Quality Assurance and Safety of Crops & Foods, 2019; 11 (5): 411-419

Wageningen Academic Publishers

Analysis of acrylamide from potato chips using an amino column followed by PDA as the detection system in HPLC

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Received: 8 September 2018 / Accepted: 9 January 2019

RESEARCH ARTICLE

Abstract

This study aimed at developing a new method for measuring acrylamide, the toxic compound formed during the thermal processing of carbohydrate-rich food components, using high performance liquid chromatography coupled to a photodiode array detector (HPLC-PDA) equipped with an amino column. The results of the initial tests showed that using methanol as the extraction solvent, defatting the samples with *n*-hexane, conducting a pre-concentration factor of 5 (by evaporation) and ultrasound treatment for 8 min were optimal conditions for acrylamide extraction from potato chips. The calibration curve was plotted to measure acrylamide with the proposed amino column and to investigate the accuracy of the results by the optimised method, which was linear in the range of 100 to 5,000 µg/l (R^2=0.997) and also the recovery ratios were in the range of 95.9-102.2%. The results of method validation indicated appropriate repeatability of the experimental method (RSD: 4.39%). Limit of detection (LOD) and limit of quantification (LOQ) were found at 9 and 27 µg/kg levels, respectively. Use of the current amino column along with the optimisation of the extraction process resulted in an easy, affordable and reliable method for measuring acrylamide in potato chips by HPLC-PDA.

Keywords: acrylamide, potato chips, amino column, taguchi design, liquid chromatography-photodiode array detector

1. Introduction

Acrylamide is a highly polar compound with a low molecular weight (71.079 g/mol) that is formed during thermal processing (above 120 °C) of foods rich in carbohydrates with moderate levels of protein (Bedia-Erim and Tezcan, 2008; Bent *et al.,* 2012; Chang *et al.,* 2007). Unfortunately, based on numerous studies, acrylamide has been identified to be neurotoxic, genotoxic and also carcinogenic (Bent *et al.,* 2012; Bermudo *et al.,* 2007; Feng *et al.,* 2013; Ren *et al.,* 2007). The main acrylamide formation mechanism is through the Maillard reaction between asparagine and glucose or any other reducing sugar (Bent *et al.,* 2012; Chang *et al.,* 2007; Dong *et al.,* 2006; Radecka *et al.,* 2007). Given the importance of measuring acrylamide in the food products, chromatographic techniques have been considered as the main methods dealing with its determination (Chang *et al.,* 2007; Fernandes and Soares, 2007; Gokmen *et al.,* 2005; Morales and Rufian, 2006). The necessity for the derivatisation by bromination to increase the molecular weight and decrease the polarity of acrylamide leads to prolonging the procedure required by gas chromatography (GC) method (Bent *et al.,* 2012; Dong *et al.,* 2006; Ren *et al.,* 2007). Fortunately, no need for a derivatisation step with liquid chromatography (LC) has resulted in the simplification of the process (Chen *et al.,* 2008b; Kontominas and Paleologos, 2005; Morales and Rufian, 2006). However, deionised water, which is normally used as the extraction solvent (Bent *et al.,* 2012; Bermudo *et al.,* 2007; Chang *et al.,* 2007) may also extract other water-soluble compounds such as mono-, di- and poly-saccharides, proteins and amino acids as well as organic acids (Fernandes and Soares, 2007; Gokmen *et al.,* 2005). Therefore, to decrease the risk of interference of sample matrix with liquid chromatography, many studies have applied a solid-phase extraction (SPE) clean-up step using SPE cartridges, which complicated the process and resulted in unnecessary longer experiments (Chen *et al.,* 2008b;

Choi *et al.,* 2013; Lehotay and Mastovska, 2006). In most reported studies, the mass spectrometry (MS) detector was used together with gas or liquid chromatography methods (Arisseto *et al.,* 2006; Bermudo *et al.,* 2006b; Fernandes and Soares, 2007; Lehotay and Mastovska, 2006) to determine acrylamide in different samples. Despite the sensitivity and accuracy of the results obtained by MS detector, it is very expensive and not always available in some analytical laboratories. Therefore, due to the availability of a UV-Vis/photodiode array detector (PDA) detector in most analytical laboratories, it is an advantage to assess the possibility of such detector for acrylamide analysis (Chen *et al.,* 2008a; Choi *et al.,* 2013; Dong *et al.,* 2006). On the other hand, chromatographic columns used together with HPLC are often based on octadecyl-silica (ODS-C_{18}), hydrophilic-interaction or ion exclusion principles. However, since acrylamide is a very polar compound with poor retention and separation property on the conventional liquid chromatographic columns, in most studies, SPE clean-up steps were also applied in the procedure (Bermudo *et al.,* 2006a; Chen *et al.,* 2008b; Choi *et al.,* 2013; Gokmen *et al.,* 2005; Morales and Rufian, 2006). However, considering the chemical similarities between sugars and acrylamide especially in the polarity and water-solubility, applying the amino columns, as reported for the separation of mono- and oligo-saccharides, has been neglected (Acworth *et al.,* 2011b). The Asahipak NH2P-50 4E is a new generation of amino columns with polyamine active groups attached to a hard hydrophilic polymer gel called polyvinyl alcohol gel (instead of the traditional silica gel) as a basic compound (Showa-Denko *et al.,* 2013). Unlike other silica-based columns, this amino column also has a high chemical stability, which solves the problem with the decrease in the power of retention and separation that occurred over time in the previous columns (Acworth *et al.,* 2011a; Showa-Denko *et al.,* 2013). Therefore, considering the above matters, the objective of the current study was to optimise the sample preparation and extraction process of acrylamide applying the Taguchi's experimental design along with the one-variable-at-a-time approach to determine acrylamide using a simple, reliable, and cost-effective method in potato chips using liquid chromatography equipped with an NH2P-50 4E amino column coupled with a PDA detector without conducting the time-consuming and costly SPE clean-up steps. On this aspect, extraction solvent, defatting solvent, sonication time and pre-concentration factor were used as the main operational conditions to be optimised.

2. Materials and methods

Materials

All the samples of potato chips that were considered as the sources of acrylamides were purchased from several most-known brands available in the retail market in Tehran, Iran. Electrophoresis-grade acrylamide (99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All HPLC-grade solvents (methanol, acetonitrile, acetone, and deionised water) were purchased from Daejung Chemicals and Metals (Gynogyi-do, Korea). Other chemical compounds including potassium hexacyanoferrate trihydrate, zinc sulphate heptahydrate, dichloromethane and *n*-hexane were of laboratory grade and were obtained from Merck KGaA (Darmstadt, Germany). Polypropylene falcon tubes (25 and 50 ml in volume) were purchased from Isolab Laborgerate (Wertheim, Germany). Cellulose acetate syringe filters (0.22 µm) were obtained from CNW Technologies (Dusseldorf, Germany) and glass vials with screw caps were purchased from Agilent Technologies (Wilmington, DE, USA). Chromabond C18ec (3 ml/500 mg) SPE cartridges were purchased from Macherey-Nagel (Duren, Germany) and primary secondary amine (PSA) sorbent powder for dispersive SPE was purchased from Varian (Harbor City, BC, Canada).

Standard solutions and reagents

Acrylamide stock solution with a concentration of 1000 mg/l was prepared through dissolving 100 mg of acrylamide in 100 ml of deionised water. Afterwards, acrylamide standard solutions (at 100, 500, 1,500, 2,500, and 5,000 µg/l concentrations) were prepared by diluting the stock solution with deionised water in glass vials and stored in a refrigerator at 4 °C until used in the analysis. Moreover, the Carrez I solution was made from dissolving 7.5 g of potassium hexacyanoferrate trihydrate in 50 ml of deionised water and the Carrez II solution was made by dissolving 15 g of zinc sulphate heptahydrate in 50 ml deionised water.

Method development

The Taguchi's experimental design (Table 1) was used to investigate the effects of four different parameters including the effects of extraction solvent, defatting stage and type of solvent, pre-concentration by drying the solution at different ratios and treatment with ultrasound waves at specified times. Such approach has successfully been applied for designing numerous studies in the food area (Abbasi *et al.,* 2008; Ghasemian *et al.,* 2014; Kazazi and Rezaei, 2009). All samples of potato chips were spiked at the level of 1,200 µg/kg by the acrylamide standard solution and conducting nine different series of experiments based on Taguchi's experimental design (Table 1). The one-variable-at-a-time approach was conducted to examine the effects of SPE clean-up on the optimisation of the sample preparation and extraction method. To this purpose, according to the results obtained from the Taguchi's experimental design, the proper extraction method was selected to evaluate the effect of SPE clean-up in three levels: without conducting SPE clean-up steps, conducting clean-up step with Chromabond C18ec SPE cartridge and conducting clean-up step by

Table 1. Taguchi's experimental design using four variables at three levels each applied in this study.

Run no.	Extraction solvent	Defatting solvent	Pre-concentration factor (times)	Ultrasound treatment (min)
1	methanol	no defatting	0	0
2	methanol	dichloromethane	3	4
3	methanol	*n*-hexane	5	8
4	acetonitrile	no defatting	3	8
5	acetonitrile	dichloromethane	5	0
6	acetonitrile	*n*-hexane	0	4
7	acetone	no defatting	5	4
8	acetone	dichloromethane	0	8
9	acetone	*n*-hexane	3	0

dispersive solid phase extraction with PSA. Each of these treatments was carried out in duplicates. Thereafter, the samples were injected into the HPLC-PDA system and peak areas related to acrylamide were calculated for the injected samples. The optimised method for the preparation and extraction of acrylamide in potato chips was chosen by evaluating the relative peak areas obtained by the methods applied.

Sample preparation

The general procedure for the extraction of acrylamide, described in previous literature (Gokmen *et al.,* 2005) was applied for the preparation of the samples. Briefly, samples of potato chips were completely milled using a mixer (Moulinex, Ecully, France) and 2.00 g of the milled samples were placed in centrifuge tubes and, to conduct the recovery test, they were spiked with certain amounts of acrylamide standard solutions. Afterwards, 10 ml methanol was added to each centrifuge tube as solvent and the tube was shaken for 3 min on a vortex and then sonicated in an ultrasound bath for 8 min followed by 20 min additional shake and centrifugation for 15 min at 1,900×*g* (PE Co. Centrifuge, Novin Tashkhis, Shiraz, Iran). Afterwards, 6 ml of the upper transparent layer was taken and 240 µl Carrez I and 240 µl Carrez II solutions were added (for protein precipitation purposes) and centrifuged. To perform the pre-concentration step, 5 ml of the upper transparent layer was dried at 60 °C in a water bath equipped with a nitrogen flow for 1 h. Then, 1 ml deionised water was added as the final extraction solvent and 1 ml *n*-hexane was added to the sample for defatting and the sample was shaken for 3 min on a vortex. After this stage, the final filtration was conducted using a 0.22 µm syringe filter and the sample was prepared for injection into the liquid chromatography system. To carry out SPE clean-up with Chromabond C18ec (3 ml/500 mg), initially the cartridge was conditioned using 3 ml methanol and then 3 ml deionised water at a rate of two drops per second. Then, 1.00 ml of the extracted solution was passed through the cartridge at a rate of one drop per second and the cleaned solution was collected into the glass vial. To conduct dispersive-SPE clean-up, initially the PSA sorbent powder was washed using 1.0 ml methanol and then 1.0 ml deionised water and dried under the fume hood. Fifty mg of PSA was poured into a 2.0 ml mini-falcon and then 1.00 ml of the extracted solution was added. After shaking the sample, it was centrifuged for 15 min at 12,000×*g* (Wise Spin CF-10, Daihan Scientific, Seoul, South Korea). Then, the transparent solution was collected into a glass vial for future injection in the HPLC system.

Sample analysis with HPLC-PDA

The samples were analysed for their acrylamide contents on a Waters 717 liquid chromatography system (Waters Corporation, Milford, MA, USA) equipped with a binary pump, a vacuum degasser and an autosampler coupled to a Waters 996 photodiode array detector (Waters Corporation). Applying PDA detector in this research was due to its availability in our laboratory. The reversed-phase chromatographic separations were performed using an Asahipak NH2P-50 4E amino column (250×4.6 mm × 5 µm; Shodex Group, Kawasaki, Japan). An isocrtic elution using a mixture of deionised water and acetonitrile at 94.4:5.6 ratio (v/v) and a flow rate of 0.5 ml/min was applied during the HPLC analysis. The column temperature was set at 25 °C and the detection was performed at 200 nm. The injection volume was 50 µl.

Method validation

A 5-point calibration curve was plotted and applied for the quantification of acrylamide in the unknown samples of potato chips. Afterwards, the regression equation (y=ax+b) was obtained in the linear range of the calibration curve and the slope of the curve (a) and the intercept (b) were obtained. After injecting the unknown samples of potato chips containing acrylamide, peak areas (y) from the chromatographic data were applied in the regression equation to determine the acrylamide concentrations (x). To determine the recovery ratio, the samples of potato chips were spiked at 0, 500, 1,200 and 2,400 µg/kg for two times (n=2) using the acrylamide standard solutions and the final concentration was determined by applying the pre-concentration factor and subtracting acrylamide concentration in the sample blank. Then, the recovery ratio was obtained by dividing final concentration on the spiked concentration. To validate and investigate the accuracy of the results, the inter-day repeatability test was conducted using the samples of potato chips spiked with 1,200 µg/kg acrylamide during a 30-d period (with 6-d time intervals) and RSD was determined based on the peak areas obtained. To determine the limit of detection (LOD) and limit of

quantification (LOQ), the samples of potato chips were spiked with 0, 50, 100, and 250 µg/kg acrylamide solutions (n=2). Then, the calibration curve was plotted. The LOD and LOQ were determined according to the following equations:

$$LOD = 3.3 \times \frac{s}{S} \quad (1)$$

$$LOQ = 10 \times \frac{s}{S} \quad (2)$$

where s is the standard deviation of the intercepts and S is the slope of the calibration curve (Fernandes and Soares, 2007; Kim *et al.,* 2013).

Statistical analysis

All the analytical experiments on spiked potato chips were carried out in duplicate and the mean values of the results as well as their standard deviations were calculated. For investigating the Taguchi's statistical analysis, the Analysis of variance (ANOVA) for each of four variables were conducted applying Minitab version 15 Statistical Software (Minitab Inc., State College, PA, USA) to evaluate the significant statistical differences among the results (at $P<0.05$ level).

3. Results and discussion

Calibration curve of acrylamide standard solutions

The acrylamide peak was identified at 7.7 min (Figure 1A). The standard calibration curve was linear in the concentration range of 100 to 5,000 µg/l with a regression equation of Y = 893.25X – 11,154 and the correlation coefficient (R^2) of 0.997 (Figure 1B). Therefore, this range was considered as the studied linear range and the above regression equation from the calibration curve was applied for the quantification of acrylamide in the unknown samples of potato chips. Furthermore, considering the UV spectrum of 1 µg/ml acrylamide standard solution, it was well shown that acrylamide has the highest absorption at 200-210 nm.

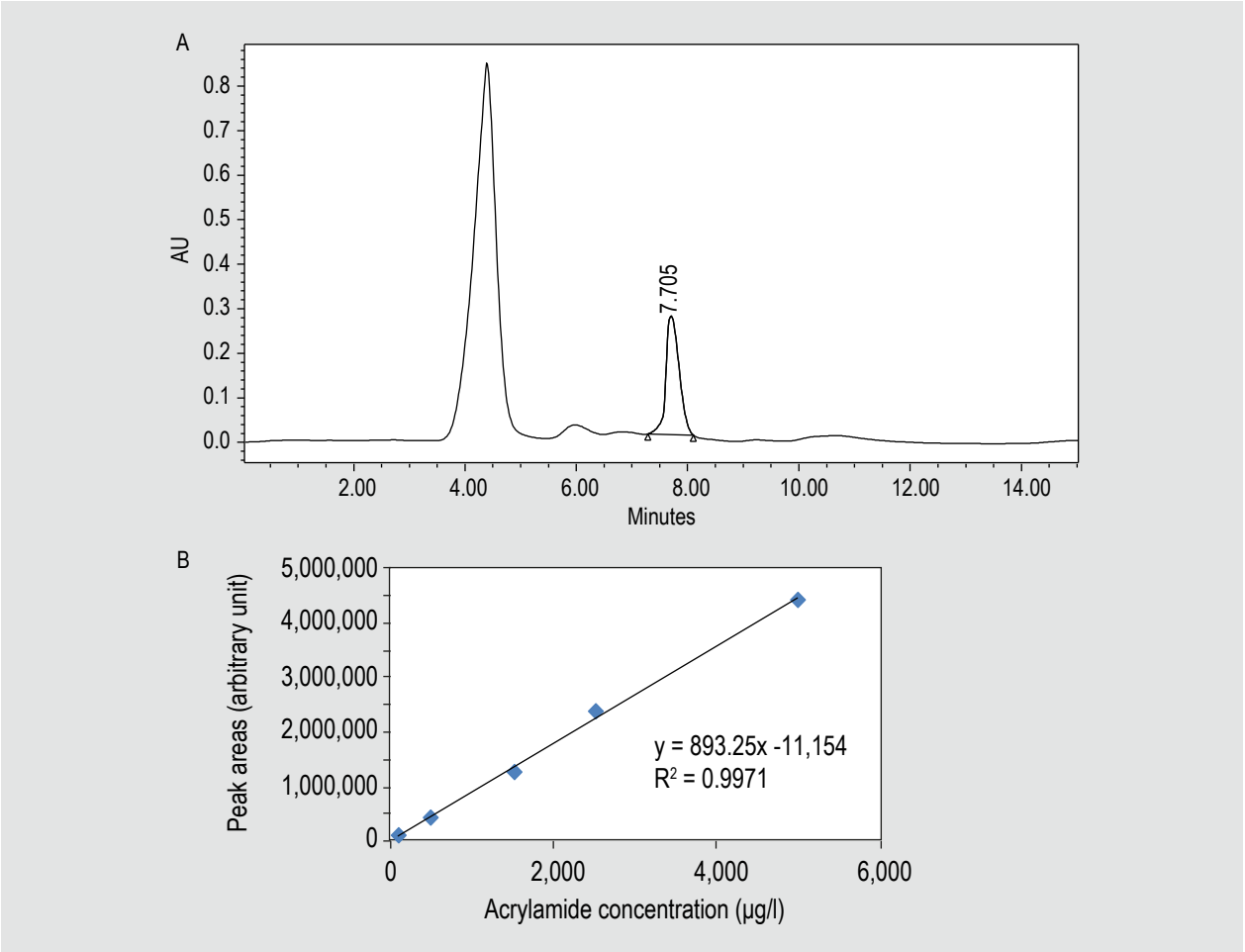

Figure 1. (A) High performance liquid chromatography chromatogram of acrylamide standard solution at 5,000 µg/l using NH2P-50 4E column detected at 200 nm using water and acetonitrile at 94.4 to 5.6 ratio (v/v) and 0.5 ml/min flow rate. (B) The 5-point calibration curve of acrylamide standard solutions at 100-5,000 µg/l concentrations.

Table 2. Analysis of variance of four main variables affecting the sample preparation and extraction method of acrylamide from potato chips including extraction solvent, defatting solvent, pre-concentration factor and ultrasound treatment provided by the Taguchi's experimental design.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-value	*P*-value
Extraction solvent	2	2.98319E+12	47.61%	2.98319E+12	1.49160E+12	*	*
Defatting solvent	2	7.07186E+11	11.29%	7.07186E+11	3.53593E+11	*	*
Preconcentration factor	2	1.47554E+12	23.55%	1.47554E+12	7.37770E+11	*	*
Ultrasound wave	2	1.09979E+12	17.55%	1.09979E+12	5.49894E+11	*	*
Error	0	*	*	*	*		
Total	8	6.26571E+12	100.00%				

Model summary

S	R-sq	R-sq(adj)	PRESS	R-sq(pred)
*	100.00%	*	*	*

Therefore, 200 nm was selected as a proper wavelength for acrylamide analysis in this study.

Effect of extraction and defatting solvents, pre-concentration factor and sonication time on the efficiency of acrylamide extraction

Using the Taguchi's experimental design, the effects of four main variables from Table 1 were investigated at three levels using nine series of experiments. The analysis of variance of four main variables from Taguchi's statistical analysis (Table 2) indicated a significant statistical difference among the results for each of four main variables including extraction solvent, defatting solvent, pre-concentration at different ratios and ultrasound treatment at various lengths of time (0-8 min) (P<0.05) (Figure 2).

Methanol as the extraction solvent resulted in higher acrylamide extraction from potato chips compared to the other extraction solvents (acetonitrile and acetone) (Figure 2A). Also, defatting with *n*-hexane improved the efficiency of acrylamide extraction from potato chips compared to the other defatting methods (defatting with dichloromethane and that with no defatting step) (Figure 2B). The effects of the pre-concentration method on the acrylamide extraction was also investigated by drying the solution in a water bath to three target concentration levels, i.e. without carrying out a pre-concentration step, experiments with a target pre-concentration factor of 3 times and experiments with a target pre-concentration factor of 5 times. As the pre-concentration level increased, the extraction level was also increased (Figure 2C). The effect of ultrasound treatment at different lengths (0, 4 and 8 min) was also examined by placing the samples inside the ultrasound bath and it was found that treating with ultrasound waves resulted in an increase in the acrylamide extraction with minimum amount of acrylamide extraction for the experiments with no ultrasound treatment and maximum for the sonication at 8 min (Figure 2D).

Effect of solid-phase extraction clean-up pretreatment on the efficiency of acrylamide extraction

The results of the Taguchi's experimental approach (previous sections) showed that the highest level of acrylamide was obtained when methanol was used as the extraction solvent, defatting was conducted using *n*-hexane, a 5-time pre-concentration was applied and when the samples were treated with ultrasound for 8 min. These conditions were then applied for the sample preparation followed by the extraction procedure to examine the effect of SPE clean-up process on the analytical results using one-variable-at-a-time method under three different conditions of SPE application, described in section 2.3. Examining the data indicated significant differences (P<0.05) among the results (Figure 3).

Chromabond C18ec SPE cartridge resulted in no acrylamide peak on the chromatogram suggesting that a large amount of acrylamide was adsorbed by this sorbent not allowing to pass through the cartridge under the pre-wash conditions described in Section 2. Therefore, this cartridge was not appropriate for a clean-up procedure of aqueous acrylamide solutions. The clean-up procedure carried out with PSA dispersive-SPE was not appropriate either since it resulted in a weak acrylamide peak on the chromatogram (peak area: 694,588±7,232), which was smaller than that obtained when no clean-up procedure was applied (peak area: 2,658,947±12,840) indicating that PSA adsorbed a considerable amount of acrylamide.

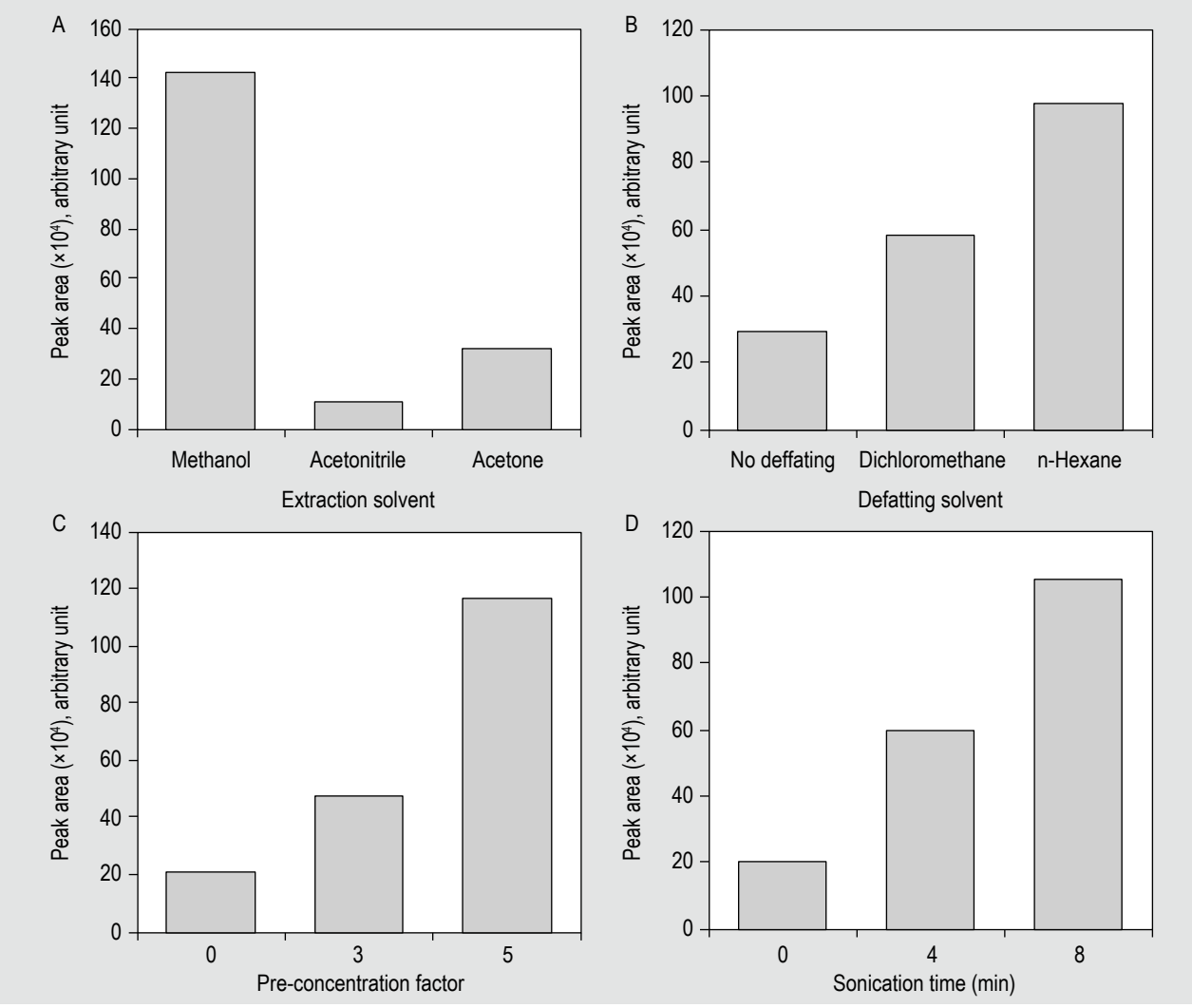

Figure 2. Effects of extraction solvent (A), defatting solvent (B), pre-concentration factor (C), ultrasound treatment (D) based on Taguchi's experimental design on the peak area of extracted acrylamide from potato chips.

Recovery test for validation of the optimised preparation method

Overall, the results of the Taguchi's experimental approach followed by the one-variable-at-a-time method applied in this study showed that the highest recovery of acrylamide was obtained when using methanol as the extraction solvent, defatting the sample with *n*-hexane, conducting a 5-time pre-concentration and treating samples with ultrasound for 8 min and not carrying out any SPE clean-up procedure(s). To validate the accuracy of the results of acrylamide extraction from potato chips using the optimised preparation method, the recovery ratio test was conducted in four levels of acrylamide concentration at 0; 500; 1,200 and 2,400 µg/kg each being carried out in two replicates. The mean recovery ratios for potato chips spiked with 500; 1,200; and 2,400 µg/kg with acrylamide were obtained at 95.9±1.3% to 102.2±1.6% (Table 3). The chromatogram related to the potato chips spiked at 1,200 µg/kg is presented in Figure 4. The desirable separation of acrylamide peak at about 7.6 min without any interferences from other components of the sample matrix (sugars, proteins, amino acids and organic acids) and the appropriate results obtained from the recovery ratio test conducted on the test samples of potato chips demonstrated the fact that through applying a new generation of amino chromatographic column together with optimising the sample preparation and extraction steps such as sedimentation of protein by Carrez I and Carrez II solutions, defatting with *n*-hexane, use of ultrasound treatment to improve the extraction efficiency, and carrying out a 5-time pre-concentration step in the extraction procedure, acrylamide can easily, accurately and affordably be quantified by HPLC-PDA system without the need to conduct time consuming and costly steps of SPE clean-up or complicated process of derivatisation.

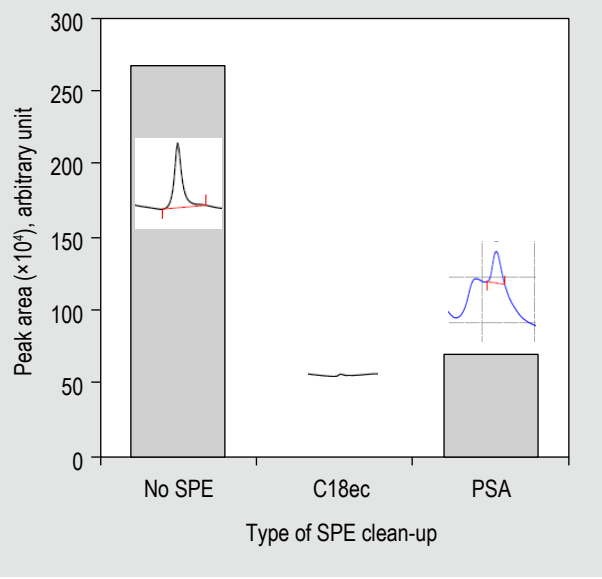

Figure 3. Effect of solid-phase extraction (SPE) clean-up method on the peak area of extracted acrylamide from potato chips. PSA = primary secondary amine.

Repeatability test

The inter-day repeatability test was conducted to validate and investigate the accuracy of the obtained results. Samples of potato chips were spiked at 1,200 µg/kg during a 30-d period with a 6-d time intervals. The mean and standard deviation of the peak area results were respectively at 1,526,792 and 67,046 providing an RSD of 4.39%. Such finding indicated a desirable repeatability for the test method applying the NH2P-50 4E amino column with the HPLC procedure.

Limit of detection and limit of quantification determination

To determine LOD and LOQ of the obtained chromatographic method, the samples of potato chips were spiked with 0, 50, 100, and 250 µg/kg acrylamide with two replicates and all the previous steps for the extraction and analysis were carried out using the NH2P-50 4E amino column. Then, the recovery ratios for the spiked samples were determined (Table 3). The LOD and the LOQ obtained for this method were 9 and 27 µg/kg, respectively.

Overall, chromatography techniques have been applied for quantifying acrylamide in food products (Chang *et al.,* 2007; EFSA, 2009; Ghasemian *et al.,* 2014; Kontominas and Paleologos, 2005; Mousavinejad *et al.,* 2015). On that aspect, gas chromatography coupled to an electron capture detector with an LOQ of 10 µg/kg (Feng *et al.,* 2013), HPLC system coupled to a diode array detector with an LOQ of 25 µg/kg (Chen *et al.,* 2008a), GC-MS with an LOQ of 38.8 µg/kg (Dong *et al.,* 2006), GC coupled to a flame ionisation detector with an LOQ of 6 µg/kg (Fang *et al.,* 2012), and LC-MS with an LOQ of 6 µg/kg (Senyuva and Gokmen, 2006) have been used for measuring acrylamide in food products.

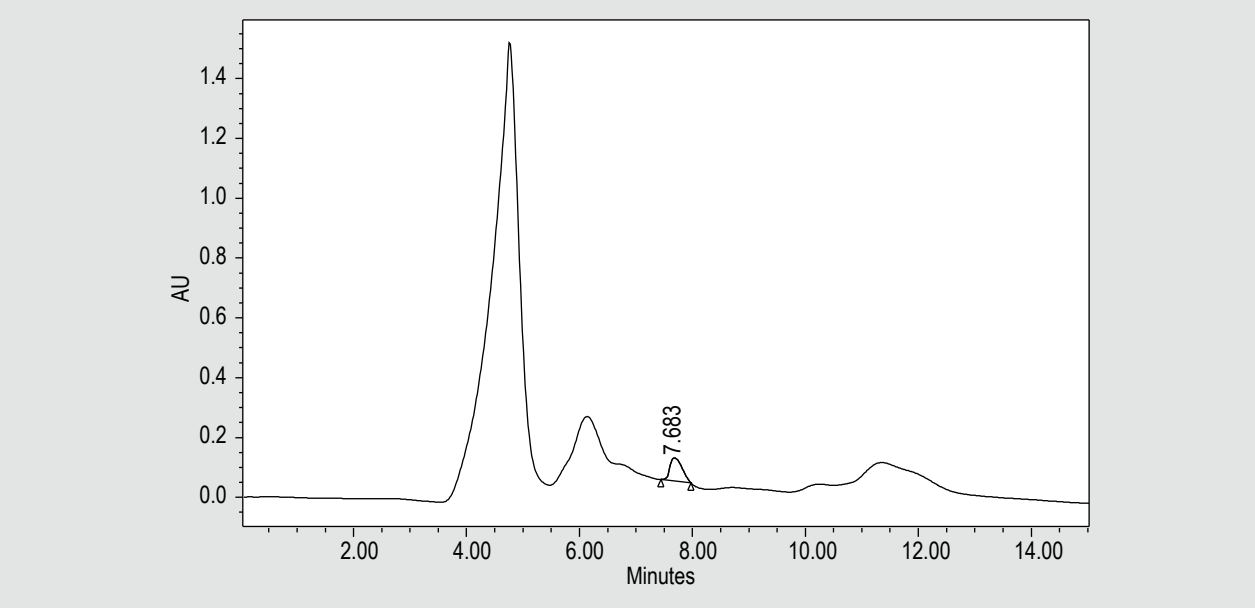

Figure 4. High performance liquid chromatography chromatogram of potato chips spiked with 1,200 µg/kg acrylamide using NH2P-50 4E column, wavelength: 200 nm, flow rate: 0.5 ml/min, mobile phase: water: acetonitrile (94.4:5.6%, v/v).

Table 3. Recovery ratios obtained for the analysis of potato chips spiked with 50, 100, 250, 500, 1,200, and 2,400 µg/kg acrylamide (n=2).

Spiked potato chips concentration µg/kg	Initial concentration[1] µg/kg	Final concentration[2] µg/kg	Recovery ratio[3] %
Sample blank[4]	111±14	111±14	N/A
50	82±3	82±3	165±5.7
100	191±2	106±2	106.1±1.6
250	329±4	243±4	97.4±1.7
500	600±6	480±6	95.9±1.3
1,200	1,347±19	1,227±18	102.2±1.6
2,400	2,433±7	2,313±6	96.4±0.2

[1] Initial concentration was determined directly from the calibration curve using regression equation (mean±SD).
[2] Final concentration was obtained by considering the pre-concentration factor and subtracting acrylamide concentration in the sample blank (mean±SD).
[3] Recovery ratio was determined through dividing final concentration by the spiked concentration levels (mean±SD).
[4] Sample blank was milled potato chips without spiking by acrylamide; N/A = not applicable.

4. Conclusions

Optimisation of the sample preparation using the Taguchi's experimental design and extraction of acrylamide from potato chips applying the one-variable-at-a-time approach were examined in the current study using a new generation of amino column (Asahipak NH2P-50 4E) in a liquid chromatographic system. Use of this column based on polyvinyl alcohol gel solved the problem with the decrease in the power of retention and separation in the previous columns that occurred over time. Afterwards, acrylamide content in potato chips was measured by using HPLC-PDA system with appropriate recovery ratio (95.9-102.2%) and LOD and LOQ at 9 and 27 µg/kg levels, respectively. In most of the past studies using liquid chromatography often an SPE clean-up step was required due to the poor retention and separation for acrylamide on the conventional liquid chromatographic columns. But, the optimised sample preparation method followed by the liquid chromatography applying the new generation of amino columns coupled with PDA detector from the current study can be suggested as an easy, affordable, and reliable method for measuring acrylamide in potato chips without the need to conduct the time consuming and expensive steps of SPE clean-up or complicated process of derivatisation.

Acknowledgements

The authors would like to acknowledge the support provided by 'The Department of Food Industries and Agriculture of Standard Research Institute-SRI' (Karaj, Iran) and 'The Research Council of College of Agriculture and Natural Resources of University of Tehran' (Karaj, Iran).

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