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Analytical evaluation of a high-throughput enzyme-linked immunosorbent assay for acrylamide determination in fried foods

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A B S T R A C T

The analytical performance and evaluation of a kit-based ELISA for the determination of acrylamide in fried potato and corn chip samples are described. The sample homogenate is subjected to clean-up using SPE, followed by analyte derivatization and ELISA detection. Accuracy, precision and linearity of the ELISA procedure have been validated using spiked samples. Analytical recovery ranged from 91.8% to 96.0% with coefficients of variation below 15%. Good linearity over a wide range of dilution and minimal assay drift was observed within a microtiter plate. IC_{50} value of the calibration curve was 110 ng/mL, with the limit of detection about 5 ng/mL and dynamic range from 10 to 1000 ng/mL. The high specificity of the ELISA was demonstrated by cross-reactivity study using 11 potential cross-reactants. A good correlation between the results obtained from the ELISA and GC–MS within the concentration range 120–1500 μg/kg was found in the chip samples (r=0.992, n=120). The data demonstrate that the evaluated and validated ELISA has a potential utility in a quick, simple and reliable acrylamide screening analysis for the medium- and large-sized food companies, as well as for residue laboratories and the food industry dealing with improving the chemical safety of foods available to the consumer.

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1. Introduction

Acrylamide is found in foods as a natural byproduct of the cooking process. Methods in which temperature exceeds 120 °C, such as baking, frying, grilling, and toasting can cause the amino acid asparagine (found in certain foods) to react with reducing sugars via the Maillard reaction to produce acrylamide [1–3]. The reducing sugars (glucose, fructose) and asparagine are natural compounds of plants and plant-derived ingredients used in the preparation of foods. They are prevalent in cereals and potatoes, while asparagine seems to be the limiting factor in cereal products [4]. The acrylamide content of food(s) varies widely within the same food product, within the same manufacturing facility, production lines and at different times, as well as between manufacturers, due to different formulations and processing conditions. High carbohydrate foods including potato, beetroot and certain heated commercial products such as crispbread that are baked or fried at high temperatures contains the highest levels of acrylamide.

Acrylamide is a controversial food contaminant with unclear effects on human health. Due to the uncertainties in exposure and the potential exposure to sources other than food, national and food safety regulatory bodies have hold the opinion that currently, it is not possible to draw any reliable conclusions from the available data regarding the cancer risk of acrylamide in food. Therefore, large number of research projects and surveys on acrylamide are funded to improve the understanding of how the contaminant is formed and to develop measures to reduce or mitigate it. Large databases of occurrence data are maintained by the European Commission [5] and the US Food and Drug Administration [6].

Currently, the food industry and food safety regulatory bodies have been cooperating closely on approaches aimed at reducing acrylamide levels in processed foods to decrease human health risk. In the EU, the Confederation of the Food and Drink Industries (CIAA) has released a series of "Toolbox" guides advising manufactures on how they can reduce acrylamide by up to 80 percent [7]. There is a clear need for an effective screening using rapid and reliable analysis of acrylamide level in food matrices for quality control purposes. Up to now, mainly GC/MS and LC–MS methods have been used for acrylamide determination in foods; however, these procedures are relatively expensive and limited in terms of timeframe per sample. Moreover, some promising approaches to acrylamide quantification in foods such as electrochemical biosensors and immunoassays were recently reported [8].

Immunochemical methods such as ELISA (enzyme linked immunosorbent assay) are rapid, robust and a high-throughput

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analytical alternatives to instrumental methods used in food and environmental trace analysis. ELISA satisfies the performance and validation criteria for screening methods and has been used for detection of many food and environmental toxic contaminants. The objective of this work was to evaluate the analytical performance and reliability of the developed ELISA kit for acrylamide determination in selected fried foods.

2. Experimental

2.1. Chemicals, materials and instrumentation

Basic assay solutions and reagents used in this study were provided in the Acrylamide ELISA, Microtiter plate kit (Abraxis). Acrylamide Standards 0 (0 ng/mL), 1 (5 ng/mL), 2 (10 ng/mL), 3 (25 ng/mL), 4 (50 ng/mL), 5 (100 ng/mL), 6 (250 ng/mL), and 7 (1000 ng/mL) were diluted in 60% methanol/de-ionized water and used for analyte derivatization. The compounds used for cross-reactivity study were from Sigma. Single-Block Cold Box, used to cool plate during assay incubation at 4 °C, and Digital Four Block Heater, Cat# 12621-096, used to derivatize sample/standard at 50 °C were obtained from VWR. A Negative Pressure VisiPrep DL Manifold System, used in sample clean-up procedure, was obtained from Supelco. Costar Stripwell Microplate, 1 × 8 Well, Flat Bottom, Certified High Binding, Polystyrene, Cat# 2592, was used as immunoassay solid-phase medium. SPE columns (M-M Multimode 1 g/6 mL and Envi+ Biotage 500 mg/6 mL) were provided with the ELISA kit.

2.2. Sample pre-treatment

2.2.1. Sample extraction

Samples obtained from local stores were dried and homogenized using mortal and pestle. 1 g of the samples was weighed into 50 mL plastic centrifuge tubes. 20 mL of 0.1% formic acid/deionized water was added to the samples and shaked on a vertical shaker for 60 min. Samples were centrifuged for 30 min, 1600 × *G* at 4 °C and supernatant obtained was further used for cleaning up with SPE columns.

2.2.2. Sample clean up

Biotage Multi-Mode columns were conditioned with 3 mL of methanol and 10.0 mL of deionized water. 12.5 mL of each extracted supernatant was passed through the Biotage Multi-Mode column and the eluent was collected. The columns were rinsed with 1 mL of deionized water and the column eluent was collected. Then, Biotage Envi+columns were conditioned with 5 mL of methanol and 5 mL of deionized water to waste. The eluent collected from the first column was passed through Biotage Envi+column to waste. The columns were then rinsed with 4 mL of deionized water to waste. The captured acrylamide was then eluted with 5 mL of 60% methanol/deionized water and the collected eluent was used for derivatization step (at this stage, the extraction and sample clean-up involve 8-fold dilution of the sample supernatant).

2.2.3. Sample derivatization

50 μL of derivatization reagent was added to each 500 mL of calibration standard or sample eluate in glass vial with screw cap. The vials with standards and samples were vigorously vortexed for 10 s and incubated at 50 °C for 60 min. The derivatized samples and calibration standards were then diluted 1:7.5 in assay buffer and used for ELISA analysis.

2.3. ELISA performance

50 μL of derivatized sample and standard, 50 μL of peroxidase conjugate diluted (1:10,000) and 50 μL of antibody diluted (1: 2500) were added to the wells of plate and swirled gently on a countertop for 60 s to mix the reaction mixture. After 60 min incubation in a cold box at 4 °C, the contents of wells were decanted and blotted face-down on stack of paper towels. Then, 250 μL of ice cold washing solution was added to wells and contents of plate were decanted and blotted face-down on a stack of paper towels. The plates were washed an additional three times with cold washing solution. Followed by 150 μL of color solution was added and the plates were swirled gently on a countertop to mix contents for approximately 60 min. After 20 min incubation at 23 °C, 100 μL of stop solution was added. The absorbance signal was read in a plate reader at 450 nm. To get the final result in μg/kg, the concentration read from calibration curve in μg/L is multiplied by dilution factor 8.

2.4. GC–MS analysis

A modification of USEPA Method 8032 using GC–ECD after bromination of the acrylamide was employed as a standard reference method for sample analysis [1]. Briefly, samples were carried through the derivatization and extraction procedure and 2 μL of sample was injected into a GC/MS system under conditions described in [1]. Acrylamide-d3 was added as an internal standard prior to sample extraction.

3. Results and discussion

3.1. Analytical characterization of ELISA

The immunoassay described in this work uses a competitive assay format. Since the peroxidase-labeled acrylamide conjugate competes with the analyte in the samples for the available antibody binding sites, the color developed is inversely proportional to the concentration of acrylamide. It is common to report displacement in terms of B/B_0 measurement to describe color inhibition. B/B_0 is defined as the absorbance observed for a sample or standard divided by the absorbance at zero analyte concentration. A calibration curve for the ELISA was constructed from 7 calibration standards carried out through the above sample preparation procedure (Fig. 1). Due to lack of true blank samples, the assay sensitivity, established as the lowest concentration (LOD) that can be distinguished from zero, was calculated by determining the 90%

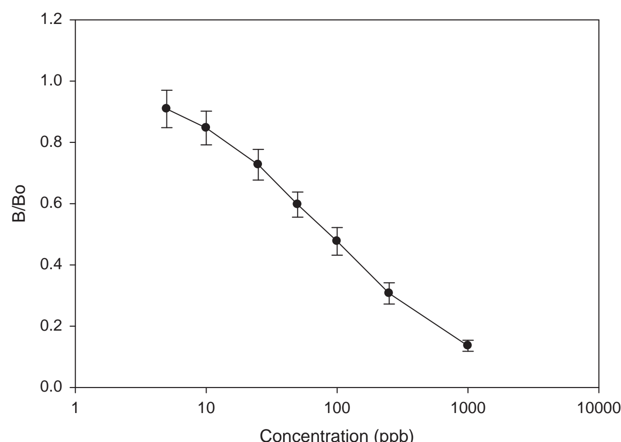

Fig. 1. Standard calibration curve for acrylamide. The bars show the standard deviations SD ± (0.06–0.018) for 42 replicate assays performed over 4 months.

B/B_0 concentration. The calculated LOD using the 90% B/B_0 method was approximately 6 ng/mL. The B/B_0 values for the acrylamide standards were linearly transformed using a log/logit curve. The dynamic range of the transformed curve producing inhibition signal between 15% and 90% B/B_0 was 5–1000 ng/mL. Taking into consideration that the final dilution factor was 8-fold (including extraction with water and clean up using SPE) the corresponding dynamic range in analyzed (processed) samples was 40–8000 μg/kg. Limit of quantification for food samples is 40 μg/kg.

3.2. Assay drift examination

The incubation times used in the assay represent non-equilibrium conditions. Affinity and kinetics constants of the antibody used can also influence assay results. In addition there could be a significant lapse in time between the addition of reagents between the first samples and the last samples. To determine the effect of these conditions and factors upon sample values in the ELISA method, a drift study was performed. Two separate assays were run following the acrylamide ELISA assay protocol. Forty samples (prepared in 60% methanol/water) with a concentration 0 ng/mL acrylamide and forty samples with a concentration of 100 ng/mL acrylamide were assayed at a maximum assay preparation/pipetting time interval of 150 s. Analysis of the data was performed by plotting well number versus absorbance for the 0 ng/mL sample assay, and by plotting well number versus concentration for the 100 ng/mL spiked sample assay. Linear regression was utilized to determine the slope of the line of best-fit. The linear regression line of the 0 ng/mL sample assay had a slope +0.0021 absorbance units per well number with a mean absorbance value of 1.139 absorbance units (Fig. 2). The change in absorbance of the 0 ng/mL sample across the assay was calculated to be a B/B_0 of 0.93, which is greater than the B/B_0 of the first standard (5 ng/mL) of 0.91 indicating that negative samples will remain negative or below the limit of quantitation of the assay if pipetting of the antibody solution step is kept under 150 s. The linear regression line of the 100 ng/mL sample assay had a slope of −0.2493 ng/mL per well number with a mean result of 99 ng/mL (Fig. 3). The change in result of the 100 ng/mL sample across the assay was calculated to be −9.972 ng/mL (−10.1%). The difference in recovery from samples at the beginning and the end of the assay was within ±10%; therefore assay drift was considered to be acceptable if the pipetting of the antibody step is kept under 150 s. It is recommended, however, if using manual or semi-automatic pipettes to only analyze up to 20 samples per run. If automatic pipettes are used then the difference in concentration between beginning and end of the assay plate should be negligible even with 40 samples assayed in the same run (Fig. 3).

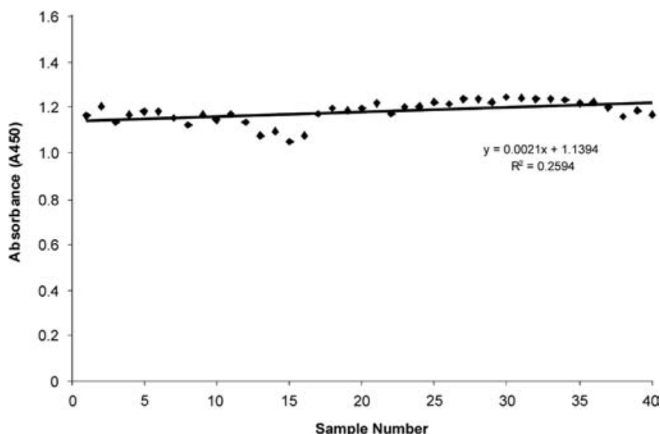

Fig. 2. Plate drift for 0 ng/mL sample assay.

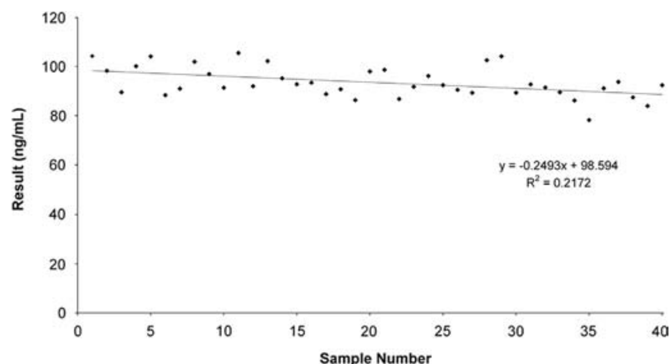

Fig. 3. Plate drift for the 100 ng/mL spiked sample assay.

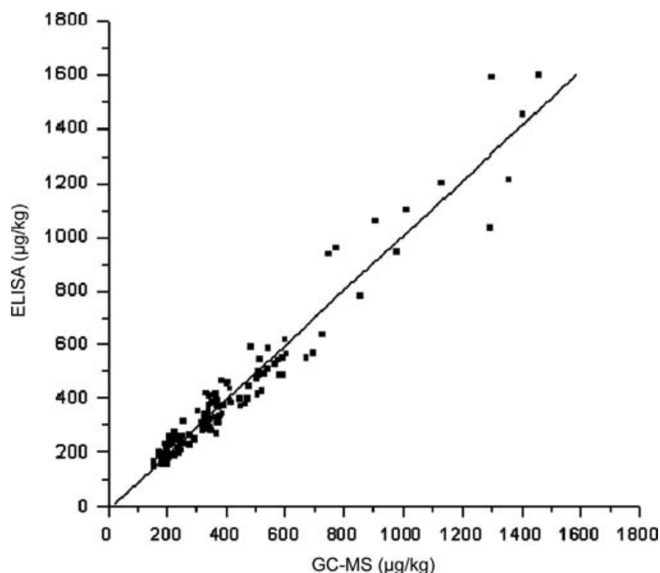

Fig. 4. Correlation between ELISA and GC results in the chip samples.

3.3. Assay specificity

Specificity of the assay was tested by the measurement of cross-reactivity using acrylamide and related compounds. The IC_{50}, defined as the inhibitory concentration estimated at 50% $B/B0$, was determined by estimating the concentration of acrylamide necessary to displace 50% of the acrylamide–HRP conjugate. The cross-reactivity of twelve potentially interfering compounds was determined by testing 10, 100, 1000, and 10,000 ng/mL concentrations. Interfering dilutions were prepared in 60% methanol/deionized water. Data analysis was obtained by calculating the IC_{50} for acrylamide and the twelve potentially interfering compounds. The IC_{50} for acrylamide was 170 ng/mL. Table 1 shows that the antibody incorporated into the ELISA kit was very specific for acrylamide as illustrated by the low cross-reactivity of the methyl acrylate, methacrylamide, acrylonitrile, acrylic acid, 2-pyrrolidinone, acrolein, 2-pyroglutamic acid, glutamic acid, glutamine, asparagine, aspartic acid, and aspartamine.

3.4. Precision and accuracy

Precision studies using spiked samples were performed to measure the percent coefficient of variation (% CV) within and between assays. Following the acrylamide standard ELISA protocol, four samples were spiked with 25, 50, 125, and 400 ng/mL of

acrylamide in 60% methanol/deionized water, and analyzed over a 5-day period with five assays per day in singlicate. Data was analyzed using an ANOVA statistical model. The mean analytical recovery of the 25 total assays for the spiked samples was 91.8–96.0 with coefficients of variation below 13% for all concentrations tested. Results obtained are presented in Table 2.

3.5. Linearity and matrix effect assessment

Test for linearity determines the extent of the assay range to which the dose–response of the analyte is linear at a particular sample dilution. The assay response was assessed for the linearity and recovery by evaluating five serial dilutions from spiked fried potatoes and fried corn chips. The samples were spiked with 800 ng/mL of acrylamide, followed by extraction, sample clean-up and derivatization procedures. Five serial dilutions (1:1) of both sample extracts were prepared in 60% methanol/de-ionized water and assayed for linearity. The initial concentration of the spiked fried corn chip and fried potato chip sample was 1116 ng/mL and 1288 ng/mL, respectively. As shown in Table 3, the recoveries of the 1:1 to 1:16 serial dilutions of the fried corn chip and the fried potato chip spiked samples varied from 87.0% to 115.6% with a correlation of variation less than 11.8%.

Table 1
Cross-reactivity between acrylamide and related compounds.

Compound	IC_{50} (ng/mL)
Acrylamide	170
Acrolein	> 10,000
Acrylic acid	> 10,000
Acrylonitrile	6,000
Asparagine	> 10,000
Aspartamine	> 10,000
Aspartic acid	> 10,000
Glutamic acid	> 10,000
Glutamine	> 10,000
Methacrylamide	> 10,000
Methyl acrylate	> 10,000
2-Pyrrolidinone	> 10,000
2-Pyroglutamic acid	> 10,000

Table 2
Precision and accuracy of the ELISA.

Added standard	25 ng/mL	50 ng/mL	125 ng/mL	400 ng/mL
Assays	5	5	5	5
Days	5	5	5	5
n	25	25	25	25
Average result	23.8	48.0	116.2	367.2
% Recovery	95.1	96.0	93.0	91.8
% CV (within assay)	9.7	6.7	5.0	3.5
% CV (between assay)	12.4	8.3	5.7	7.9

3.6. Correlation study

A comparison of 128 fried potato and corn chips samples analyzed using the ELISA method and a GC method showed very good correlation. Data was analyzed by plotting the concentration determined by the GC method (x-axis) versus the concentration determined by the ELISA method (y-axis). Correlation was determined using linear regression. The slope of the linear regression line was 1.01 with a y-intercept of -14.53. The slope of the linear regression line is approximately equal to the theoretical desired slope of 1, which would indicate a 1:1 relationship between the results of the two quantitative methods. The r value of 0.97 also suggests that there was very good correlation between the two sets of data (Fig. 4).

4. Conclusions

Several antibodies have been produced and employed in immunoassays for the detection of acrylamide; however, they lack appropriate sensitivity to be used in real screening test [9–11]. It is demonstrated in this study that the kit-based ELISA enables the reliable quantitation of acrylamide in potato and corn chip samples in working concentration range of 40–8000 μg/kg with very good precision and accuracy. The analytical sensitivity of the presented ELISA method is well compatible with an EU recommendation on investigations into the levels of acrylamide in certain food categories that specifies indicative values that are comparable to the German signal values [12]. The indicative values were recommended by the European Commission for foodstuffs such as baby food and processed cereal based foods (80 and 100 μg/kg, respectively), soft bread, biscuits and breakfast cereals (150, 250 and 400 μg/kg, respectively) and for the highest levels in french fries and potato chips (600 and 1000 μg/kg, respectively). These indicative values are not maximum residual limits and are aimed at performing investigation in cases where the levels of acrylamide in a foodstuff, identified by ongoing routine monitoring, exceed the relevant indicative value.

Although sample pre-treatment requires clean up with SPE cartridges prior to acrylamide derivatization, the ELISA-based method offers advantages such as high detection specificity, cost-effective and relatively rapid testing as well as high sample throughput and field portable testing. The validated ELISA kit can be used for screening of acrylamide in fried and baked foods and is aimed to the medium- and large-sized food industry companies as well as for residues laboratories and regulatory authorities dealing with improving the chemical safety of food available to the consumer.

Table 3
Linearity of serial sample dilution.

Dilution	Fried corn chips (ng/mL)				Fried potato chips (ng/mL)			
	Expected	Measured	% CV	% REC	Expected	Measured	% CV	% REC
Undiluted[a]	–	1116	3.4	–	–	1288	0.9	–
1:1	558	511	8.4	91.6	644	639	11.8	99.2
1:2	279	243	6.4	87.0	322	305	4.1	94.8
1:4	140	130	11.4	93.3	161	175	0.3	108.7
1:8	70	70	2.5	99.9	80	83	0.9	103.2
1:16	35	36	4.1	102.0	40	47	4.5	115.6

[a] Spiked sample.

Acknowledgments

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