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HPLC–MS validation of QualisaFoo® biosensor kit for cost-effective control of acrylamide levels in Italian coffee

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Abstract

Acrylamide is a carcinogenic and mutagenic compound found in many industrially processed foods and beverages, including coffee. The aim of this work is to determine the acrylamide level in some Italian coffees by using a mass spectrometry method and an enzymatic test kit. Comparison of average values in four Italian coffees determined using the two methods permitted us to validate the results obtained with the kit, hence the kit itself, showing that acrylamide is present in low amount.

The amount of acrylamide was also determined in other foods, by using the kit. This work shows that there is a correspondence between the two methods and that the kit provides a cost-effective method to determine the amount of acrylamide in food.

Keywords: Food; Safety; Acrylamide; Enzymatic test; HPLC–MS analysis; QualisaFoo® kit

1. Introduction

Acrylamide (2-propenamide) is considered an environmental risk factor. It has been shown that prolonged expositions to it produce neurotoxic effects in both animals and humans (Dybing & Sanner, 2003; Le Quesne, 1980). This compound is a highly hydrosoluble substance, the formation of which is mainly the result of Maillard reaction between the amino acid asparagine and reducing sugars (Yarnell, 2002) which may be incidentally taken from foods and beverages.

Acrylamide has been labelled by the International Agency for Research on Cancer (IARC) as a probable human carcinogen (IARC, 1994); very recently it has been classified as a carcinogen and mutagen by the European Union (http://ecb.jrc.it/classification-labelling/). It is present in several industrial products and processes, and can be found in a variety of products (Rothweiler, Kuhn, & Prest, 2004). Recently, researchers from Sweden reported finding acrylamide in certain baked and fried foods such as potato crisps and French fries (Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2000).

Although considerable controversy exists regarding the acrylamide exposure levels relevant to carcinogenicity in humans (Blasiak, Gloc, Wozniak, & Czechowska, 2004; Yagi et al., 2001), the reports on the presence of acrylamide in European food prompted the U.S. Food and Drug Administration (FDA) to analyze a variety of foods for the presence of acrylamide. Gas chromatography–mass spectrometry (GC–MS) methods have been used for the detection of acrylamide (Andrzejewski, Roach, Gay, & Musser, 2004; Murkovic, 2004). In addition, liquid chromatography–mass spectrometry (LC–MS) or LC–MS/MS analytical techniques were applied for acrylamide determination in specific food groups or a limited variety of

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foods (Granby & Fagt, 2004; Pittet, Pèrisset, & Oberson, 2004).

During the course of initial literature survey, one food commodity, coffee, consistently proved to be difficult to be analyzed for the presence of acrylamide (Delatour, Périsset, Goldmann, Riediker, & Stadler, 2004). Due to the use of roasted coffee beans in making coffee, the probability of significant levels of acrylamide being present was considered to be high. In addition, the high consumption of coffee by people living in the United States and in many other countries may make coffee a significant daily source of acrylamide. Recently, several studies have been published reporting data on acrylamide levels in coffee (Andrzejewski et al., 2004; Granby & Fagt, 2004). However, these studies present data for only a small variety of coffee and do not report any of the analytical difficulties associated with the analysis (Delatour et al., 2004). In this paper, we report data on acrylamide levels from a variety of ground coffees, discussing also some analytical problems encountered during analysis. In this work we report also for the first time, on the quantitation of acrylamide in some Italian coffee brands by using the QualisaFoo® test kit. QualisaFoo® kit is a new, enzyme based, cost-effective method for food sample preparation for spectrophotometric determination of acrylamide levels. The results of QualisaFoo® test procedure have been validated analyzing the same coffee samples with a well established HPLC–MS method.

2. Materials and methods

2.1. Materials

Acrylamide (2-propene amide) [CAS No. 79-06-1] (>99.5%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Individual stock of acrylamide solutions were prepared by dissolving 100 mg of compound in 100 ml of water and stored in a glass-stoppered bottle at 4 °C. Standard working solutions at various concentrations were daily prepared by appropriate water dilution of stock solution aliquots.

HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). HPLC grade acetic acid was purchased from J.T. Baker. Deionized water (<18 MΩ/cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

All the solvents were filtered through a 0.2 μm cellulose filter (RC 58) from Schleicher and Schuell before use.

The solid phase extraction (SPE) columns were STRATA-X-C 33 μm cation mixed-mode polymer, 200 mg/6 ml, purchased from Phenomenex USA (distributed by Chemtek Analytica, Bologna, Italy).

2.2. Samples

A total of four different brands of ground coffee were used. All of them are commercial italian brands of coffee nationwide sold.

Two samples are ground coffees for electric drip coffee maker (Illy and Lavazza). The others are ground coffees for moka machine (Kimbo and Lavazza Dek), one of which is decaffeinated (Lavazza Dek).

2.3. HPLC–MS Analysis

A Hewlett Packard (Palo Alto, CA, USA) HP-1100 series LC–MSD system equipped with a binary solvent pump, an autosampler with the volume injection set to 20 μl, and a mass selective detector (MSD) with electrospray ionisation (ESI) coupled with an HPLC/MSD Chem Station (Rev.A.08.03) was used.

Operating conditions of the ESI interface in positive ion mode were the followings: nebulizer gas (nitrogen) pressure: 40 psi; drying gas (nitrogen) flow rate: 13 l/min; drying gas temperature: 350 °C; capillary voltage: 4000 V.

Separation was performed on a Synergi 4 μ Polar-RP 80 A (150 mm × 4.6 mm ID, particle size 4 μm) protected by a securityguard cartridge Polar RP (4 mm × 4.6 mm ID), both from Phenomenex USA (distributed by Chemtek Analytica, Bologna, Italy).

The mobile phase was H_2O (+0.2% acetic acid): CH_3CN (+0.2% acetic acid) 99:1, in isocratic mode. The flow rate was 0.6 ml/min. Acrylamide was detected using the selective ion monitoring mode at $m/z = 72$ for acrylamide and $m/z = 94$ for sodium adduct.

Time-scheduled selected-ion monitoring (SIM) of the most abundant ions of acrylamide was performed as reported in Table 1, using high resolution settings.

2.4. Sample preparation

Each analyzed sample (9 ml) was taken from a mixture of 10 coffees of the same brand previously prepared and stored at room temperature. Coffee from moka machine was prepared as follows: 7 g of ground coffee was loaded in a single sized moka coffee machine, together with 80 ml of distilled water, then the coffee machine was heated on an electric heater. An average of 71 ml of coffee was made. Regarding coffee for electric drip coffee maker, operation with the coffee maker yielded coffees of 20 ml each.

Operations were repeated 10 times for each brand. 9 ml of brewed coffee was centrifuged at 4000 rpm for 15 min, and 5 ml of the supernatant was purified by using SPE cartridges (STRATA-X-C, 200 mg/6 ml).

The SPE cartridges were conditioned applying 2 × 2 ml of MeOH, followed by 2 × 2 ml of water at a flow rate of

Table 1
Time scheduled SIM conditions for HPLC–MS acrylamide analysis conditions

Compound	Time	SIM ion	Gain	Fragmentor (V)	Dwell time (ms)
Acrylamide	4.0–5.5	72	3	50	289
Acrylamide + sodium		94	3	50	289

2 ml/min. The supernatant (prepared as described above) was applied at a flow rate lower than 0.5 ml/min, drying under vacuum (10–12 in. Hg) for 30 s; after that the elution was performed with 2 × 1 ml of water at a flow rate lower than 0.5 ml/min. The eluate was collected in a sample vial and any residual water was dried from the sorbent by applying full vacuum. This eluate was used for LC/MS analysis.

2.5. Recovery, precision and linearity studies

The recoveries were determined by adding 44 μl of the appropriate acrylamide standard solution (50 mg/kg) to 9 ml of brewed coffee before the centrifugation step. The recovery ranged between 52% and 78% with most samples having a recovery of 70%. The linearity was tested at the following concentrations: 0.025, 0.1, 0.5 and 1 mg/kg; the excellent linearity is demonstrated by R^2 of 0.9998 (Fig. 1). The reproducibility of the whole analysis was tested with nine repeated experiments giving a relative standard deviation of 5.4%. The limit of quantification (LOQ) was estimated on the basis of 10:1 signal to noise ratio obtained with standard acrylamide at a low concentration level and was 0.025 mg/Kg.

2.6. Procedure of QualisaFoo® test

The kit provides all reagents for total extraction and quantitative determination of acrylamide from foods such as coffee, pasta, biscuits, and fried or backed potatoes. Each sample should be conserved dry and possibly microbiologically uncontaminated. The test is based on a thermostable amidase from *Sulfolobus solfataricus* (SsAH) and its mutants according to a previous report (Cilia, Fabbri, Uriani, Scialdone, & Ammendola, 2005).

2.7. QualisaFoo® Sample preparation

The analysis was performed by using from 3 to 20 g of a ground sample (depending from concentration of acrylamide) that was dissolved in 0.2 M NaOH. The solution was stirred for about 20 min (at 50 °C for coffee and at room temperature for other samples) and, after centrifugation at 9000*g*, the supernatant fraction was incubated for 20 min at 80 °C with a first enzyme, to eliminate some interferences. After the reaction, the sample was quickly cooled and an aliquot of 4 ml of it was loaded onto a first SPE column specific for acrylamide absorption (1 g/6 ml). Acrylamide was eluted and again loaded on a smaller SPE column (0.5 g/2 ml). Then the collected acrylamide solution was concentrated and divided into two aliquots of 0.5 ml each, which were used as sample and control, respectively.

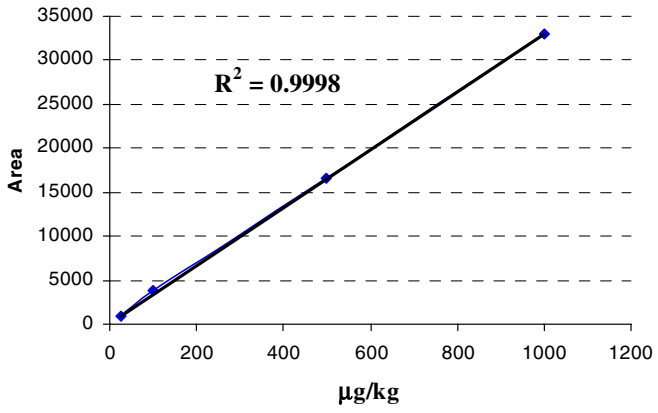

Fig. 1. Calibration curve with standards of 0.025, 0.1, 0.5 and 1 mg/kg.

2.8. Recovery, precision and linearity studies

The recoveries were determined by adding 40 ng/ml of analyte standard solution at the sample.

The recovery of acrylamide from SPE column was followed by reverse-phase HPLC chromatography on a column Inertisil ODS-3 (150 mm × 4.6 mm ID, particle size 5 μm, G.L. Sciences). Twenty milliliters of the sample was loaded at a flux of 1 ml/min; the analyte was eluted at 40 °C in TFA-0.1% in water and detected at 210 nm. When recovery was ascertained, the test sensibility was established spectrophotometrically using the stoichiometric relation between acrylamide and ammonium. The minimun detectable ammonium value resulted in 20 ng, corresponding to about 100 ng/g of acrylamide.

The medium recovery of analyte was 68% with a sensibility of 0.2 μg/ml. Finally, since the determination of analyte is evalued as the amount of NH_4^+ produced from enzyme reaction, a curve of calibration for ammonium has to be constructed. By using a standard solution of 30% ammonium, seven working dilutions were prepared in the range from 0.0454 μg/ml upto 4.04 μg/ml and the absorbance was read at 630 nm. In this range, which is in that of acrylamide values we found in the analysis, the test showed an excellent linearity, as demonstrated by R^2 of 0.9962 and a detection limit of 0.05 μg/ml (data not shown).

To determine the final acrylamide concentration in the sample, a corrective factor was used.

2.9. Enzyme determination

The sample was incubated with the SsAH enzyme for 30 min at 70 °C. The amide hydrolysis produced a stoichiometric amount of dissolved NH_4^+,which, after enzyme reaction, was determined according to Berthelot method (Cilia et al., 2005). Within 30 min, a colour developed and the intensity was determined by data interpolation with respect to a calibration curve for NH_4^+. The absorbance of developed colour is read at $\lambda = 630$ nm. Finally, the value of absorbance is corrected by a factor, introduced to take into account the percentage of acrylamide recovery and the mass of the sample used for the analysis.

The results obtained with this kit were validated with LC–MS experiments repeated five times.

3. Results and discussion

Four different Italian coffees, produced with different industrial technologies, as suggested by the presence of a decaffeinated coffee, were assayed for their acrylamide contents, using two different methods. In both analytical systems, the sample required the selective extraction of acrylamide from the matrix. After extraction and purification procedures, the sample was directly analysed on HPLC–MS according to a reported method (Granby & Fagt, 2004). The obtained results are shown in Table 2.

On the contrary, the analysis with enzymatic test required a careful treatment of the sample taking also into account that the enzyme has to be specific for the target substrate. In addition, the enzymatic test requires that acrylamide is concentrated in a solvent compatible with enzyme activity. For this reason, the kit is based on a thermo-stable engineered enzyme, which exhibited a reasonable solvent resistance, maintaining its activity in solvents other than water. This enzyme works on several substrates, thus decreasing the interference with the broad spectrum of substrates of SsAH enzyme. After the unconverted target molecule was purified by the first enzymatic reaction, it was concentrated with a second column. Finally, reacting with a specific amidase acrylamide produced an equivalent NH_4^+ concentration that was determined by spectrophotometric analysis. The analysis results of the tested four coffee samples obtained in the enzymatic test were compared to data from HPLC–MS experiments, showing a good agreement (Fig. 2, Table 2). In detail, Fig. 2 shows the superimposition of two normalized HPLC chromatograms: one (dotted line) from a solution purified with the procedure of the QualisaFoo® kit, thus ready for enzymatic colour development, and the other (full line) from the solution purified for LC–MS analysis according to the literature (Granby & Fagt, 2004). As shown, the two purified samples show a very similar pattern, indicating a very close composition.

Table 2
Acrylamide concentration in coffee

Coffee brand	HPLC–MS		QualisaFoo®	
	Acrylamide level (μg/kg)[a]	RSDs (%)	Acrylamide level (μg/kg)[a]	RSDs (%)
Kimbo	186	6	190	3
Lavazza	88	12	250	9
Lavazza Dek	<120[b]	–	109	11
Illy	<120[b]	–	118	8

[a] Mean value of six determination.
[b] In these cases it has not be possible to fully quantify acrylamide levels because of matrix interferences.

Data from our experiments demonstrated also that acrylamide content does not correlate with lot number or production date (data not show).

Acrylamide concentrations found in this analysis demonstrate that acrylamide levels in the analyzed italian coffees are quite low; hence, taking also into account the Italian habit of having short coffees, i.e. small volume coffees, the acrylamide intake from that beverage is very low. Values reported in Table 2 (HPLC–MS and QualisaFoo® kit) are, for each sample, very similar; in fact, Kimbo values are identical, whereas some differences has been found in the case of Lavazza. For the other two samples (Lavazza Dek and Illy), QualisaFoo® kit demonstrated to be a better tool for determining acrylamide concentrations in coffee: in fact, while in the case of HPLC–MS procedure it was not possible to fully quantify acrilamide value below 120 μg/kg, due to matrix interference, the purification procedure of the QualisaFoo® kit gave a sample in which quantification was possible, yielding data that, once again, are in agreement with HPLC results.

All analyzed coffees are quite low in acrylamide content, with Illy and Lavazza dek brands being quite low, showing a concentration close to 100 μg/kg.

To assess whether our data were similar to acrylamide levels in coffee already reported, we compared our results with some public databases and two scientific papers. As shown in Table 3, acrylamide range determined in Italian coffee was practically superimposable with those reported by FAO/WHO and literature 1; as far as FDA and literature 2, ranges are clearly wider, but this is mainly due to the

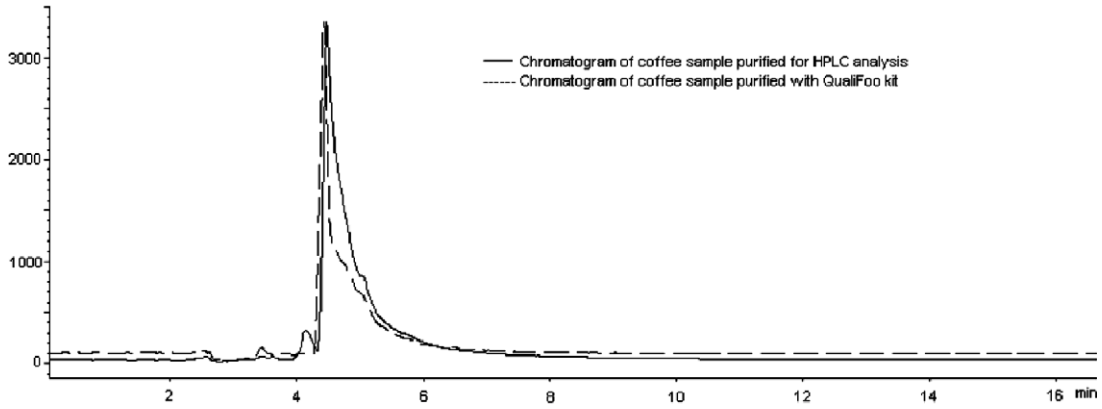

Fig. 2. HPLC–MS comparison of samples recovered using the two different purification methods.

Table 3
Acrylamide content (μg/kg) in coffee assayed with QualisaFoo® kit in comparison with FDA, FAO/WHO and selected literature data

QualisaFoo® kit	FDA[a]	Joint FAO/WHO consultation[b]	Literature 1[c]	Literature 2[d]
109–250	27–609	170–230	150–340	45–374

[a] FDA data are from the 2004 survey reported in http://www.cfsan.fda.gov/~dms/acrydata.html#u1004.
[b] Data are from report of a Joint FAO/WHO consultation: ftp://ftp.fao.org/es/esn/jecfa/acrylamide_2002-09-16.pdf.
[c] Murkovic (2004).
[d] Andrzejewski et al. (2004).

fact that a large number of samples was analyzed. However, also in these last two reports, the mean value of acrylamide concentration is very similar to the one found by us.

4. Conclusion

With this paper we have shown that acrylamide levels in four Italian brands of coffee are quite low: a moderate consumption of coffee does not account for a relevant intake of acrylamide.

We have also demonstrated that QualisaFoo® kit is a reliable tool for a quick and affordable determination of acrylamide in coffee and other edible goods; the kit could be a inexpensive and easy to use alternative to HPLC–MS or GC–MS methods for process control during food production.

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