BASELINE

SELECTION AND IMPROVING OF FIT-FOR-PURPOSE SAMPLING PROCEDURES FOR SPECIFIC FOODS AND RISKS

PROJECT DELIVERABLE

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SUMMARY

In this deliverable an analytical methodology based in liquid chromatography coupled with tandem mass detection is described for the quantification of acrylamide in food and, in particular, in plant products with a high content of starch and aminoacids.

The deliverable is divided in different sections describing in detail the different stages of the procedure: extraction of samples, calibration, reporting and validation. The quality assurance and quality control procedures necessary to improve reliability and assure the performance of the method are also described as well as all the practical requirements needed to trustfully apply the method in a laboratory.

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AIM

The aim of this method is to describe a procedure to determine acrylamide in food products with a high content of starch and aminoacids by liquid chromatography coupled with tandem mass detection and the quality requirements for its practical implementation in a laboratory.

BASIS

Acrylamide is extracted in aqueous solution, impurities are cleaned up with solid phase extraction and analyte measured by HPLC-MS/MS.

MATERIALS AND EQUIPMENT

APPARATUS AND EQUIPMENT

- Ultraturrax homogeniser/Disperser
- Mincer/grinder
- Micropipettes of different volumes
- Precision balance
- Centrifuge
- Orbital shaker
- MilliQ equipment for ultrapure water
- Vacuum collector
- High performance liquid chromatography equipment coupled with mass detector.

MATERIALS

- Falcon tubes of 50 ml
- Pasteur pipettes
- PVDF filters 0.45 µm
- General laboratory material (flasks, volumetric flasks, etc...)
- Chromatographic vials and micro-vials
- SPE Bond Elut Accucat cartridge (200 mg and 3 ml) from Varian
- SPE Oasis HLB cartridges (6cc and 200 mg) from Waters
- Test tubes
- Glass syringes.

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REAGENTS

- MilliQ water
- Methanol (HPLC grade)
- Formic acid (MS quality)
- Dichloromethane
- Argon
- Acrylamide standard $^{13}C_3$ 99% (isotopically marked acrylamide)
- Acrylamide standard 99%

PROCEDURE

STANDARDS PREPARATION

Acrylamide

Solution A, 10 mg/ml: weight and dissolve 0.01 g of acrylamide in MilliQ water and take to a final volume of 1 ml in a volumetric flask.

Solution B, 10 µg/ml: take 25 µl of solution A and take to a final volume of 25 ml with MilliQ water.

Isotopically marked acrylamide

Solution A*, 10 mg/ml: weight and dissolve 0.01 g of $^{13}C_3$ acrylamide in MilliQ water and take to a final volume of 1 ml in a volumetric flask.

Solution B*, 10 µg/ml: take 25 µL of solution A* and take to a final volume of 25 ml with MilliQ water.

SAMPLE EXTRACTION

Extraction

1. Samples are homogenised in a grinder.
2. Weight 2.5±0.05 g of sample in a 50 ml falcon tube (3 replicates).
3. Add 10 ml of MilliQ water, 1 ml of dichloromethane and 140 µl of solution B* (in all the 3 tubes). Fat content of the samples will be separated in the dichloromethane phase.
4. Add 140 µl of solution B in one of the tubes (spiked sample).

5. Mix with the ultraturrax and add 9 ml of MilliQ water to clean the ultraturrax dispersing element.
6. Shake for 20 minutes in orbital stirrer.
7. Centrifuge for 20 minutes at 10000g.
8. Filter the aqueous phase through 0.45 µm PVDC filter (up to 3 ml of extract).

Purification

9. Conditioning of SPE Oasis cartridge: pour 3.5 ml of methanol (cartridge solvation) followed by 3.5 ml of MilliQ water (cartridge equilibration).
10. Sample loading: pour 1.5 ml of filtered extract in the SPE cartridge.
11. Wash the cartridge with 0.5 ml of MilliQ water. Avoid the cartridge to become dry.
12. Elute the retained acrylamide of the sample with 2.5 ml of MilliQ water (Elution A).
13. Conditioning of SPE Accucat cartridge: 2.5 ml of methanol (cartridge solvation) followed by 2.5 ml of MilliQ water (cartridge equilibration).
14. Elution A (2.5 ml) is loaded in the Accucat cartridge. Avoid the cartridge to become dry and collect the eluted sample.
15. Elute with 1.5 ml of MilliQ water and collect the total final volume of 4 ml in a test tube.
16. Transfer the elution to a vial for chromatographic analysis. If the sample cannot be immediately analysed, it can be stored in refrigeration.

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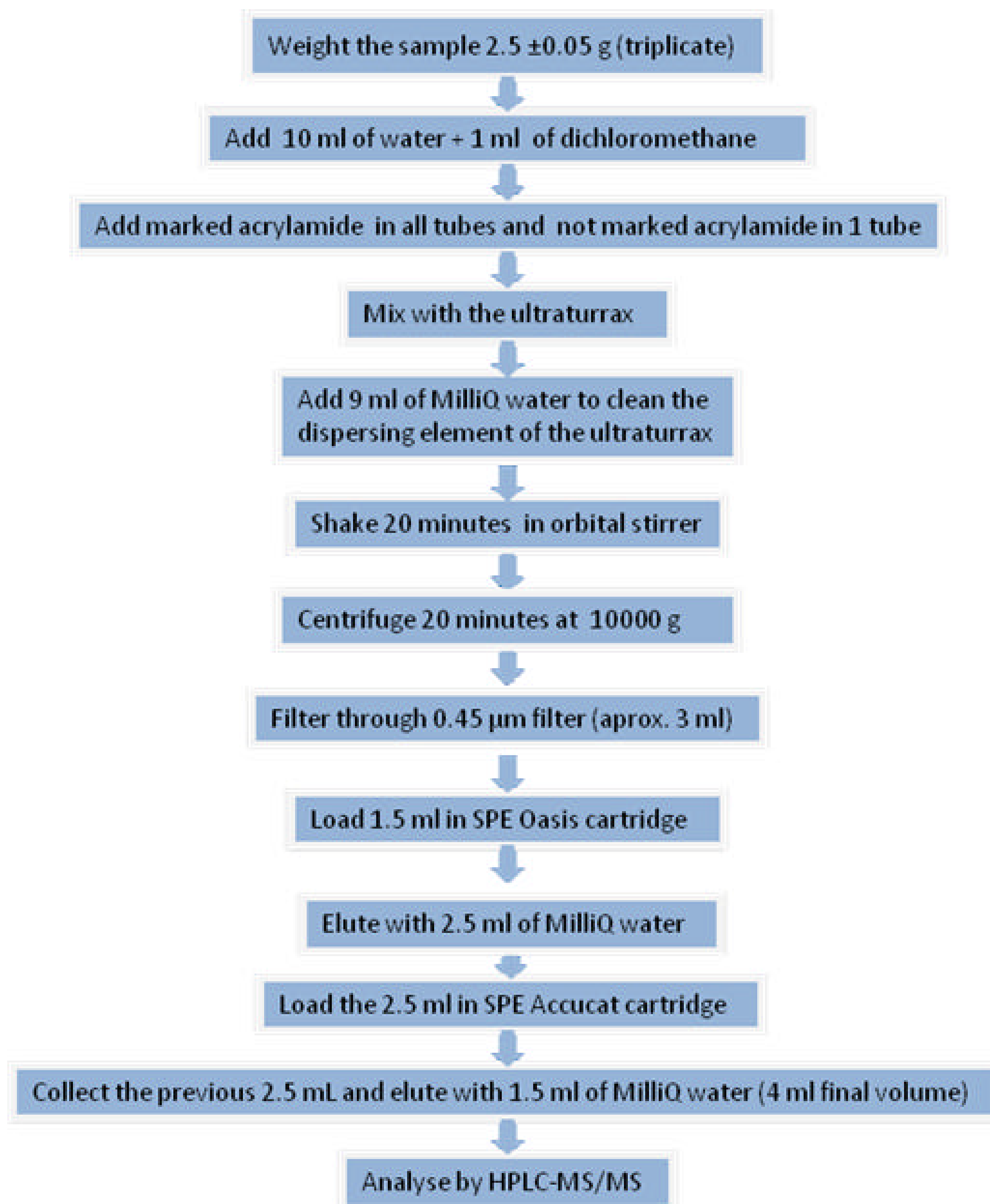
Flow chart of the process



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INSTRUMENTAL ANALYSIS

HPLC conditions

- C18 reverse phase column
- Injection volume: 20 µL
- Flow rate: 0.25 ml/min
- Mobile phase composition and gradient:
  - A: MilliQ water with formic acid (0.25%)
  - B: Methanol

Table 1. HPLC gradient conditions.

Time (min)	% A	%B
0	99	1
4.5	99	1
7	20	80
9	20	80
10	99	1
13	99	1

Mass detector conditions

- Source temperature: 120°C
- Desolvation temperature: 350°C
- Cone gas flow: 50 L/h
- Desolvation gas flow: 700 L/h

Table 2. Mass detector conditions.

Compound	Transition	Cone voltage (V)	Collision energy (V)
Acrylamide	**72>55**	20	10
	72>27		14
Marked acrylamide	**75>58**	20	10
	75>29		19

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CALIBRATION CURVE

Calibration standards are prepared from standard solution of acrylamide and isotopically marked acrylamide (solution B and B*).

500 µL of each solution (B and B*) are transferred to a 25 ml volumetric flask and take to the final volume with MilliQ water. Final concentration for each of them is 200 µg/l (solution C). The lowest calibration level (LCL) is 2 µg/l for both compounds.

Calibration curve is prepared directly in vials according to the table below:

Table 3. Calibration standards.

Number	Final concentration/vial (µg/l)	Solution C volume (µl)	H_2O volume (µl)
1	2.0	10	990
2	5.0	25	975
3	10	50	950
4	30	150	850
5	50	250	750
6	100	500	500
7	200	1000	0

RESULTS

Identification and Confirmation

Tandem mass technology allows a high selectivity in the determination of analytes. Identification and confirmation of a positive result will be performed at the same time using the instrumental software.

Identification and confirmation of acrylamide is performed through the retention time and the comparison between ion ratios in the sample and the standard.

Quantification and Reporting

Results are obtained from interpolation in the obtained external calibration curve.

Calibration curve must be daily injected and fulfil that minimal correlation coefficient (R^2) is 0.99. Calibration standards include acrylamide and marked acrylamide as was described before.

Two calibration curves are computed from internal standard (marked acrylamide) and acrylamide. First one is used to compute recovery in the samples, and second one to quantify acrylamide in samples.

Final concentration of acrylamide in the samples will be expressed as the predicted value (using the calibration curve) corrected by the corresponding recovery coefficient.

METHOD VALIDATION

Method validation should be performed on the most representative matrixes to be analysed in the laboratory.

In particular the representative matrixes that may be considered are:

- Low water- high fat content products (i.e. nuts, coffee, potato crisps).
- Low water- low fat content products (i.e. soft bread, biscuits, crackers, breakfast cereals).
- High water-high fat content products (i.e. French fries from potato, prepared meals).

Linearity

Linearity should be studied through 7 different calibration levels of acrylamide and marked acrylamide within the calibration range. Standards' concentrations are 2, 5, 10, 30, 50, 100 and 200 µg/l.

At least three linearity tests at different sessions should be performed. Slope (m) and intercept (b) are calculated as follows:

$$y = mx + b$$ where,

y: response area

x: acrylamide concentration

Linearity can be computed using the formula:

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$$C_m = \left[1 - \frac{S_m}{m}\right] * 100$$

where,

S_m: standard deviation of the slope

m: slope

C_m: linearity coefficient.

Linearity function must be =95% in a chromatographic method. Criterion for residuals in each of the calibration levels should be ±20% for each of the calibration curves.

Selectivity and specificity

Using the linearity studies and, once determined the retention times and characteristic mass for each compound, it must be checked that no interferences in the mixture are found.

Possible interferences in the samples may also be checked through the recovery data obtained in the spiked replicate sample (spiked with not marked acrylamide).

Limit of Quantification (LOQ)

LOQ correspond with the LCL for acrylamide. Signal to noise ratio must be at least 3:1 .

LOQ should be verified through additions at the LCL in the samples. The recoveries should meet the requirements of trueness and precision described next.

Trueness

It is defined as the closeness of agreement between the average value obtained from a series of test results (i.e. the mean recovery) an accepted reference or true value (ISO 5725-1). The measure of trueness is normally expressed as 'bias'.

Trueness must be determined through the study of recoveries of marked acrylamide in the samples (all samples analysed should be spiked).

Once obtained the concentration values, average, standard deviation, recovery and compatibility index will be computed. Compatibility index is a parameter to show if there

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are significant differences between the theoretical concentration of the added standard and the recovery value obtained. The following formulas should be applied:

$\bar{x} = \frac{\sum x_i}{n}$	$SD \cdot (standard \cdot deviation) = \sqrt{\frac{\sum (x_i - \bar{x})^2}{n}}$	$CompatibilityIndex = \frac{\lvert V_{Ri} - \overline{x_i} \rvert}{\sqrt{U^2_{V^P_{Ri}} + \left(t \cdot \frac{S_i}{\sqrt{n}} \right)^2}} \leq 1$
$Trueness = \frac{V_{Ri} - \overline{x_i}}{V_{Ri}} \cdot 100$	$100 - \text{Trueness} = \text{Recovery}$	

x: obtained concentration values

n: number of repetitions

V_R: reference value (real concentration added)

$U_{V^P_{Ri}}$ =uncertainty of the reference value with a confidence interval of 95%

t: students value for n=10 measures and a=0.05.

Results should be reported corrected for recovery.

Precision

The precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions. The measure of precision usually is expressed in terms of imprecision and computed as standard deviation of the test results (ISO 5725-1). It is usually calculated from recovery or analysis of reference materials, obtained using the same method on the same sample(s) in a single laboratory over a short period of time, during which differences in the materials and equipment used and/or the analysts involved will not occur.

Data obtained from recovery studies can be used to obtain mean value and standard deviation for each concentration and variation coefficients according to the formula:

$$CV = \frac{S}{\bar{x}} * 100$$

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Uncertainty

It is defined as a range around the reported result within which the true value can be expected to lie with a specified probability (confidence level, usually 95%). Uncertainty data should encompass trueness (bias) and reproducibility.

As the process includes multiple independent steps (standards weight, standards mixture preparation, volume addition, repetitions, etc...), uncertainty was computed using the validation process data. Uncertainty of each step was computed and global uncertainty was computed as square root of the sum of individual quadratic uncertainties.

$$U = \sqrt{U_{VR}^2 + \left(W_R \cdot \frac{S_R}{\sqrt{n_R}}\right)^2 + \left(\frac{W_R \cdot S_M}{\sqrt{n_M}}\right)^2}$$

where,

U_{VR}: reference value for uncertainty

S_R: standard deviation of reference samples (obtained in precision tests)

S_M: standard deviation of ordinary samples

W_R: W factor applying depending on the number of repetitions performed to obtain S_R.

n_R: number of repetitions to compute S_R.

n_M: number of repetitions to be computed according to method.

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