QUANTITATIVE ANALYSIS OF ACRYLAMIDE IN TOBACCO, TOBACCO PRODUCTS, FIBRE-BASED MATRICES AND TOBACCO DERIVED PRODUCTS WITH (b) (4)

Purpose

To determine the concentration of acrylamide (AA) in tobacco, tobacco products, fibre-based matrices and tobacco derived products (also called purified products) wit (b) (4)

Applies to

APS

General information

Principle of the method

Acrylamide is extracted after the addition of internal standard (b) (4)

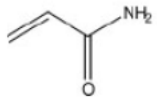


Figure 1. Chemical structural formula of acrylamide

Note: All reference documents and additional information stated "available upon request" are in Swedish. They are available upon request but need to be translated into English first.

Method scope, measurement range and measurement uncertainty

The method is used for quantitative analysis of acrylamide in tobacco, tobacco products, fibre-based matrices and tobacco derived products.

Method's calibration range
(b) (4) ng/ml

Method's measurement range
(b) (4) ppb (ng/g)

In exceptional cases, the measurement range can be increased to (b) (4) ng/ml, which corresponds to (b) (4) ppb.

Measurement uncertainty

The combined relative measurement uncertainty for acrylamide is stated with a coverage factor of 2. **Table 1** shows the combined relative measurement uncertainty for single, duplicate and triplicate samples.

Table 1. The combined relative measurement uncertainty for single, duplicate and triplicate samples with a coverage factor of 2

Analyte	Single sample (%)	Duplicate samples (%)	Triplicate samples (%)
Acrylamide	(b) (4)		

Measurement uncertainty contributions are greatest from bias followed by uncertainty in the standard curve's linearity. In order to reduce the uncertainty contribution from linearity, it is important to have chromatography and sensitivity in MS/MS that corresponds to **Fig. 2**.

Literature references

1. (b) (4)
2.
3.
4.
5.

Internal reference documents (available upon request)

1. (b) (4)
2.

Risk assessment and safety instructions

For risk and safety phrases, see the safety data sheet in (b) (4).

Summarised risk assessment

Preparation of stock and standard solutions of acrylamide (carcinogenic) and (b) (4) must be performed in a fume cupboard. Wear suitable protective clothing and gloves. The transfer of acetonitrile between glass vessels takes place in a fume cupboard or on a draw bench.

Substances hazardous to the environment

None

Flammable substances

(b) (4)

Equipment

Apparatus

(b) (4)

Table 2. General quantification parameters

Name	Retention time (min)	Quantification Trace (m/z)	Qualification Trace (m/z)	Internal Standard Reference
(b) (4)				

Other equipment

(b) (4)

(b) (4)

Solid phase extraction with SPE robot

(b) (4)

(b) (4)

(b) (4)

Chemicals, reagents and solvents

(b) (4)

For certificates of analysis for acrylamide, see binder labelled with "*Acrylamide Analysis Certificate*" in room 206.

Check samples and reference materials

As a check sample, tobacco flour is used and divided into 10 gram packages. (b) (4) no. 24 in room 203. (b) (4) Use one bag at a time until they run out. Description of the check sample and its storage location id available upon request.

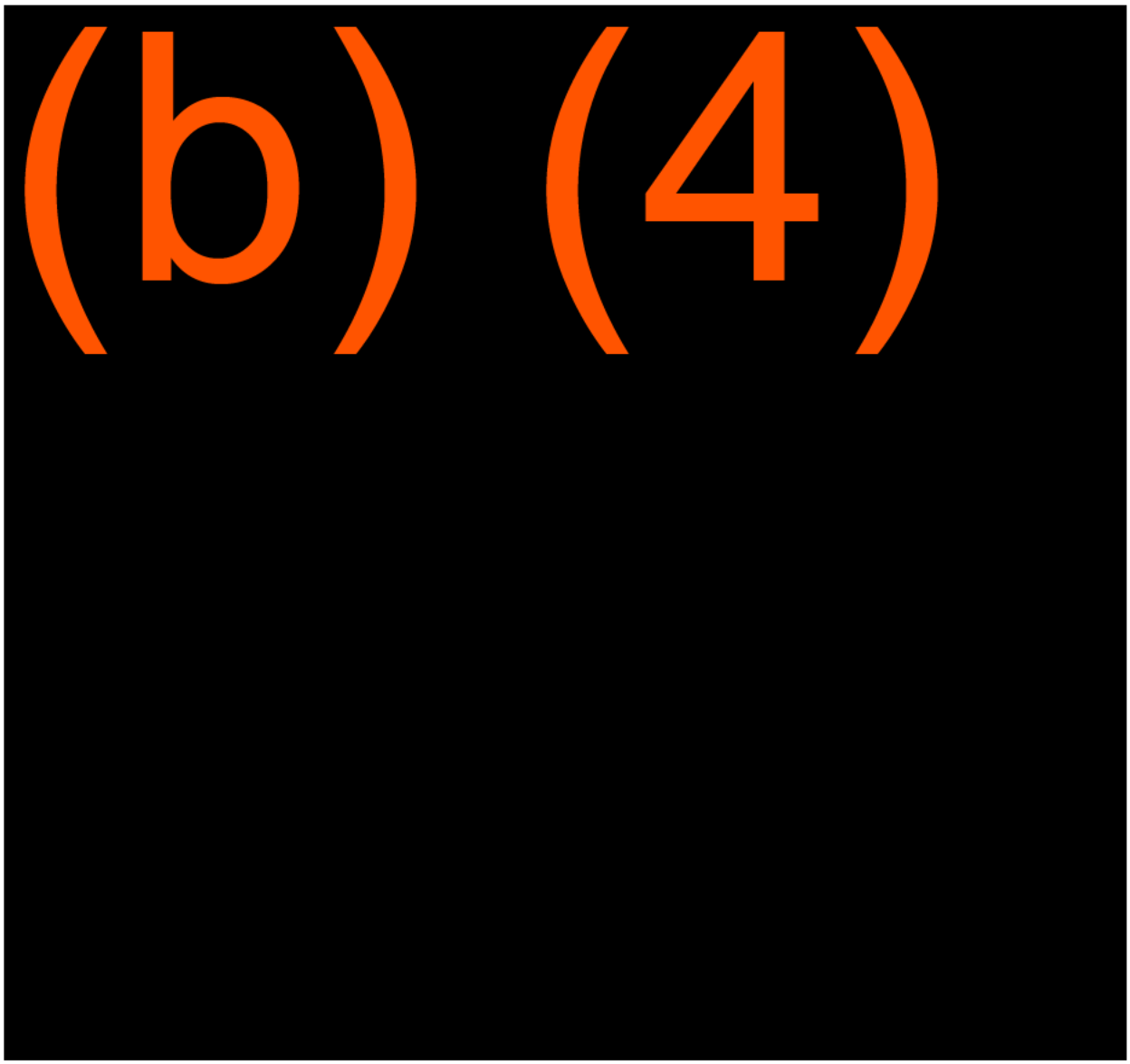
Preparation of standard solutions

General information

All solutions are taken out and allow to reach room temperature before use. Stock solutions, interim standard solutions and calibration standard solutions are stored in a refrigerator.

Preparations of stock, standard and calibration standard solutions

(b) (4)



Calibration standards

Five calibration standards are prepared in five separate 50 ml volumetric flasks (dark) according to **Table 3.** Dilute each solution to 50 ml with MQ water. Shelf life: 1 month in a refrigerator.

Hazard symbols: Not subject to labelling

Table 3. Preparation of calibration standards

(b) (4)

Each calibration standard contains 10 ng/mL of internal standard.

Comparison of new and old calibration standards

When new calibration standards have been prepared, both the new and old calibration standards are analysed. The slope of the new and the old standard curve may not vary more than ± 10%. If it differs by more than ± 10 %, contact the person responsible for the method to discuss measures to take.

Preparation of other solutions

(b) (4)

Sample handling

Sample storage and preparation

Tobacco, tobacco products, fibre-based matrices and tobacco derived products are stored and prepared according to the instruction for preparation of samples (b) (4)

Sample amount

The minimum required amount of sample for all matrices is 6 g in order to perform duplicate analysis and re-analyses. The minimum amount of sample for performing one analysis is around 1 g.

Analysis

Calibration and verification of apparatus

The system's chromatographic performance is evaluated by visually checking the analyte signal/noise ratio (S/N) and peak shape from an injection of calibration standard 1 (Cal1). Compare with **Fig. 2** in the validation report. Start by injecting at least three Cal 1 to equilibrate the system. A standard curve is generated in each sequence by injecting all the calibration solutions first and last in the sequence.

Each sequence is built up as follows:

- Blank (replaces eluent and purges)
- Blank (wash 1: washes the column with isopropanol)
- Blank (replaces eluent and purges)
- Blank (wash 2: equilibrates the column back to methanol)
- Blank (MQ water)
- Cal 1 x3
- Cal 1–5
- Blank (MQ water)
- Check sample 1
- Check sample 2
- Blank (MQ water)
- Samples
- Blank (MQ water)
- Check sample 1
- Check sample 2
- Blank (MQ water)
- Cal 1–5
- Blank (replaces eluent and purges)
- Blank (wash 1: washes the column with isopropanol)

- Blank (replaces eluent and purges)
- Blank (wash 2: equilibrates the column back to methanol)
- Blank (MQ water)

Sample stability

The shelf life of prepared samples in vials that are stored in a refrigerator or sample manager is 7 days.

Analytical procedure

Sample preparation

Samples stored in a freezer can be thawed and weighed out the day before preparation. Flasks with samples are stored in a refrigerator overnight.

1. Weigh out 1.00 ± 0.10 g (to 4 decimal place accuracy) sample in a 50 ml Falcon tube (or 100 ml Erlenmeyer flask).
2. 1 ml of internal standard (interim standard solution 4 (2000 ng/ml)) is added with multipipette (10 ml).
3. Add 19 ml of MilliQ water with a dispensette.
4. Shake the samples for 20 min on a shaker at 130 rpm.
5. Centrifuge the Falcon tubes for 10 min at 2,500 rpm. If Erlenmeyer flasks have been used: Pour about 5–10 ml of extract into the centrifuge tubes and centrifuge the samples for 10 min at 2,500 rpm.

Solid phase extraction, normal pH, with (b) (4)

(b) (4)

Solid phase extraction at pH 12 with (b) (4) (only done on request)

(b) (4)

Special instructions

(b) (4)

Documentation

Write on the work list who has weighed out and prepared the samples, the names of the calibration standards used, and the internal standard solution, results file (if different from the batch name), who evaluated the analysis and any comments on the analysis or control chart.

Data

Collection and storage of data

(b) (4) The samples are injected with auto injector. In order to control the injector, a sample/work list is created in which the sample identity is specified along with the methods to be used.

Calculations

(b) (4)

For calculation of sample concentration:

$$\text{Amount in sample (ppb as is)} = \frac{C}{M} \times 20 \qquad \text{Equation 1}$$

where:
C = the concentration estimated from the calibration curve (ng/ml)
M = weighed out amount of sample (b) (4) (g)
20 = conversion factor/dilution factor (ml)

Quality assurance

Control chart

For the internal quality control, two check samples are analysed in each sequence according to (b) (4) (available upon request) and ensures that sample preparation has been undertaken as planned. The results are given in ppb as is. (b) (4) Note that a comment must be entered in the control chart for any value that falls outside the limit, even if the analysis is approved.

If the analysis results can not be approved in compliance with the criteria in (b) (4) (b) (4) (available upon request), the following measures must be taken:

- The Cone Gas Nozzle and Sample Cone in the MS instrument must be cleaned.
- A new internal standard solution for spiking of samples is to be prepared.

Comments regarding the issue are entered in the control chart and in the event of extended comments, refer to the work list.

Standard curve criteria

For each quantification batch, the linearity and accuracy (% CV) of the standard curve are verified in (b) (4) The correlation coefficient should be ≥ 0.995 and the accuracy should be between 85 and 115% for each calibration standard in order for the standard curve to be approved. If the standard curve does not meet the necessary requirements, up to three injections at the same or different levels of concentration may be removed from the standard curve, if this improves accuracy. However, at least one point of Cal 1 must be approved, as well as one point higher than the highest quantified concentration of a sample. If the standard curve does not meet the necessary requirements contact the person responsible for the method.

Duplicate and triplicate samples

In the duplicate samples analysis, the difference between the samples should not exceed 20%. When analysing triplicate samples, the difference between samples should not exceed 22%. If this is exceeded, the samples must be weighed out again, re-prepared and re-analysed. If a value < LOQ and a value > LOQ is obtained for duplicate samples, then a re-analysis should be done if the last mentioned value is greater than 3 standard deviations above LOQ (as it is calculated for repeatability).

Confirmation

(b) (4)

If the response in a sample is higher than the highest standard

Concentrations above 100 ng/ml

When concentrations exceed 100 ng/ml, in exceptional cases, the measurement range can be increased to 500 ng/ml. Xevo TQ-S uses linear curve fitting while (b) (4)

Concentrations above 500 ng/ml

If the concentrations in a sample is greater than 500 ng/ml, the analysis is not accepted and the sample is re-weighed, but with a smaller amount, for a new analysis. Only the results from the newly prepared sample are reported. The sample injected after the sample with high analyte concentration may, in the case of suspected carry-over, be re-analysed to exclude carry-over.

Reporting of analysis results

(b) (4)

Revision history

(b) (4)

Person responsible
Director APS

Validation

The matrices and samples that are used for validation are listed in **Table 4**.

Supporting documentation for validation
Calculations and all raw data files used are available upon request.

Table 4. Investigated matrices in the validation

Sample	Sample type	Validation
(b) (4)		

Specificity

Despite extensive sample preparation and selective detection through MS/MS good separation is needed to ensure good selectivity, see **Fig. 2.**

(b) (4)

The selectivity has been good for all the investigated matrices.

(b) (4)

Cross talk

(b) (4)

Carry-over

Carry-over was checked by injecting the strongest standard followed by a blank injection. No peak was detected in the blank injection, consequently no presence of carry-over.

Repeatability

(b) (4)

(b) (4)

Precision within the laboratory

Precision within the laboratory has been determined for (b) (4) using two different laboratory technicians. The standards were also varied by using standards prepared from two different weighings in.

Pooled RSD % for the two matrices:

(b) (4)

At the additional validation in 2013, (b) (4). The combined results from the original and additional validation are presented below.

RSD pool for all matrices:

(b) (4)

Reproducibility/Sampling comparison

(b) (4)

Evaluation of sample comparisons with other laboratories

(b) (4)

Accuracy

(b) (4)

Table 5. Accuracy for spiked samples

(b) (4)

Bias from accuracy

The estimated concentration error in the method to the true (spiked) concentration in % (bias) is calculated as the square root of the sum of squares of accuracy -100 (see **Table 4**) and the uncertainty in the addition of the analyte. Bias from accuracy is used for calculating measurement uncertainty. (b) (4)

Matrix effect

(b) (4)

Yield

The "true" yield (independent of matrix effects in the detector) for the extraction process has been determined by comparing the areas of (b) (4)

(b) (4)

Process efficiency

(b) (4)

Limit of detection (LOD)

(b) (4)

Limit of quantification (LOQ)

(b) (4)

Linearity

(b) (4)

Robustness

(b) (4)

Stability

Stock standards of (b) (4) have been shown to be stable for at least two years when stored in a refrigerator. Both standards and samples have been shown to be stable in storage in the auto-injector (4°C) for at least a week.

Measurement range and measurement uncertainty

The measurement range of the method was determined to 60 - 10000 ppb. The relative measurement uncertainty for all matrices from the original validation and additional validation 2013 are merged. (b) (4) The largest contributions to measurement uncertainty come from bias followed by the uncertainty in the linearity of the standard curve.

Additional validation of new instrumen(b) (4)

Repeatability

(b) (4)

Table 6. (b) (4) .

Sample	Concentration AA (mean value) ppb	RSD (%)	RSD pool (%) All three samples
(b) (4)			

Linearity for (b) (4)

(b) (4)

LOD) and LOQ for (b) (4)

(b) (4)

The cross validation analysis of the same samples for (b) (4)

(b) (4)

Conclusion

(b) (4)

Appendix 1. Analysis on (b) (4)

UHPLC system: (b) (4)

Detector: (b) (4) .

UHPLC data system: (b) (4)

UHPLC parameters:

Eluent agent
Eluent A: MQ water
Eluent B: Methanol
Eluent B: Isopropanol (wash of column)

Injection volume: 1 µl

Programming of the UHPLC pumps in (b) (4) for chromatographic separation and washing of column is performed as shown in **Tables 7**, **8a and 8b.**

Table 7. Time programming of (b) (4) pumps

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Gradient type
(b) (4)				

(b) (4)

(b) (4)

When analysing acrylamide, the UHPLC column needs to be washed after about 20 samples in order for the peak symmetry to be good. When washing the column, two wash programs after another are needed. (b) (4) (**Table 8b**). Isopropanol and MQ water are used as eluents.

The switching of mobile phase at the beginning and end of the gradient is made with a switch valve built-into the MS. Settings for the method:

Initial flow state: Waste

Event	Time(min)	Name	Action
(b) (4)			

Table 8a. Time programming of (b) (4) pumps, Wash 1

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Gradient type
(b) (4)				

Table 8b. Time programming of (b) (4) pumps, Wash 2

Time (min)	Flow (ml/min)	% Eluent A	% Eluent B	Gradient type
(b) (4)				

MS/MS-parameters

Polarity: ESI positive mode

NOTE: The parameters below may be changed if necessary, for example, following service and cleaning when technicians re-optimise certain parameters or if the sensitivity changes during operation and needs to be adjusted. The parameters should therefore be considered as approximate or as initial values.

(b) (4)

MRM Functions

Function 1

(b) (4)

Data processing:

(b) (4)

The substance-specific parameters for acrylamide and (b) (4) are summarised in **Table 9**.

Table 9. Multiple Reaction Monitoring (MRM) parameters for detection

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Dwel time (sec)	Cone (V)	Collision (eV)
(b) (4)					

Appendix 2. (b) (4)

Apparatus

UHPLC system: (b) (4)

UHPLC column: (b) (4)

Detector: (b) (4) .

UHPLC data system: (b) (4)

UHPLC parameters

Eluent agent
Eluent A: MQ water.
Eluent B: Methanol
Eluent B: Isopropanol (wash)

Injection volume: 1 µl

Programming of the UHPLC pumps in (b) (4) for chromatographic separation and washing of column is performed as shown in **Tables 7**, **8a** and **8b** in **Appendix 1.**

(b) (4)

(b) (4)

MS/MS-parameters

Polarity: ESI positive mode

NOTE: The parameters below may be changed if necessary, for example, following service and cleaning when technicians re-optimise certain parameters or if the sensitivity changes during operation and needs to be adjusted. The parameters stated below should therefore be considered as approximate or as initial values.

(b) (4)

(b) (4)

Multiple Reaction Monitoring (MRM)

Function 1

Type MRM

(b) (4)

Data processing:

(b) (4)

The substance-specific parameters for acrylamide and (b) (4) are summarised in **Table 10**.

Table 10. Multiple Reaction Monitoring (MRM) parameters for detection with (b) (4)

(b) (4)