EURL-MP-method_007 (version 1)
Determination of opium alkaloids in poppy seeds and poppy seed containing bakery products by LC-MS/MS

Analyte group:	**Plant toxins** – opium alkaloids
Analyte(s):	Morphine Codeine
Commodity group:	Poppy seeds, poppy seeds containing bakery products
Commodities validated:	Poppy seeds, bakery products containing poppy seeds (bread, cake, biscuits)
Technique:	Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:
Not applicable

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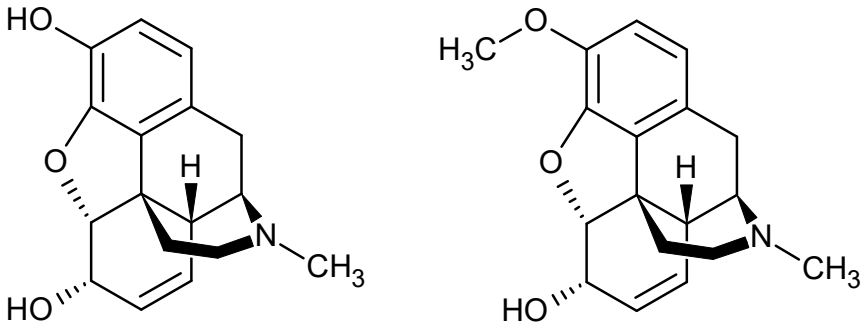
1 Introduction

Opium alkaloids (OAs) are secondary metabolites that are stored and metabolised in the latex of the poppy plant (*Papaver somniferum* L.). Except for the seeds, the latex is present in all parts of the plant, and in particular in the pericarp of the seed capsule. The dried latex of the immature capsules, which is released by incisions, is called opium. Opium contains approximately 20 – 25% alkaloids, of which around 50 different alkaloids have been isolated in pure form.

The alkaloid content of the poppy plant depends on various factors such as variety, location, soil conditions, fertilisation, climate, weather and harvesting time. Opium alkaloids can be divided into two distinct chemical classes: the phenanthrenes, including morphine, codeine, thebaine and oripavine and the benzylisoquinolines, including noscapine and papaverine. Morphine is generally the predominant alkaloid (Figure 1). It is also the pharmacologically most active opiate, having strong narcotic properties. Codeine itself is not pharmacologically active but it is partly metabolised in the liver to morphine [1,2]. For this reason morphine and codeine, but also thebaine and oripavine, have been included in the list of drug abuse substances.



(a) (b)

Figure 1 Chemical structures of morphine (a) and codeine (b)

Poppy seeds are used in bakery products, as decoration of dishes, in fillings of cakes and in desserts and to produce edible oil. Poppy seeds do not contain OAs, but they can become contaminated with alkaloids from the latex as a result of insect damage, or through poor harvesting practices.

Consumption of foods containing poppy seeds that are contaminated with opium alkaloids can lead to adverse health effects and to detectable contents of free morphine in blood as well as measurable concentrations in urine, sufficient to interfere with drug abuse testing. Currently, the EU is considering for poppy seeds a maximum limit of 20 mg/kg morphine equivalents, for the combination of morphine and codeine, in which the toxicity factor of morphine is set at 1.0 and that of codeine at 0.2 [2]. For food products, such as bakery products, a limit of 1.5 mg/kg morphine equivalents is being considered.

2 Scope

This method describes the quantitative determination of two opium alkaloids, morphine and codeine, in poppy seeds and bakery products. The method was developed and validated for the individual compounds in the range from 0.25 to 50 mg/kg in poppy seeds and in the range of 0.1 to 10 mg/kg in bakery products. The method is also applicable for the analysis of thebaine, oripavine, papaverine and noscapine, but this not part of this method.

3 Principle

The opium alkaloids are extracted from poppy seeds and bakery products and stabilised using a mixture of methanol/water/formic acid 60/40/0.4 (v/v/v). An aliquot of the supernatant is diluted with methanol/water 25/75 (v/v) and spiked with internal standards (morphine-d3 and codeine-d3). The sample is analysed by LC-MS/MS using a gradient of acetonitrile and ammonium carbonate buffer pH 9. Quantification is performed on the basis of matrix matched calibration in blank extract with internal standard correction.

4 Reagents

Use only reagents of recognised analytical grade. Solvents shall be of quality for LC analysis, unless otherwise specified.

4.1 Analytical standards

4.1.1 **Morphine**, e.g. reference solution 1000 µg/mL in methanol or solid substance

4.1.2 **Codeine,** e.g. reference solution 1000 µg/mL in methanol or solid substance

4.1.3 **Morphine-d3,** reference solution 1000 µg/mL in methanol

4.1.4 **Codeine-d3,** reference solution 1000 µg/mL in methanol

4.2 Chemicals & solutions

4.2.1 **Water,** deionised Milli-Q and with a minimal resistance of 18.2 MΩ/cm

4.2.2 **Acetonitrile**, LC-MS grade

4.2.3 **Methanol**, LC-MS grade

4.2.4 **Formic acid**, 99-100%

4.2.5 **Ammonia,** 25%, p.a. quality

4.2.6 **Ammonium carbonate,** HPLC quality

Note 1: Ammonium carbonate is offered in different forms by suppliers. Ammonium carbonate with CAS No 506-87-6 has shown to work well.

4.2.7 **Extraction solvent:** 0.4% formic acid in methanol/water (60/40, v/v)
Mix 600 ml of methanol (4.2.3) with 400 ml of water (4.2.1) and 4 ml of formic acid (4.2.4). This solution is stored at room temperature and can be used for 3 months.

4.2.8 Dilution solvent: methanol/water (25/75, v/v)
Mix 25 ml of methanol (4.2.3) and 75 ml of water (4.2.1). This solution is stored at room temperature and can be used for 3 months.

4.2.9 Mobile phase A, 10 mM ammonium carbonate in water, pH 9.0
Dissolve 0.96 g of ammonium carbonate (4.2.6) in 1000 ml of water (4.2.1). Check pH and adjust, when necessary, to pH 9.0 ± 0.1 by adding formic acid (4.2.4) or 25% ammonia (4.2.5) with a positive displacement pipette (5.12). This solution is stored at room temperature and can be used for 1 month.

4.2.10 Stock solutions (200 µg/ml)
Accurately weigh (± 0.02 mg) into separate amber coloured glass bottles (5.3) between 3 and 5 mg (5.1) of the opium alkaloids (4.1.1 and 4.1.2). However, when the standard is only available in a quantity of 3 mg or less, the entire content of the container is used. In that case the weight reported by the supplier is used. Flush the contents of the bottle three times with methanol (4.2.3) to dissolve and collect all material. Add a volume of methanol (4.2.3) in such a way that the concentration of the solution is 200 µg/ml. Take into account the weight, the purity and the appearance form of the standard. The solutions can be used for 24 months when stored in the dark at ≤-20°C.

4.2.11 Mixed standard solution (20 µg/ml)
Pipette 2 ml of each of the stock solutions (4.2.10) of morphine and codeine in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.2.12 Mixed standard solution (1000 ng/ml)
Pipette 1 ml of the mixed standard solution 20 µg/ml (4.2.11) in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.2.13 Mixed standard solution (100 ng/ml)
Pipette 2 ml of the mixed standard solution 1000 ng/ml (4.2.12) in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.2.14 Mixed internal standard (IS) solution (20 µg/ml)
Pipette 1 ml of morphine-d3 1000 µg/ml (4.1.3) and 1 ml of codeine-d3 1000 µg/ml (4.1.4) in a calibrated volumetric flask of 50 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 60 months when stored in the dark at ≤-20°C.

4.2.15 Mixed internal standard (IS) solution (500 ng/ml)
Pipette 500 µl of mixed internal standard solution 20 µg/ml (4.2.14) in a calibrated volumetric flask of 20 ml and make up the volume with methanol (4.2.3) and mix. The solution can be used for 12 months when stored in the dark at ≤-20°C.

4.2.16 Working standard solution (10 ng/ml)
Pipette 10 µl of the mixed standard solution 1000 ng/ml (4.2.12) and 20 µl of the mixed IS solution 500 ng/ml (4.2.15) in a vial and add 970 µl of dilution solvent (4.2.8) and mix. Prepare a fresh solution every new day of analysis.

5 Equipment

Any reference to type and/or product is only to inform the user and to identify the equipment and does not imply exclusion of similar equipment.

Usual laboratory glassware and equipment, in particular, the following, can be used:

5.1 **Analytical balance**, accuracy: 0.01 mg

5.2 **Laboratory balance**, accuracy: 0.01 g

5.3 **Glass bottle**, 4, 20, 30 or 60 ml, amber coloured, with screw cap

5.4 **Mechanical vertical or horizontal shaker or rotary tumbling machine**, adjustable

5.5 **Laboratory shaker** (vortex)

5.6 **Bottle**, 250 ml

5.7 **Centrifuge tubes**, 50 ml, polypropylene, with screw cap

5.8 **Centrifuge** suitable for 50 ml centrifuge tubes

5.9 **Mini-Uniprep™ PTFE filter** vial, 500 µl

5.10 **Compressor for filter vials**

5.11 **pH meter**

5.12 **Various pipettes** -use positive displacement pipettes for solutions prepared in methanol

5.13 **LC-MS/MS** system with the following components:

5.13.1 **LC pump,** capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy

5.13.2 **Injection system**, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0.1%.

5.13.3 **Analytical column**, capable of retaining the target OAs and capable of baseline separation of OAs.

5.13.4 **Column oven**, capable of maintaining a constant temperature of 50°C.

5.13.5 **Tandem mass spectrometer (MS/MS)**, capable of ionisation of the compounds in positive mode, performing Multiple Reaction Monitoring (MRM), and with a sufficiently wide dynamic range and capable of unit mass separation and equipped with a computer-based data processing system. Any ionisation source giving sufficient yield may be employed.

6 Procedures

This document describes the quantification of opium alkaloids in poppy seeds and in poppy seed containing bakery products. The target level in poppy seeds is 20 mg/kg and in bakery products 1.5 mg/kg. The steps described in section 6.3 are presented in the format of a checklist in Annex A.1 and Annex A.2.

6.1 Preparation of the test sample

Poppy seeds and bakery products are cryogenically ground to ≤1 mm. Dry products are stored at 4-6°C and moist products are stored at ≤-20°C.

6.2 Test portion

For common poppy seeds serving as blank sample the amount of homogenised test material is 2.0 ± 0.05 g.
For ground poppy seeds the amount of homogenised test sample examined is 10.0 ± 0.1 g.
For ground bakery products the amount of homogenised test sample examined is 4.0 ± 0.05 g.

6.3 Extraction, clean-up and preparation of test solutions

6.3.1 Poppy seeds

6.3.1.1 Preparation of matrix matched calibration standards (MMC(P))

The calibration standards are prepared by addition of standard solutions to blank sample extract. Use as blank sample ground seeds of the common poppy (*Papaver rhoeas*), which is devoid of the OAs determined in this method.

Weigh a test portion of 2 ± 0.05 g (5.2) ground common poppy seeds in a PP tube of 50 ml (5.7). Add 20 ml of extraction solvent (4.2.7) to the test portion, shake vigorously and extract for 30 minutes on a shaking machine (5.4). Centrifuge the sample for 10 minutes at 3500 rpm (5.8) and transfer 8 aliquots, 10 µl each, of the supernatant to filter vials (5.9). Add mixed standard solutions 1000 ng/ml or 100 ng/ml (4.2.12, 4.2.13), the mixed internal standard solution 500 ng/ml (4.2.15) and dilution solvent (4.2.8) according to Table 1. Mix and close the vial with the help of a compressor (5.10).

Table 1: Preparation of matrix-matched calibration standards for poppy seeds, MMC(P)

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Sample extract (6.3.1.1) (µl)	Mixed standard solution 100 ng/ml (4.2.13) (µl)	Mixed standard solution 1000 ng/ml (4.2.12) (µl)	Mixed internal standard solution 500 ng/ml (4.2.15) (µl)	Dilution solvent (4.2.8) (µl)
MMC(P) 1	0	0	10	0	-	10	480
MMC(P) 2	1	0.5	10	5	-	10	475
MMC(P) 3	2.5	1.25	10	12.5	-	10	467.5
MMC(P) 4	5	2.5	10	25	-	10	455
MMC(P) 5	10	5	10	50	-	10	430
MMC(P) 6	20	10	10	-	10	10	470
MMC(P) 7	50	25	10	-	25	10	455
MMC(P) 8	100	50	10	-	50	10	430

6.3.1.2 Quality control sample recovery poppy seeds (MMRS(P)) (QC 10 mg/kg)

Weigh a test portion of 2 ± 0.05 g (5.2) ground common poppy seeds into a 50 ml PP tube (5.7). Add 100 µl of the stock solutions of morphine and codeine 200 µg/ml (4.2.10) and mix the sample. Wait 30 minutes before starting the extraction procedure. Add 19.8 ml of extraction solvent (4.2.7) to the test portion and shake vigorously. Extract for 30 minutes on a shaking machine (5.4) and centrifuge for 10 min at 3500 rpm (5.8). Transfer 10 µl of the supernatant to a filter vial (5.9), add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 480 µl dilution solvent (4.2.8). Mix and close the vial with the help of a compressor (5.10).

6.3.1.3 Sample preparation procedure for poppy seeds

Weigh a test portion of 10 ± 0.1 g (5.2) ground seeds in a bottle of 250 ml (5.6). Add 100 ml of extraction solvent (4.2.7) to the test portion and shake vigorously. Extract for 30 minutes on a shaking machine (5.4). Let the sample settle for 15 min and transfer of the supernatant an aliquot of 40 ml to a 50 ml PP tube (5.7). Centrifuge the tube for 10 minutes at 3500 rpm (5.8). Transfer 10 µl of the supernatant to a filter vial (5.9) and add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 480 µl dilution solvent (4.2.8). Mix and close the vial with the help of a compressor (5.10).

6.3.2 Bakery products

6.3.2.1 Preparation of matrix matched calibration standards (MMC(B))

The calibration standards are prepared by addition of standard solutions to blank sample extract. Choose a blank material, in which according to previous analyses no opium alkaloids were detected. The blank material should match with most of the samples to be analysed (e.g. bread, cake or cookies).

Weigh a test portion of 4 ± 0.05 g (5.2) blank sample in a PP tube of 50 ml (5.7). Add 40 ml of extraction solvent (4.2.7) to the test portion, shake vigorously and extract for 30 minutes on a shaking machine (5.4). Centrifuge the sample for 10 minutes at 3500 rpm (5.8) and transfer 8 aliquots, 50 µl each, of the supernatant to filter vials (5.9). Add mixed standard solutions 1000 ng/ml or 100 ng/ml (4.2.12, 4.2.13), the mixed internal standard solution 500 ng/ml (4.2.15) and dilution solvent (4.2.8) according to Table 2. Mix and close the vial with the help of a compressor (5.10).

Table 2: Preparation of matrix-matched calibration standards for bakery products, MMC(B)

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Sample extract (6.3.2.1) (µl)	Mixed standard solution 100 ng/ml (4.2.13) (µl)	Mixed standard solution 1000 ng/ml (4.2.12) (µl)	Mixed internal standard solution 500 ng/ml (4.2.15) (µl)	Dilution solvent (4.2.8) (µl)
MMC(B) 1	0	0	50	0	-	10	440
MMC(B) 2	1	0.1	50	5	-	10	435
MMC(B) 3	2.5	0.25	50	12.5	-	10	427.5
MMC(B) 4	5	0.5	50	25	-	10	415
MMC(B) 5	10	1	50	50	-	10	390
MMC(B) 6	20	2	50	-	10	10	430
MMC(B) 7	50	5	50	-	25	10	415
MMC(B) 8	100	10	50	-	50	10	390

6.3.2.2 Quality control sample recovery bakery products (MMRS(B)) (QC 1 mg/kg)

Weigh 4 ± 0.05 g (5.2) of blank sample into a 50 ml PP tube (5.7). Add 200 µl of the mixed standard solution 20 µg/ml (4.2.11). Wait 30 minutes before starting the extraction procedure. Add 40 ml of extraction solvent (4.2.7) to the test portion and shake vigorously. Extract for 30 minutes on a shaking machine (5.4) and centrifuge for 10 min at 3500 rpm (5.8). Transfer 50 µl of the supernatant to a filter vial (5.9), add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 440 µl dilution solvent (4.2.8). Mix and close the vial with the help of a compressor (5.10).

6.3.2.3 Sample preparation procedure for bakery products

Weigh a test portion of 4 ± 0.05 g (5.2) g in a tube of 50 ml (5.7). Add 40 ml of extraction solvent (4.2.7) to the test portion and shake vigorously. Extract for 30 minutes on a shaking machine (5.4) and centrifuge for 10 minutes at 3500 rpm (5.8). Transfer 50 µl of the supernatant to a filter vial (5.9) and add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 440 µl dilution solvent (4.2.8). Mix and close the vial with the help of a compressor (5.10).

7 LC-MS/MS analysis

Chromatographic and mass spectrometric conditions may be chosen freely. The optimal measurement conditions strongly depend on the instrumentation used. However, important criteria and parameters with respect to the chromatographic separation and detection of the analytes are:

The chosen column dimensions and chromatographic conditions should be appropriate to obtain base line separation of morphine and codeine from compounds with the same mass-to-charge ratio. For chromatographic separation mobile phases may be used over the range pH 2 to 12. It should be noted that an analytical column containing high pH-resistant cross-linked C18 reversed phase packing material is required for use with a mobile phase of pH higher than 7.

The injection volume should be optimised for the column dimension and the sensitivity of the mass spectrometric system. The use of large volume injections may result in distorted peak shapes.

The chosen mass spectrometric conditions should be appropriate to measure the analytes with sufficient sensitivity and specificity. Preferably, the protonated molecular parent ion should be selected as precursor ion and the product ions should be specific for the compound. Preferably, product ions that are formed by the loss of water from the protonated molecular parent ion should not be selected. Select at least two precursor-to-product ion combinations to be included in the multiple reaction monitoring (MRM) method. Each chromatographic peak should be composed of at least 10 data points.

The analytical series should not be started before it is verified, by injecting the working standard solution of 10 ng/ml (4.2.16) at least three times, that the system produces stable analyte retention times and that the sensitivity of the detector is sufficient and stable. The system should be able to detect the product ion with the lowest intensity with an S/N ratio of at least 500 in the working standard solution of 10 ng/ml (4.2.16).

Example LC-MS/MS conditions and example LC-MS/MS chromatograms are given in Annex B.

7.1 Injection sequence

Analyse the sample extracts in the order as given below.

- Standard working solution 10 ng/ml (4.2.16)
- Dilution solvent (4.2.8)
- Calibration standards MMC(P) (6.3.1.1) or MMC(B) (6.3.2.1)
- Dilution solvent (4.2.8)
- Quality control sample recovery MMRS(P) (6.3.1.2) or MMRS(B) (6.3.2.2)
- Dilution solvent (4.2.8)
- Sample extracts (6.3.1.3 or 6.3.2.3)
- Dilution solvent (4.2.8)
- Calibration standards MMC(P) (6.3.1.1) or MMC(B) (6.3.2.1)

Optionally: inject dilution solvent (4.2.8) between the different samples or every 10-20 samples.

8 Evaluation and calculations

Peak areas are used for all subsequent calculations. For each injection, check peak assignment and integration for all measured transitions and adjust if needed.

8.1 Verification of linearity of LC-MS/MS measurement

The matrix-matched calibration standards (MMC, Table 1 and Table 2) are used to determine the linearity of the LC-MS/MS system and to determine if the sample pre-treatment is done correctly.

For the calibration series, the response factor derived from the peak areas of the non-labelled analyte and the isotopically labelled analogue (Equation IV, section 8.3.1) is plotted as function of the added concentration in the sample (mg/kg). Apply linear regression using the least squares method. The correlation coefficient of the line should be ≥ 0.990. The deviation of the back calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, should not exceed 20%.

8.2 Identification of opium alkaloids in the samples

Identify opium alkaloids in the samples by comparing retention time and ion ratio with that of the calibration standards (MMC) according to SANTE/12682/2019 [3].

Calculate for each analyte the deviation of the retention time (Equation 1) and the deviation of the ion ratio (Equation II, III). When for an analyte the deviation of the retention time does not exceed 0.1 min, the deviation of the ion ratio does not exceed 30% and the concentration exceeds the LOQ of 0.5 mg/kg in case of poppy seeds or the LOQ of 0.1 mg/kg in case of bakery products, the identity of the analyte in the sample is confirmed. The identity of the analyte in MMC(P)2 (0.5 mg/kg) for poppy seeds or MMC(B)2 (0.1 mg/kg) for bakery products should be confirmed as a verification of the LOQ.

Use the following equations:

Equation I Deviation of the retention time (ΔRT)

$$\Delta RT = RT_{sample} - RT_{avg}$$

where:

ΔRT	is the deviation of the retention time of the analyte in the sample extract, compared to the average retention time in the MMC(P) or MMC(B) (min)
RT_{sample}	is the retention time of the analyte in the sample extract (min)
RT_{avg}	is the average retention time of the analyte in the MMC(P) or MMC(B) (min)

Equation II Ion ratio (IR)

$$IR = \left(\frac{A_{low}}{A_{high}}\right) x\ 100\%$$

where:

IR	is the ion ratio (%)
A_{low}	is the area of the product ion with the lowest intensity
A_{high}	is the area of the product ion with the highest intensity

Equation III Relative deviation of the ion ratio (D)

$$D = \left(\frac{IR_{sample} - IR_{average}}{IR_{average}}\right) x\ 100\%$$

where:

D	is the relative deviation of the ion ratio of the analyte in the sample, compared to the average ion ratio of the analyte in the MMC(P) or MMC(B) (%)
IR_{sample}	is the ion ratio of the analyte in the sample (%) (Equation II)
$IR_{average}$	is the average ion ratio of the analyte in the MMC(P) or MMC(B) (%) (Equation II)

Note 2: for calculation of the reference ion ratio use only responses with an S/N > 10. For the higher concentrations, exclude peak areas exceeding the linear range of the mass detector from calculation of the reference ion ratio.

8.3 Quantification of opium alkaloids in the samples

8.3.1 Quantification by means of isotope labelled internal standard correction

Quantification of morphine and codeine is based on isotope labelled internal standard correction. Calculate the response factor (RF) of the analyte in the sample and in the MMC according to Equation IV. Calculate the concentration (C) of the analyte in the sample according to Equation V (see note 3 and note 4).

Equation IV Response factor (RF)

$$RF = \frac{A_x}{A_{IS}}$$

where:

RF	is the response factor
A_x	is the sum of the peak areas of the product ions of the analyte in the sample
A_{IS}	is the peak area of the product ion of the isotope labelled analogue in the sample

Equation V Concentration in the sample (C)

$$C_{sample} = \left(\frac{RF_{sample} - b}{a}\right) x \frac{1}{R}$$

where:

C_{sample}	is the concentration of the analyte in the sample (mg/kg)
RF_{sample}	is the response factor obtained for the analyte in the sample
b	is the intercept of the MMC(P) or MMC(B)
a	is the slope of the MMC(P) or MMC(B)
R	is the recovery of the QC sample MMRS(P) or MMRS(B) (see Equation VI, section 8.3.2)

Note 3: in case no isotope labelled internal standards can be used, the response factor (RF) can be replaced by the sum of the peak areas of the product ions (A_x).

Note 4: optionally, the area of the quantifier ion only may be used in the calculations of analyte concentrations.

8.3.2 Recovery

Calculate for poppy seeds the recovery of the opium alkaloids with Equation VIa and for bakery products with Equation VIb.

Equation VIa Recovery for poppy seeds:

$$R = \left(\frac{RF_{MMRS(P)}}{RF_{MMC(P)\,6}}\right) \times 100\%$$

Where:

R	is the recovery (%)
$RF_{MMRS\,(P)}$	is the response factor of the analyte in recovery sample MMRS(P) fortified at 10 mg/kg (6.3.1.2)
$RF_{MMC(P)\,6}$	is the response factor of the analyte in MMC(P) 6 of 20 ng/ml (6.3.1.1)

Equation VIb Recovery for bakery products:

$$R = \left(\frac{RF_{MMRS(B)}}{RF_{MMC(B)\,5}}\right) \times 100\%$$

Where:

R	is the recovery (%)
$RF_{MMRS\,(B)}$	is the response factor of the analyte in recovery sample MMRS(B) fortified at 1 mg/kg (6.3.2.2)
$RF_{MMC(B)\,5}$	is the response factor of the analyte in MMC(B) 5 of 10 ng/ml (6.3.2.1)

Note 5: in case no isotope labelled internal standards can be used, the response factor (RF) can be replaced by the sum of the peak areas of the products ions (A_x).

8.4 Final result

The concentration of morphine and codeine in the sample is expressed as mg/kg.
Total opium alkaloid content according to regulation (EC) No 1881/2006 should be expressed in mg/kg as the sum of the concentration of morphine and 0.2 x concentration of codeine.

9 References

[1] EFSA, Scientific Opinion on the risks for public health related to the presence of opium alkaloids in poppy seed. EFSA Panel on Contaminants in the Food Chain. EFSA Journal, 2011. 9(11): 2405. pp. 150.

[2] EFSA, Update of the Scientific Opinion on opium alkaloids in poppy seeds. EFSA Panel on Contaminants in the Food Chain (CONTAM), EFSA Journal 2018. 16(5): 5243. pp. 119.

[3] DG_SANTE, Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed SANTE/12682/2019. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_mrl_guidelines_wrkdoc_2019-12682.pdf

Annex A.1 Checklist for sample preparation poppy seeds

Technician:	
Date:	
Lab. journal / page:	

Annex A.1 Checklist for sample preparation poppy seeds

A.1.1 Preparation of matrix matched calibration standards for poppy seeds MMC(P) (6.3.1.1)

- ☐ Weigh 6 portions of 2.0 ± 0.05 g (5.2) blank poppy seed in 50 ml polypropylene tubes (5.7);
- ☐ Spike the samples according to Table A.1;
- ☐ Add 20 ml of extraction solvent (4.2.7) to the test portion;
- ☐ Shake vigorously and extract for 30 minutes on a shaking machine (5.4);
- ☐ Centrifuge the sample for 10 minutes at 3500 rpm (5.8);
- ☐ Transfer 8 aliquots,10 µl each, of the supernatant to filter vials (5.9);
- ☐ Add standard solutions 1000 ng/ml or 100 ng/ml (4.2.12, 4.2.13), mixed internal standard solution 500 ng/ml (4.2.15) and dilution solvent (4.2.8) according to Table A.1;
- ☐ Mix and close the vial with the help of a compressor (5.10).

Table A.1 Preparation of matrix-matched calibration standards for poppy seeds

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Sample extract (6.3.1.1) (µl)	Mixed standard solution 100 ng/ml (4.2.13) (µl)	Mixed standard solution 1000 ng/ml (4.2.12) (µl)	Mixed internal standard solution 500 ng/ml (4.2.15) (µl)	Dilution solvent (4.2.8) (µl)
MMC(P) 1	0	0	10	0	-	10	480
MMC(P) 2	1	0.5	10	5	-	10	475
MMC(P) 3	2.5	1.25	10	12.5	-	10	467.5
MMC(P) 4	5	2.5	10	25	-	10	455
MMC(P) 5	10	5	10	50	-	10	430
MMC(P) 6	20	10	10	-	10	10	470
MMC(P) 7	50	25	10	-	25	10	455
MMC(P) 8	100	50	10	-	50	10	430

Annex A.1 Checklist for sample preparation poppy seeds

A.1.2 Quality control sample recovery MMRC(P) (10 mg/kg) (6.3.1.2)

- ☐ Weigh 2.0 ± 0.05 g (5.2) of the blank poppy seed material in a tube of 50 ml;
- ☐ Add 100 µl of morphine and codeine stock solution 200 µg/ml (4.2.10) and wait 30 minutes before starting the extraction procedure;
- ☐ Add 18 ml of extraction solvent (4.2.7) to the test portion and shake vigorously;
- ☐ Extract for 30 minutes on a shaking machine (5.4) and centrifuge for 10 min at 3500 rpm (5.8);
- ☐ Transfer 10 µl of the supernatant to a filter vial (5.9);
- ☐ Add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 480 µl dilution solvent (4.2.8);
- ☐ Mix and close the vial with the help of a compressor (5.10).

A.4 Sample preparation for analysis (6.3.1.3)

- ☐ Weigh a test portion of 10 ± 0.1 g (5.2) g ground poppy seeds in a bottle of 250 ml (5.6);
- ☐ Add 100 ml of extraction solvent (4.2.7) to the test portion and shake vigorously;
- ☐ Place the tube for 30 minutes on a shaking machine (5.4);
- ☐ Let the sample settle for 15 min and transfer of the supernatant an aliquot of 40 ml to a 50 ml PP tube (5.7);
- ☐ Centrifuge the tube for 10 minutes at 3500 rpm (5.8);
- ☐ Transfer 10 µl of the supernatant to a filter vial (5.9);
- ☐ Add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15)
- ☐ Add 480 µl dilution solvent (4.2.8);
- ☐ Mix and close the vial with the help of a compressor (5.10).

Annex A.2 Checklist for sample preparation bakery products

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Annex A.2 Checklist for sample preparation bakery products

A.1 Preparation of calibration standards for poppy seeds MMC(B) (6.3.2.1)

- ☐ Weigh a test portion of 4 ± 0.05 g (5.2) blank bakery product sample in a PP tube of 50 ml (5.7);
- ☐ Add 40 ml of extraction solvent (4.2.7);
- ☐ Shake vigorously and extract for 30 minutes on a shaking machine (5.4);
- ☐ Centrifuge the sample for 10 minutes at 3500 rpm (5.8);
- ☐ Transfer 8 aliquots, 50 µl each, of the supernatant to filter vials (5.9);
- ☐ Add standard solutions 1000 ng/ml or 100 ng/ml (4.2.12, 4.2.13), mixed internal standard solution 500 ng/ml (4.2.15) and dilution solvent (4.2.8) according to Table A.2;
- ☐ Mix and close the vial with the help of a compressor (5.10).

Table A.2: Preparation of matrix-matched calibration standards for bakery products

Code	Concentration in extract (ng/ml)	Concentration in sample (mg/kg)	Sample extract (6.3.2.1) (µl)	Mixed standard solution 100 ng/ml (4.2.13) (µl)	Mixed standard solution 1000 ng/ml (4.2.12) (µl)	Mixed internal standard solution 500 ng/ml (4.2.15) (µl)	Dilution solvent (4.2.8) (µl)
MMC(B) 1	0	0	50	0	-	10	440
MMC(B) 2	1	0.1	50	5	-	10	435
MMC(B) 3	2.5	0.25	50	12.5	-	10	427.5
MMC(B) 4	5	0.5	50	25	-	10	415
MMC(B) 5	10	1	50	50	-	10	390
MMC(B) 6	20	2	50	-	10	10	430
MMC(B) 7	50	5	50	-	25	10	415
MMC(B) 8	100	10	50	-	50	10	390

Annex A.2 Checklist for sample preparation bakery products

A.2 Quality control sample recovery (MMRC(B)) (1 mg/kg) (6.3.2.2)

- ☐ Weigh 4 ± 0.05 g (5.2) of blank sample into a 50 ml PP tube (5.7);
- ☐ Add 200 µl of mixed standard solution 20 µg/ml (4.2.11) and wait 30 minutes before starting the extraction procedure;
- ☐ Add 40 ml of extraction solvent (4.2.7) to the test portion and shake vigorously;
- ☐ Extract for 30 minutes on a shaking machine (5.4);
- ☐ Centrifuge for 10 min at 3500 rpm (5.8);
- ☐ Transfer 50 µl of the supernatant to a filter vial (5.9);
- ☐ Add 10 µl of mixed internal standard solution 500 ng/ml (4.2.15) and 440 µl dilution solvent (4.2.8);
- ☐ Mix and close the vial with the help of a compressor (5.10).

A.4 Sample preparation for analysis (6.3.2.3)

- ☐ Weigh a test portion of 4 ± 0.05 g in a tube of 50 ml (5.7);
- ☐ Add 40 ml of extraction solvent (4.2.7) to the test portion and shake vigorously;
- ☐ Extract for 30 minutes on a shaking machine (5.4);
- ☐ Centrifuge for 10 minutes at 3500 rpm (5.8);
- ☐ Transfer 50 µl of the supernatant to a filter vial (5.9);
- ☐ Add 10 µl of the mixed internal standard solution 500 ng/ml (4.2.15) and 440 µl dilution solvent (4.2.8);
- ☐ Mix and close the vial with the help of a compressor (5.10).

Annex B Example of LC-MS/MS conditions

B.1 UPLC conditions

The equipment and measuring conditions shown here are provided as an example. Other analytical equipment, columns, mobile phases and gradient conditions may work equally well.

Example conditions for the UPLC system

LC system:	Xevo TQ-S
Analytical column:	Waters BEH C18 1.7 µm, 100 x 2.1 mm
Column temperature:	50°C
Vial tray temperature:	10°C
Mobile phase solvent A:	10 mM ammonium carbonate in water pH 9 (4.2.9)
Mobile phase solvent B:	Acetonitrile
Flow rate:	0.4 ml/min
Injection volume:	2-5 µl
Gradient program:	Table B.1

Table B.1 Gradient for the UPLC system

Time (min)	Mobile phase A (4.2.9) (%)	Mobile phase B (4.2.2) (%)
0.0	90	10
7.0	60	40
9.5	30	70
9.7	90	10
12.0	90	10

See Annex B.3 for an example LC-MS/MS chromatogram.

B.2 MS conditions

The conditions given in Table B.2.1 are guidelines; in practice adjusted settings may be required to obtain an optimal performance of the LC-MS/MS system.

Table B.2.1 Example for MS conditions

Tune parameter	Xevo TQ-S
Ionization mode	ESI positive
Capillary voltage	3.0 kV
Cone voltage	30 V
Source temperature	150°C
Desolvation temperature	600°C
Cone gas flow	150 L/hr
Desolvation gas flow	800 L/hr
CID gas, pressure	Argon; 4,3 10^{-3} mbar
Solvent discard	0-1.5 and 10.5-12 min

The precursor ions fragment to structurally related products ions. In Table B.2.2 the theoretical masses of the precursor ion and corresponding product ions are shown. Depending on the instrument, a deviation of ± 0.3 Da is allowed. All transitions shown in Table B.2.2 are included in the MS method installed on the LC-MS/MS. The retention times can differ from column to column and between UPLC systems. The retention times shown in Table B.2.2 are therefore indicative.

Table B.2.2: MS/MS fragmentation conditions for opium alkaloids

Component	Indicative RT (min)	Precursor ion (m/z)	Cone voltage (V)	Product ion 1 (m/z)	Collision energy 1 (eV)	Product ion 2 (m/z)	Collision energy 2 (eV)	Product ion 3 (m/z)	Collision energy 3 (eV)
Morphine	2.70	286.2	40	153.0	40	165.0	30	201.0	25
Morphine-d3	2.60	289.2	40	153.0	40	165.0	30		
Codeine	4.30	300.2	40	153.0	40	165.0	35	215.0	25
Codeine-d3	4.20	303.2	40	153.0	40	165.0	35		

B.3 LC-MS/MS example chromatogram of a bakery product sample (cake) fortified at 1 mg/kg