APPLICATION NOTE 22061

An improved UHPLC-MS/MS assay for the determination of acrylamide from foodstuffs

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Keywords: Acrylamide, Supported Liquid Extraction (SLE), Hypercarb, TSQ Endura, Vanquish Horizon, UHPLC MS/MS, food, potato chips, coffee, baby food

Application benefits

- Simple two stage extraction
- Stage one removes fats and lipophilic compounds for assay robustness
- Automation of stage two Supported Liquid Extraction (SLE) is possible using a 96-well plate format
- Unique selectivity and exceptional robustness using Thermo Scientific™ Hypercarb™ HPLC columns
- 4.5 min run time ensures high throughput
- Excellent recovery and no ion suppression

Goal

To develop and test an improved assay for the quantitative determination of acrylamide from a range of foodstuffs

Introduction

Acrylamide was discovered in foodstuffs in 2002 and was shown to form due to a Maillard reaction between amino acids and reducing sugars. This means carbohydrate rich foods are particularly susceptible and high concentrations have been determined in a variety of heat-processed food. This caused widespread alarm due to potential health risks,

which is not entirely surprising, given that the toxicological properties of acrylamide were studied long before its discovery in foodstuffs.

Acrylamide is classified as probably carcinogenic to humans according to the International Agency for Research on Cancer and also as a reproductive and mutagenic toxicant following *in vitro* and *in vivo* mammalian studies.

Between 2007 and 2011, after EC recommendations and a substantial screening program, indicative values were set for acrylamide in a variety of foodstuffs. The year 2017 saw the introduction of benchmark levels and guidance for acrylamide reduction in heat processed food, meaning suitable analytical methods were required for acrylamide detection. This was not without difficulty given the complexity of the matrices combined with the polarity and low mass of acrylamide.

This application serves to build on experience from our customers' assays and eliminate the problem of lipids making their way through the extraction procedure, particularly in potato chips. The revised procedure removes fats and more lipophilic compounds during step 1. The use of a specific elution solvent mixture for the SLE in step 2 ensures a clean extract, excellent recovery, and more importantly, no ion suppression in a range of matrices. Other improvements include revised column dimensions and a gradient that includes an organic flush, improved k', and increased linear velocity to minimize peak widths. The higher signal/noise ratio observed as a result of these changes allows the injection volume to be minimized, further reducing the potential for column and system fouling.

Experimental

Table 1. Summary of assay results

Parameter	Value
Analyte:	Acrylamide
Analytical matrix	Potato chips, ground coffee and baby food
Calibration range	100–5000 ng/g
Lower limit of quantification (LLOQ)	100 ng/g
Sample volume	1 g
Calibration model	Linear regression
Weighting factor	$1/x^2$
Accuracy (bias) and precision	-12.5–12.7 (CV% 0.6–4.9)
Carryover	No carryover observed
Specificity	No specificity issues observed in potato chips, ground coffee, or baby food
Absolute recovery	82.5–106% at LQC

Chromatography consumables

- Thermo Scientific™ Nunc™ 15 mL conical sterile polypropylene centrifuge tubes (P/N 339651)
- Thermo Scientific™ HyperSep™ SLE 96-well plate 200 mg (pH 9)/2 mL (P/N 60109-200-2-9W)
- Thermo Scientific™ Hypercarb™ HPLC column, 100 × 2.1 mm, 5 µm (P/N 35005-102130)
- Thermo Scientific™ WebSeal™ 96-well plate, square well, V bottom 2 mL, 50 pack (P/N 60180-P135)
- Thermo Scientific™ WebSeal™ non-sterile mat, square well, 5 pack (P/N 60180-M131)
- Thermo Scientific™ HyperSep™ universal vacuum manifold, gauge and waste base (P/N 60104-231)
- Thermo Scientific™ HyperSep™ glass block vacuum manifold pumps (P/N 60104-241)

Reagents

- Thermo Scientific™ UHPLC-MS grade water (P/N W8-1)
- Thermo Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Thermo Scientific™ Optima™ UHPLC-MS grade methanol (P/N A456-1)
- Fisher Chemical™ HPLC grade ethyl acetate (P/N E/0906/17)
- Fisher Chemical™ HPLC grade tetrahydrofuran (P/N T/0706/PB17)
- Fisher Chemical™ HPLC grade dichloromethane (P/N D/1857/17)
- Fisher™ BioReagents ethylene glycol (P/N BP230-1)
- Fisher Scientific™ Optima™ LC-MS grade formic acid (P/N A117-50)

Sample preparation and analysis

Sample preparation protocol

1. Weigh 1 g ± 0.05 g of ground potato chips, ground coffee, or homogenized baby food in a 15 mL Nunc tube (P/N 339651).
2. For calibration standards and quality controls, fortify the food matrix with an appropriate volume (10 µL) of standard and internal standard (10 µL of internal standard only for single blanks and unknowns).
3. Allow 30 min to absorb and the solvent to evaporate.
4. Add 10 mL of water and place on a horizontal flatbed shaker for 30 min.
5. Add 2 mL of dichloromethane and place back on the flatbed shaker for 10 min.

6. Centrifuge at 3500 × g for 15 min.
7. Take a 200 µL aliquot of the top phase and add to the appropriate wells of a HyperSep SLE 96 well plate 200 mg (pH 9)/2 mL (P/N 60109-200-2-9W).
8. Allow to absorb for 15 min (a small pulse of vacuum may be required to draw the sample into the plate).
9. Elute with 2 × 750 µL of ethyl acetate/tetrahydrofuran (50/50, v/v) into a 2 mL 96-well plate (P/N 60180-P135) containing 20 µL of ethylene glycol.
10. Apply a pulse of vacuum to fully dry the plate.
11. Evaporate under N_2 at 40 °C for approximately 1 h.
12. Add a further 200 µL of water to each well, cap (P/N 60180-M131) and vortex mix briefly.
13. Centrifuge at 3500 × g for 15 min.

Instrumentation

- Thermo Scientific™ Vanquish™ Horizon UHPLC system consisting of the following:
  - System base Vanquish™ Horizon (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- Thermo Scientific™ TSQ Endura™ Triple-Stage Quadrupole Mass Spectrometer (P/N IQLAAEGAAXFAPJMBFU)

Separation conditions

Parameter	Value
Mobile phase A	Water/formic acid (100/0.5, v/v)
Mobile phase B	Methanol
Flow rate	0.5 mL/min
Run time	4.5 min
Column temperature	60 °C, with active pre-heating and still air mode
Injection volume	1 µL
Gradient parameters	Table 2

Table 2. Gradient parameters

Time	Flow (mL/min)	% B	Curve
0.00	0.500	0.0	5
0.50	0.500	0.0	5
2.00	0.500	10.0	5
2.00	0.500	100	5
3.00	0.500	100	5
3.00	0.500	0.0	5
4.50	0.500	0.0	5

MS/MS conditions

Table 3. MS/MS source parameters

Parameter	Value
Source	Ion Max source with HESI-II probe
Polarity	Positive
Spray voltage	3500 V
Vaporizer temperature	400 °C
Sheath gas pressure	50 Arb
Aux gas pressure	15 Arb
Ion transfer tube temperature	350 °C
CID gas pressure	1.5 mTorr

Table 4. Compound transition details

Compound	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)
Acrylamide	Positive	72.3	55.3	11
Acrylamide-d_3	Positive	75.3	58.3	12

Data processing

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.10, was used for data acquisition and analysis.

Method assessment and results

Calibration model and range

The calibration model was assessed to be a linear regression with $1/x^2$ weighting (Figure 1). Eight calibration standards over the range 100–5000 ng/g were freshly prepared in water/ethylene glycol (90/10, v/v). As acrylamide was expected to be found above the LLOQ in many matrices and determining the concentration of acrylamide in many food types in one run was the aim, a non-extracted calibration line was used. Absolute recovery was calculated intra-day for each matrix.

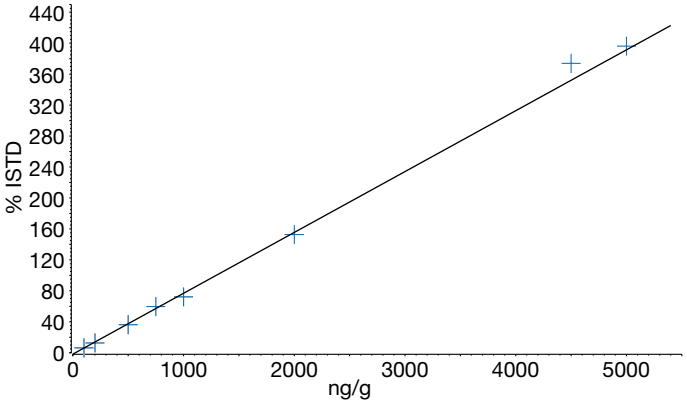

Figure 1. Calibration curve for acrylamide

Accuracy and precision

Accuracy and precision (A&P) were determined by analysis of six replicates of quality control (QC) samples at four concentrations over the calibration range tested. QCs were assessed as non extracted samples and as extracts in each of the three food types. For the food QCs, the average concentration of acrylamide was determined from three single blank (SB) samples of each food type. The average concentration measured in the three SB samples was added to the nominal concentration to give a new theoretical QC concentration for each food matrix.

The nominal concentrations were 100 ng/g (LLOQ), 250 ng/g (LQC), 800 ng/g (MQC), and 4000 ng/g (HQC). Intra-batch A&P was acceptable for all compounds with bias being <13% and CV <5% at each of the four level QC levels in both non-extracted and extracted food QCs. Accuracy and precision data (Tables 5–8) and a representative potato chip chromatogram at the LQC level (Figure 2) is presented.

Table 5. Accuracy and precision – non-extracted

	Acrylamide ng/g			
	LLOQ 100	Low 250	Medium 800	High 4000
Non-extracted	114	232	763	3760
	120	232	760	3650
	114	247	794	3560
	109	241	767	3680
	113	228	769	3670
	106	221	789	3670
Mean	113	234	774	3670
Bias %	12.7	-6.6	-3.3	-8.3
CV %	4.1	3.9	1.8	1.7

Table 6. Accuracy and precision – ground coffee

	Acrylamide ng/g			
	LLOQ 334	Low 484	Medium 1030	High 4230
Ground coffee	341	437	983	3960
	323	434	982	3900
	327	459	975	3970
	321	448	993	3810
	314	449	986	3910
	333	437	980	3950
Mean	327	444	983	3920
Bias %	-2.2	-8.3	-4.5	-7.3
CV %	2.9	2.1	0.6	1.5

Table 7. Accuracy and precision – ground potato chips

	Acrylamide ng/g			
	LLOQ 388	Low 538	Medium 1090	High 4290
Ground potato chips	397	516	1110	3880
	421	504	1040	3850
	403	497	1070	3870
	407	501	1080	3940
	407	518	1100	3850
	404	512	1030	3790
Mean	406	508	1070	3860
Bias %	4.7	-5.6	-1.8	-10.0
CV %	2.0	1.7	3.0	1.3

Table 8. Accuracy and precision – homogenized baby food

	Acrylamide ng/g			
	LLOQ 146	Low 296	Medium 846	High 4050
Homogenized baby food	147	259	775	3770
	139	264	789	3900
	138	259	762	3800
	136	252	793	3750
	136	262	779	3800
	129	261	772	3840
Mean	138	259	778	3810
Bias %	-6.0	-12.5	-8.0	-5.9
CV %	4.2	1.6	1.5	1.4

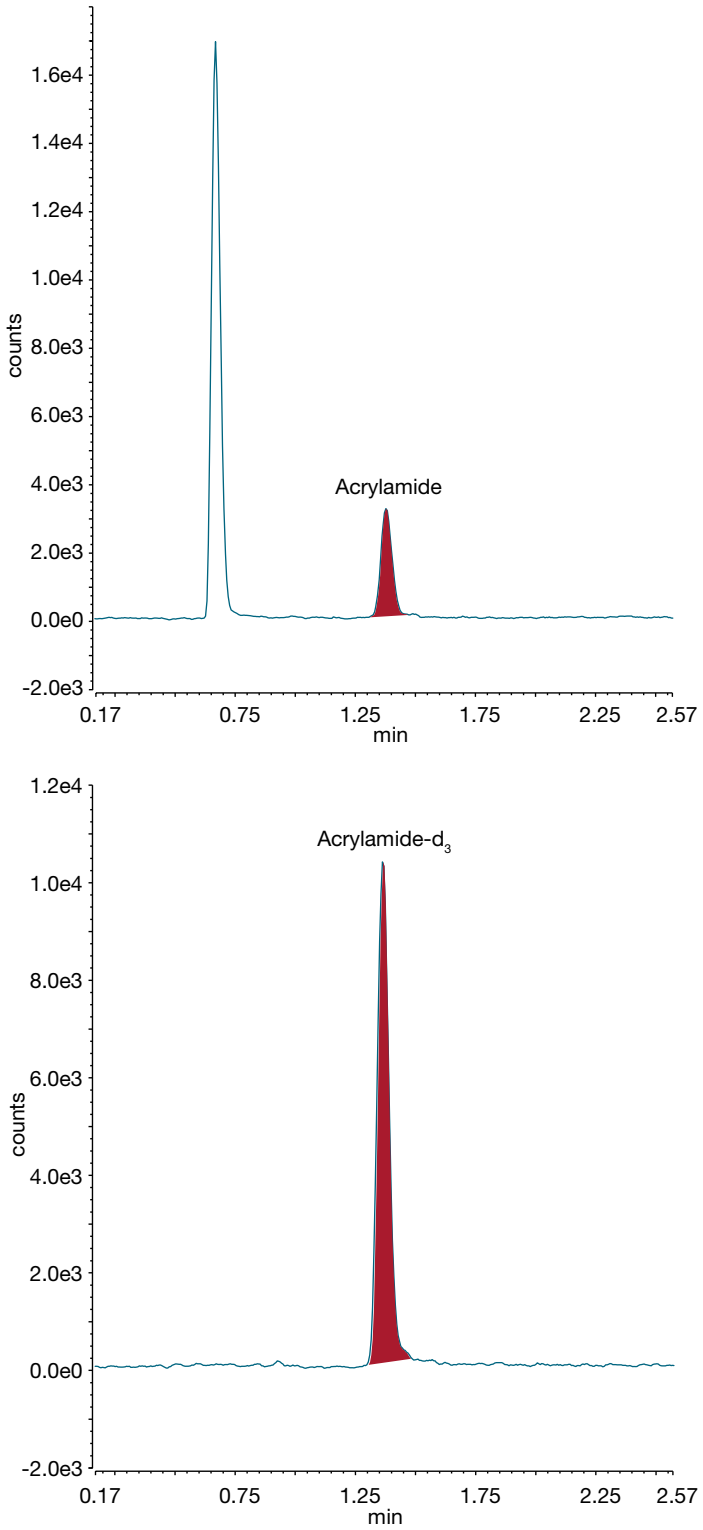

Figure 2. Example chromatogram at LQC in ground potato chips

Determination of assay specificity

As measurable concentrations of acrylamide were expected to be seen in each of the sources of food, it was only possible to check that the working concentration of internal standard did not contribute to the acrylamide channel due to isotopic impurity. One double blank sample (no acrylamide or internal standard added) prepared in water/ethylene glycol (90/10, v/v) and one single blank sample (internal standard only added) prepared in water/ethylene glycol (90/10, v/v) were chromatographed.

Acceptable specificity for acrylamide and acrylamide-d_3 was demonstrated as no interfering peaks were detected in the blank samples. A representative chromatogram of a single blank is shown in Figure 3.

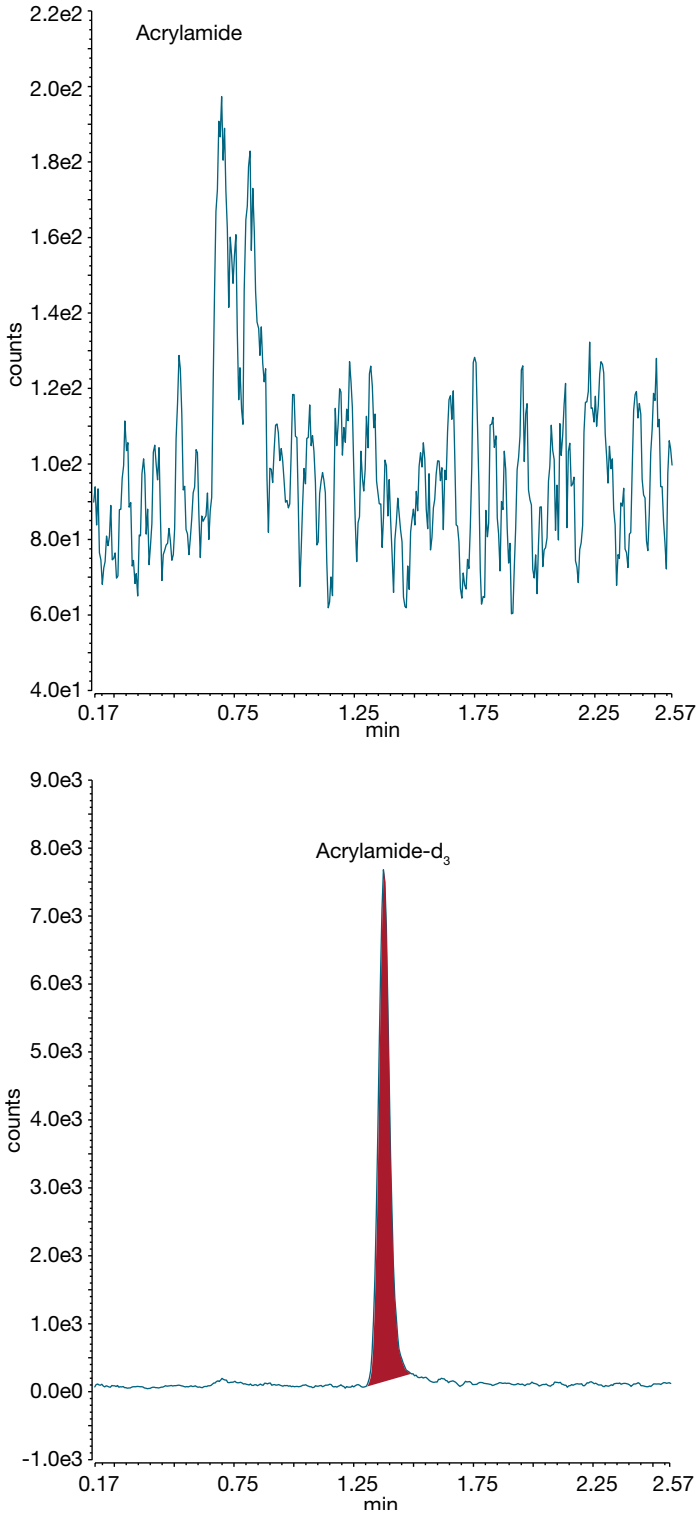

Figure 3. Example chromatogram of a single blank prepared in water/ethylene glycol (90/10, v/v)

Determination of assay carryover

The carryover was assessed by injecting two double blank samples after the injection of the highest calibration sample (ULOQ). The area response of the analyte and internal standard in the blank samples was compared to the area responses of the non-extracted LLOQ samples after them. No carryover was present using the Vanquish Horizon UHPLC system as shown in Figure 4.

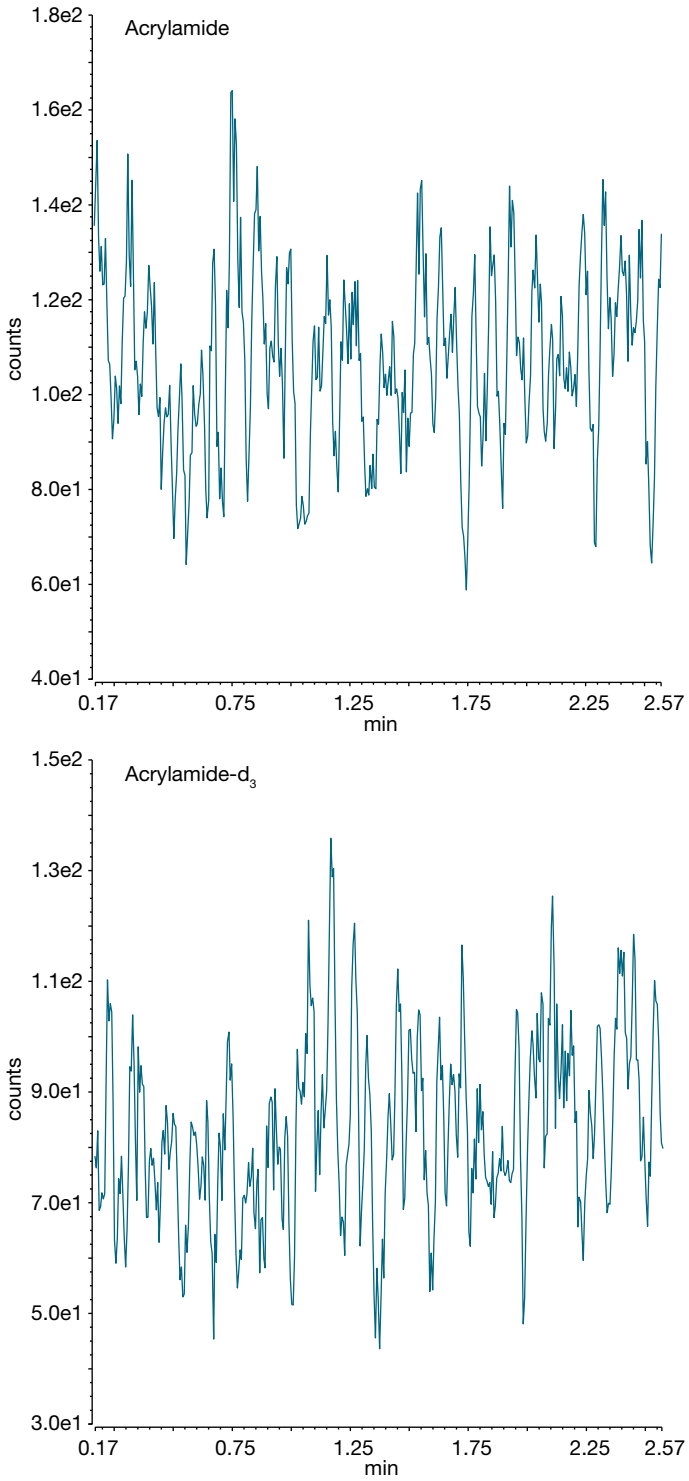

Figure 4. Chromatogram of a double blank prepared in water/ethylene glycol (90/10, v/v) following a ULOQ sample

Determination of absolute recovery

For complex matrices such as those encountered in food and beverage analysis, the use of a suitable internal standard is strongly recommended. The internal standard should be added before any manipulation of the samples where it will help to correct for small volume discrepancies during sample transfers and small variations in recovery. As the internal standard used here is also stable-labeled, it will effectively compensate for potential ion suppression or enhancement during MS/MS analysis as used for this analysis.

As acrylamide was shown to be present in each of the food types tested, it was not possible to separate the matrix effects and recovery experiments as would normally be the case. Absolute recovery (combined matrix effects and recovery) was calculated by comparing the average peak area for the internal standard in the six LQC replicates, for each of the three sources of food matrix, compared to the internal standard peak area in the non-extracted QCs. This approach is valid given that no peak is visible for the internal standard transition in double blank food matrices. It is also a stable-labeled version of acrylamide, so it will have identical properties throughout the extraction procedure and during UHPLC-MS/MS analysis.

The results for the absolute recovery assessment are shown in Table 9.

Table 9. Absolute recovery of acrylamide from foodstuffs

	ISTD peak area LQC			
	Non-extracted	Ground coffee	Ground potato chips	Homogenized baby food
	34659	29887	36593	36243
	36235	29233	36423	35793
	35130	27483	38787	33963
	34098	28958	37799	32340
	36094	29765	37829	35540
	37262	30724	38792	36434
Mean	35580	29342	37704	35052
CV %	3.3	3.7	2.7	4.5
Absolute Recovery %	-	82.5	106.0	98.5

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Application of the method for the quantitation of acrylamide from other matrices

As the method was shown to be suitable for the quantitative determination of acrylamide in three different matrices, it was subsequently applied to determine the acrylamide concentration in burnt brown and white toast.

The toast was burnt to a point where it was felt it might still be consumed (Figure 5 and Figure 6). The whole pieces were then ground to a fine powder and three replicate samples from each piece were taken for analysis and processed according to the sample preparation procedure.

The concentrations determined from the samples are presented in Table 10. The acrylamide concentration in the brown toast is double that measured in the white toast and the figure measured in brown toast is comparable to that measured in potato chips. All three replicates fall within a narrow distribution of concentration meaning that the sample was homogenous and the analysis procedure consistent and highly reproducible.

Figure 5. Burnt white toast before homogenization

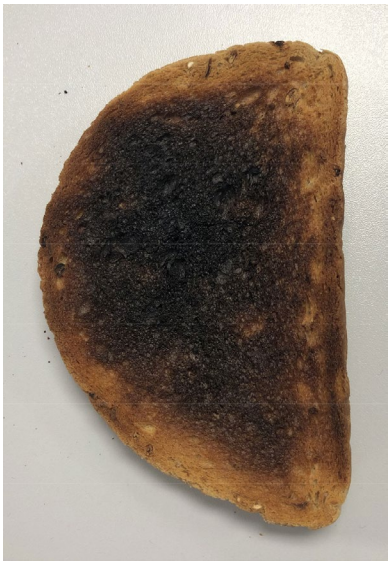

Figure 6. Burnt brown toast before homogenization

Table 10. Concentration of acrylamide in burnt toast

	Acrylamide ng/g	
	White toast	Brown toast
	148	309
	156	319
	145	325
Mean	150	318
CV %	3.8	2.5

Conclusions

- Simple, robust two-stage extraction for the quantitation of acrylamide from food matrices
- Improved clean-up eliminates matrix effects and ensures high recovery
- 4.5 min run time for high throughput
- Applicable to a wide range of foodstuffs

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