methods makes the concentration of the extract difficult and, consequently, presents problems in lowering the LOD.

Although robust methods have been reported (Roach et al. 2003; Croft et al. 2004) that can achieve good sensitivity and selectivity of acrylamide, of all the relevant food matrices, chocolate and coffee products are problematic, and selected mass transitions reveal difficulty in obtaining baseline separation in certain profiles (Roach et al. 2003). In a recent validation of a LC–MS–MS method for acrylamide in different food groups, coffee was found to be one of the more difficult matrices to analyse as it requires frequent reconditioning of the HPLC column (Roach et al. 2003). Carbohydrate foods, such as chocolate, gave large matrix effects (Ahn et al. 2002). The conclusions of an inter-laboratory trial (Fauhl et al. 2002), various updated papers on acrylamide research activities from the AOAC (Eberhart et al. 2005; Lineback et al. 2005; Taeymans et al. 2005) and the EC JRC Expert Workshop (Report of the European Workshop on Analytical Methods for the Determination of Acrylamide in Food Products 2003), have shown that many of the methods do not perform well in difficult matrices, such as chocolate and coffee. Some previous studies (Andrzejewski et al. 2004; Delatour et al. 2004) have demonstrated the quantitative analysis of acrylamide in coffee and

corresponding foods. However, the loss of the analyte was >95% in these matrices and a significant ion-suppression effect led to a low response of acrylamide under positive electrospray ionisation conditions (Riediker and Stadler 2003). The problems with coffee (Senyuva and Gökmen 2005b) and chocolate (Ren et al. 2006) have been resolved but, in general, laboratories are continuously adapting their methods to increase automation and improve the sensitivity.

The aim of this study was to develop a selective and sensitive method using PFE and isotope dilution LC–MS–MS for acrylamide quantitation in coffee and chocolate samples. The selectivity was improved through the use of florisil® in the PFE extraction cell during the simultaneous extraction and clean-up step. The sensitivity of the method was improved through the optimization of PFE parameters and through choosing the best ionization source after comparing three ionization techniques (APCI, APPI and combined APCI/APPI). Both PFE conditions and ion source parameters were optimized using statistically designed experiments, such as Plackett–Burman designs and central composite designs (Massart et al. 1997), which optimize such analytical parameters much more efficiently and in less experimental runs than the conventional approach of changing-one-factor-at-a-time (COST).

Experimental

Standards and solvents

Acrylamide (>99%) and [$^{13}C_3$]acrylamide (99% isotopic purity) were obtained from Sigma (Diesenhofen, Germany) and Cambridge Isotope Laboratories Inc. (Andover, MA, USA), respectively. Acrylamide is stable in acid, unstable in base and light sensitive. Stock solutions were prepared by dilution in water and stored in capped amber vials at 4°C. Working standard solutions for the calibration curve were prepared by dilutions in water. Concentrations of the calibration standards were 0.1, 1, 10, 25, 50, 100, 250, 500, 1000 and 2000 ng ml^{-1}, all with [$^{13}C_3$]acrylamide at 75 ng ml^{-1}.

Neutral aluminum oxide and silica gel were supplied by Fluka (Buchs, Switzerland) and florisil® was from US Silica Company (Berkeley Springs, WV, USA).

Formic acid (analytical reagent grade) was purchased from Merck (Darmstadt, Germany); methanol (gradient HPLC grade) and acetonitrile (Multisolvent® HPLC, reagent grade) were supplied by Scharlau (Barcelona, Spain). Hyflo super Cell® diatomaceous earth was supplied by Sigma-Aldrich (Steinheim, Germany). Milli-Q water was from a Millipore purification system. Acrylamide reference test material FAPAS T3008 (coffee) was provided by CSL (Central Science Laboratory, York, UK).

Statistical data manipulation and numerical analysis of data resulting from experimental design were carried out by means of the statistical package MINITAB for Windows, Release 14 (Minitab Inc., Birmingham, UK).

Food samples

Retail sampling was undertaken over the period January–April 2006. Different chocolate and coffee samples were collected by public health inspectors from local supermarkets in Valencia (Spain) and stored at room temperature until analysis.

Sample preparation

Chocolate and coffee samples were pulverized and homogenized using a laboratory mill and passed through a 1 mm mesh sieve. Sub-samples of the homogenate were stored at 4°C in high-density polyethylene bottles with plastic screw-capped lids. For sampling, 1 g of the above-mentioned samples was weighed and spiked with [$^{13}C_3$]acrylamide as internal standard to achieve a final concentration of 75 ng g^{-1}. The sample was ground with diatomaceous earth (DE) with a pestle and mortar. To perform clean-up inside the PFE extraction cell, the bottom of the 22 ml stainless-steel extraction cells (containing a cellulose filter in the cell outlet) was loaded with around 10 g of florisil®. Then, the sample mixed with diatomaceous earth was placed into the cell. The sample cells were then closed to finger tightness and placed in the carousel of a PFE accelerated solvent extractor (ASE®) system 200 from Dionex (Sunnyvale, CA, USA).

The samples were extracted with acetonitrile using the following optimized conditions: temperature of 35°C with 5 min heat-up period under a pressure of 1500 psi and three static cycles with a static period of 4 min. The flush volume was 60% of the extraction cell volume. The sample cells were purged using pressurized nitrogen (125–150 psi) for 2 min. The extracts and suspension were centrifuged at 5000 rpm for 15 min at 5°C. Then, the precipitate was washed twice with acetonitrile and the clear supernatant was evaporated in a Turbo Vap 500 (Zymark, Idstein, Germany). Final extracts were reconstituted in 1 ml of water and filtered through a 0.22 μm nylon filter prior to LC–MS–MS analysis.

APCI–MS–MS analysis

A TSQ tandem mass spectrometer instrument, together a with Surveyor LC quaternary pump and

a Surveyor autosampler from Thermo Finnigan (San Jose, CA, USA), were used.

Analytical separation was carried out on a Atlantis C18 column, 150 × 2.1 mm I.D., 5 μm from Waters Corporation (Milford, MA, USA).

The mobile phase employed for the isocratic elution of the analyte was water at a flow-rate of 100 μl min^{-1}. The LC eluent was directed to the LC–MS interface within the retention time window 1.5–10 min using a diverter valve (Rheodyne, Cotati, USA). The total run time of the chromatograms was 10 min. An injection volume of 10 μl was used. The autosampler and column temperatures were 20 and 26°C, respectively.

Acrylamide was detected in positive mode. The monitored transitions for acrylamide were m/z 72 → 55 at 11 V and 72 → 44 at 24 V. For [$^{13}C_3$]acrylamide, the monitored transition was 75 → 58 at 11 V. Collision gas pressure was 1.5 mTorr. Tube lens offset voltages were optimised for acrylamide using the automated optimisation procedure in syringe infusion mode provided by the manufacturer.

The APCI optimised parameters were as follows: discharge current, 3 A; capillary temperature, 250°C; sheath gas pressure, 25 psi; auxiliary gas pressure, 1 arbitrary units (au) and vaporisation temperature, 250°C.

Quantitation and confirmation

For quantitation, an isotope dilution approach was used. Acrylamide in incurred samples was quantified using a linear calibration function, which was established with standard solutions of acrylamide dissolved in distilled water at concentration levels in the range 0.1–2000 ng ml^{-1}. Each solution contained 75 ng ml^{-1} of [$^{13}C_3$]acrylamide as internal standard. A daily response curve was plotted of the area response ratio for m/z 55/58 vs. amount of acrylamide injected with a constant amount of [$^{13}C_3$]acrylamide. Nine-point linear calibration plots with correlation coefficient (R^2) > 0.995 were used. Recovery correction was not applied to the results as the use of [$^{13}C_3$]acrylamide as internal standard made this correction unnecessary.

Confirmation of identity of the response is based on three criteria. First, the analyte ions m/z 55 and m/z 44 and the internal standard ion m/z 58 must occur at the same retention time. Second, the relative abundance of the analyte signals for m/z 55 and 44 must be within ±20% of the observed relative abundance recorded for an acrylamide standard of a similar concentration. Third, the three ions (m/z 55, 44, 58) must have a signal-to-noise (S/N) ratio >3.

Analytical quality assurance and safety

Each set of samples was analysed under quality-assurance protocols, including duplicate samples, reagent blanks and a quality control test materials (FAPAS coffee test material T3008).

The laboratory operates under the quality assurance system established by ISO/IEC/EN 17025.

Caution: Acrylamide monomer is toxic and can be adsorbed through the skin. Gloves and safety glasses should be worn during analysis and standards and sample preparation steps should be carried out in a fume cupboard.

Results and discussion

Optimisation of the ion source settings

Acrylamide has a weak acid–base character and requires a high water content in the mobile phase for the separation in reversed-phase liquid chromatography that makes the ionisation difficult in ESI. The suitability of APCI for acrylamide analysis has been reported previously. A response 15 time higher than ESI has been obtained using APCI (Galceran 2005), but no studies have been done comparing APCI with APPI or APCI/APPI combined. To compare the analytical responses obtained with these three interfaces, a preliminary optimisation of the individual ion source settings is required.

Screening design

Considering the literature and previous studies carried out in our laboratory (Pardo et al. 2006), five factors (k = 5) were selected as potentially affecting the APCI efficiency: capillary temperature (CT), vaporisation temperature (VT), auxiliary gas pressure (AGP), sheath gas pressure (SGP) and discharge current (DC). To optimise the APPI ion source settings, four factors (k = 4) were selected: CT, VT, AGP and SGP using a light source (a krypton lamp that emits photons with energies of 10.0 and 10.6 eV). APCI and APPI sources can be combined by turning on the corona discharge current and the light source. The same factors (k = 5) used to optimise the APCI efficiency were selected to optimise the combined APCI/APPI source.

The relative influence of these factors on the analytical response (arbitrary units of peak area of ions m/z 55 and 44) was studied with a Plackett–Burman (P–B) design (Massart et al. 1997). This screening design locates the parameters with the greatest influence in a reduced number of experiments. Althought P–B designs for studying five factors require as few as eight runs, we used

12 runs plus a triplicate centre point to have sufficient degrees of freedom for testing statistical significance.

The estimated effects of the factors and their statistical significance at 95% confidence level ($\alpha < 0.05$) for responses m/z 55 and m/z 44 are shown in Table I. As can be seen, only vaporisation temperature, capillary temperature and sheath gas had a significant effect on the m/z 55 and m/z 44 responses ($P < 0.05$) using APCI, APPI or combined APPI/APCI sources. As a consequence, these factors were selected for further optimisation. For the next stage in the optimisation process, the non-significant factors: discharge current and auxiliary gas pressure were fixed at 3 A (negative effect) and 1 au (negative effect), respectively. The discharge current using APPI was turned off.

Table I. Estimated effects* and P values obtained from a Plackett–Burman design† used in the optimisation of APCI, APPI and APCI/APPI combined.

	m/z 55		m/z 44	
Factor	Effect	p	Effect	p
APCI				
Capillary temperature (V)	−1 0748 974	0.038	−420 278	0.020
Sheath gas pressure (psi)	−14 513 537	0.010	−532 181	0.006
Auxiliary gas pressure (au)	−68 309	0.988	−43 104	0.773
Vaporisation temperature (°C)	−16 308 526	0.005	−634 432	0.002
Discharge current (A)	−2 362 584	0.600	−114 582	0.451
APPI				
Capillary temperature (V)	−294 458	0.004	−925 038	0.001
Sheath gas pressure (psi)	−215 364	0.021	−698 163	0.005
Auxiliary gas pressure (au)	−56 425	0.475	−408 298	0.058
Vaporisation temperature (°C)	−337 559	0.002	−1 368 591	0.000
APPI/APCI				
Capillary temperature (V)	−25 171 861	0.035	−972 783	0.032
Sheath gas pressure (psi)	−29 857 956	0.017	−1 080 272	0.021
Auxiliary gas pressure (au)	−2 420 562	0.814	−95 316	0.807
Vaporisation temperature (°C)	−59 060 715	0.000	−2 206 099	0.000
Discharge current (A)	−8 236 912	0.432	−3 287 85	0.408

*Coded units; $\alpha = 0.05$.
†A solution containing 50 ng ml^{-1} of acrylamide and 75 ng ml^{-1} of [$^{13}C_3$]acrylamide was used.
The minimum, central and maximum values used in the P–B design were: VT (250–375–500°C); CT (150–225–300°C); SGP (25–43–60 psi); AGP (0–3–5 au); DC (3–4–5 A).

Central composite design

To obtain a more accurate optimisation of the significant source parameters (vaporization temperature, capillary temperature and sheath gas pressure), a central composite design (CCD) was used (Massart et al. 1997). This type of experimental design permits the response surface to be built and the factor settings or operating conditions that maximise acrylamide response to be found.

The CCD consisted of a full factorial 2^3 design (eight hypercube points), six axial points and six central points in cube. The 20 runs were randomized to provide protection against the effect of hidden variables. The minimum, central and maximum values used in the CCD were: VT (375–463–550°C); CT (150–250–350°C); SGP (25–43–60 psi).

The responses were fitted by a multiple regression equation, including second-order (curvature) and interaction terms. The model was validated using regression ANOVA. The next step was to select the factor settings that maximized the acrylamide response. This could be done by the ''response optimizer'' from the response surface design in the MINITAB program.

The response surfaces were similar for the two analytical signals (m/z 55, 44). Therefore, there was a factor setting to simultaneously maximise the desirability for each response (the desirability is 0.0 for the lowest values obtained in the CCD, increases as response values increase and is 1.0 for the highest response obtained in the experiments). We maximised a composite desirability, which combines the individual desirability of each response into a single measure. The optimised factor settings were: Vaporisation temperature 250°C, sheath gas pressure 25 psi and capillary temperature 350°C for APCI; vaporisation temperature 500°C, sheath gas pressure 25 psi and capillary temperature 150°C for APPI; and vaporisation temperature 450°C, sheath gas pressure 25 psi and capillary temperature 150°C for combined APCI/APPI. These conditions provide a composite desirability of 0.94, 0.96 and 0.93, respectively.

Comparison of the effect of ionisation sources on the analytical response

To compare the analytical responses obtained with the different ionisation sources, a 100 ng ml^{-1} acrylamide standard was injected using APCI, APPI and APCI/APPI combined. The analytical schedule was 3 days with five replicates each day.

Statistical analysis of the obtained data was carried out using a two-sample t-test approach at 95% confidence level (Gardiner 1997). Similar responses to APCI were obtained using APPI or APCI/APPI

combined without significant differences compared with APCI.

The second step was to compare the analytical responses of the three sources with a coffee and a dark chocolate sample. Chromatographic analysis showed similar profiles. Finally, APCI was selected because no significant signal-to-noise improvement was observed using APPI or APCI/APPI. Furthermore, the number of instruments containing APPI sources are limited at the moment.

Optimisation of PFE conditions

The effect of different solvents in the pressurised liquid extraction of acrylamide was reported by Yusà et al. (2006). Acetonitrile appeared to be a suitable solvent to extract acrylamide from the aqueous mixture; extracts produced were ''clear'' and higher signal responses were obtained due to less ion suppression effects. Acetonitrile precipitates proteins and other high molecular compounds; therefore, Carrez precipitation is not necessary.

The main parameters influencing the performance of the PFE are temperature, pressure and extraction time (Fizpatrick et al. 2000). A CCD design was chosen to study the relative influence of these three parameters on the recovery of acrylamide and was carried out with spiked coffee ($50\,\mu g\,kg^{-1}$). The design matrix and the analytical response are shown in Table II.

The responses were fitted by a multiple regression equation, including second-order (curvature) and interaction terms. Three-dimensional response surfaces display the effect of the two independent variables on the analytical response (arbitrary units of peak area of ions m/z 55, m/z 44). The ''response optimizer'' from the response surface design in the MINITAB program showed similar response surfaces for the two analytical signals and, therefore, there was a factor setting to simultaneously maximise the desirability for each response. Maximising the composite desirability, which combines the individual desirability of each response into a single measure, the optimised factor settings were: temperature 110°C, pressure 1500 psi and static time 2 min, with a composite desirability of 0.99. Figure 1 shows the m/z 55 three-dimensional response surface yielded by the model for temperature and static time, at a constant value of the pressure.

Clean-up

High background levels or interfering co-extractives, undergoing the same fragmentation reaction, were observed, especially for the SRM trace m/z $72 \rightarrow 55$, when analysing complex matrices, such as soluble coffee or cocoa powder. The challenge in this study was, therefore, to improve the acrylamide analytical signal in chocolate and coffee samples throughout an exhaustive sample clean-up and minimise interferences.

A method reported by Roach et al. (2002), employing water as extractant and a two-step SPE clean-up, was tested for coffee and chocolate, but, as shown in Figure 2(a), some co-extractives interfered with the acrylamide analytical signal, complicating correct quantitation. The method developed by Yusà et al. (2006) presented the same problem for these complex matrices (Figure 2b).

Table II. Pressurised liquid extraction conditions and analytical responses used for central composite design optimisation of the three significant factors in the analysis of acrylamide.*

	Factors			Analytical response	
Run	Temperature (°C)	Pressure (psi)	Static time (min)	Area (au)† m/z 44	m/z 55
1	58	1304	8	58 105	2 522 295
2	78	1750	6	28 696	735 152
3	78	1750	6	33 683	918 848
4	97	1304	8	43 634	1 398 094
5	97	2196	8	18 454	658 926
6	78	1000	6	23 868	810 907
7	110	1750	6	61 118	1 125 295
8	58	2196	8	41 325	984 198
9	78	1750	6	11 292	290 774
10	58	1304	4	9 430	245 312
11	78	2500	6	24 517	523 851
12	97	2196	4	21 978	639 182
13	45	1750	6	6 378	189 137
14	97	1304	4	19 765	532 499
15	78	1750	10	24 795	711 451
16	78	1750	2	24 612	872 125
17	78	1750	6	21 194	485 126
18	78	1750	6	2 766	310 507
19	78	1750	6	31 198	906 913
20	58	2196	4	19 821	610 750

*A spiked coffee at $50\,ng\,ml^{-1}$ of acrylamide and $75\,ng\,ml^{-1}$ of [$^{13}C_3$]acrylamide was used
†au: analytical response in arbitrary units.

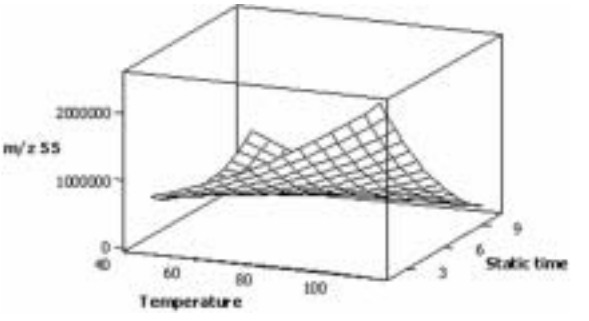

Figure 1. Response surface for acrylamide (m/z 55) obtained in the optimization of ASE parameters using a central composite design. Static time versus temperature. Hold value: pressure 1500 psi.

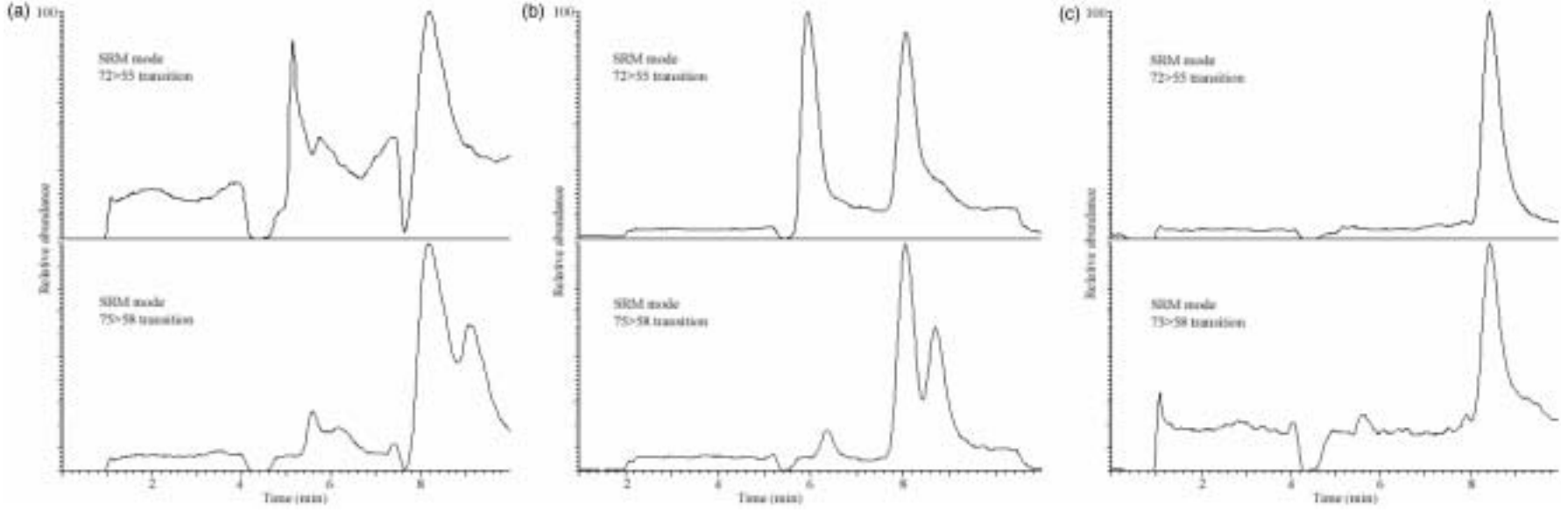

Figure 2. Chromatogram of a coffee reference test material containing acrylamide at 174 $\mu g\,kg^{-1}$. (a) Conventional extraction and SPE purification method. (b) PFE method without SPE purification. (c) Purification with florisil inside the PFE extraction cell.

To study clean-up inside the extraction cell, some different adsorbents were introduced in the pressurised liquid extraction cell: florisil®, silicagel and neutral aluminum oxide were tested.

The study was carried out with spiked coffee ($50\,\mu g\,kg^{-1}$) and five replicates of each adsorbent were prepared by introducing ~10 g inside the PFE extraction cell. The three adsorbents tested gave similar recoveries without significant differences, but florisil® completely minimized the interfering co-extractives and signal suppression disappeared (Figure 2c). Furthermore, the use of florisil® avoids the defatting step normally carried out by partitioning with hexane, petroleum ether or cyclohexane. Similar results were achieved with chocolate matrices.

Method performance

The analytical method was validated for coffee using a reference test material containing acrylamide at $174\,\mu g\,kg^{-1}$. For chocolate, a spiked dark chocolate ($200\,\mu g\,kg^{-1}$) was used. The corresponding analytical key parameters are listed in Table III. The linearity of the mass spectrometer was checked by analysing a set of standard solutions at 10 levels of concentration from 0.1 to $2000\,ng\,ml^{-1}$. The linear model was statistically validated taking into account that residual values were randomly distributed about the regression line, the P-value of the F-test statistic was <0.05 and the coefficient of determination (R^2) was better than 0.999 (Gardiner 1997; Eurachem Guide 2000).

The limit of detection (LOD) and the limit of quantification (LOQ) for acrylamide in coffee and chocolate were extrapolated from the signal-to-noise (S/N) ratios obtained for the response in the SRM trace m/z 72 → 55. The LOD (S/N = 3, five replicates) was $0.3\,\mu g\,kg^{-1}$ for coffee and $0.2\,\mu g\,kg^{-1}$ for chocolate. The LOQ (S/N = 10, five replicates) was estimated at $1\,\mu g\,kg^{-1}$ for coffee and $0.6\,\mu g\,kg^{-1}$ for chocolate. Figure 3 shows chromatograms of a dark chocolate in powder and a coffee samples spiked at LOQ levels.

Table III. Analytical features for the determination of acrylamide in chocolate and coffee samples by PFE and LC–APCI–MS–MS using dark chocolate spiked at $200\,\mu g\,kg^{-1}$ and a coffee reference test material containing acrylamide at $174\,\mu g\,kg^{-1}$.

	Chocolate	Coffee
Limit of detection* ($\mu g\,kg^{-1}$)	0.2	0.3
Limit of quantification* ($\mu g\,kg^{-1}$)	0.6	1
Intra-assay RSD† (%)	2	3
Inter-assay RSD† (%)	4	5
Recovery ± SD† (%)	96 ± 4	95 ± 5

*Five replicates; SRM transition m/z 72 → 55.
†Validation schedule: 3 days, five replicates each day.

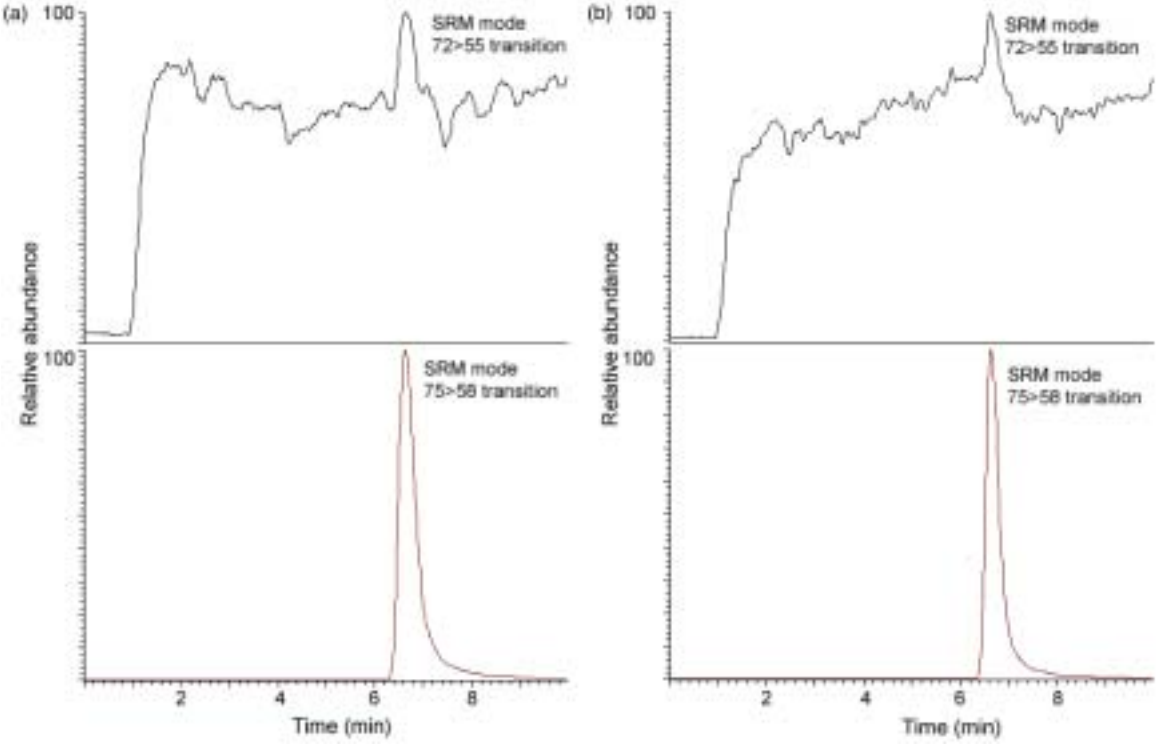

Figure 3. Chromatograms of spiked samples at LOQ levels. (a) Coffee at $1\,\mu g\,kg^{-1}$. (b) Dark chocolate in powder at $0.6\,\mu g\,kg^{-1}$.

The intra- and inter-assay relative standard deviations (RSDs) were determined using the coffee reference material and the spiked dark chocolate.

Survey data

In this study, 20 coffee and 15 chocolate samples were analysed for their acrylamide content. Different coffee and chocolate samples were selected from local supermarkets in Valencia during January–April 2006. Product sampling was not representative of all brands. The concentration of acrylamide in coffee and chocolate samples are shown in Table IV. Quantitative analysis of real samples showed concentration ranges of 14–345 $\mu g\,kg^{-1}$ for chocolates and 62–287 $\mu g\,kg^{-1}$ for coffees, with median levels of 102 and 146 $\mu g\,kg^{-1}$ in chocolate and coffee, respectively.

The levels of acrylamide found in this study are similar to those summarised by Ren et al. (2006) and Andrzejewski et al. (2004).

Conclusions

This work describes a sensitive and confirmatory method for the determination of acrylamide in problematic matrices, such as coffee and chocolate, and its suitability for monitoring the presence of this substance in foods. The PFE with purification inside the extraction cell provides an automated and rapid procedure for analysis of acrylamide in different commodities allowing the analysis of large numbers of samples with minimal labour. The use of acetonitrile as extraction solvent precipitates proteins and other high molecular co-extractives; therefore ''clear'' extracts and higher signal responses were obtained due to less ion suppression effects. The LC–MS–MS method using atmospheric pressure ionisation and florisil® inside the PFE extraction cell minimise interference in coffee and chocolate samples. The estimated LODs were 0.3 $\mu g\,kg^{-1}$ for coffee and 0.2 $\mu g\,kg^{-1}$ for chocolate, and the LOQs were estimated at 1 and 0.6 $\mu g\,kg^{-1}$, respectively. The method has been validated with coffee reference material and with a spiked dark chocolate, yielding recoveries ranging 81–105% for coffee and 87–102% for chocolate.

Using the optimized method, a survey of acrylamide levels in 20 coffee samples and 15 chocolate samples was carried out. Acrylamide median level in chocolate was 102 $\mu g\,kg^{-1}$ and 146 $\mu g\,kg^{-1}$ in coffee. The levels of acrylamide in Spanish coffee and chocolate were similar to those reported by other authors.

Table IV. Acrylamide contents ($\mu g\,kg^{-1}$) in coffee and chocolate products from the Spanish market.

Coffee type	$\mu g\,kg^{-1}$	Chocolate type	$\mu g\,kg^{-1}$
Classic roast	220	Chocolate wheat	108
Coffee mixes	153	Chocolate wheat	143
Coffee mixes	139	Chocolate wheat	<LQ
Classic roast	157	Chocolate wheat	345
Classic roast	259	Milk chocolate	15
Classic roast	261	Chocolate in powder	<LQ
Coffee mixes	199	Chocolate in powder	<LQ
Coffee mixes	134	Milk chocolate	19
Classic roast	130	Dark chocolate	18
Classic roast	120	Chocolate with almonds	51
Coffee mixes	253	Dark chocolate	102
Coffee mixes	75	Milk chocolate	49
Classic roast	105	Chocolate in powder	67
Colombia	62	Chocolate in powder	149
Classic roast	143	Chocolate in powder	297
Classic roast	149	Chocolate in powder	205
Classic roast	67		
Classic roast	98		
Coffee mixes	210		
Decaffeinated	385		

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