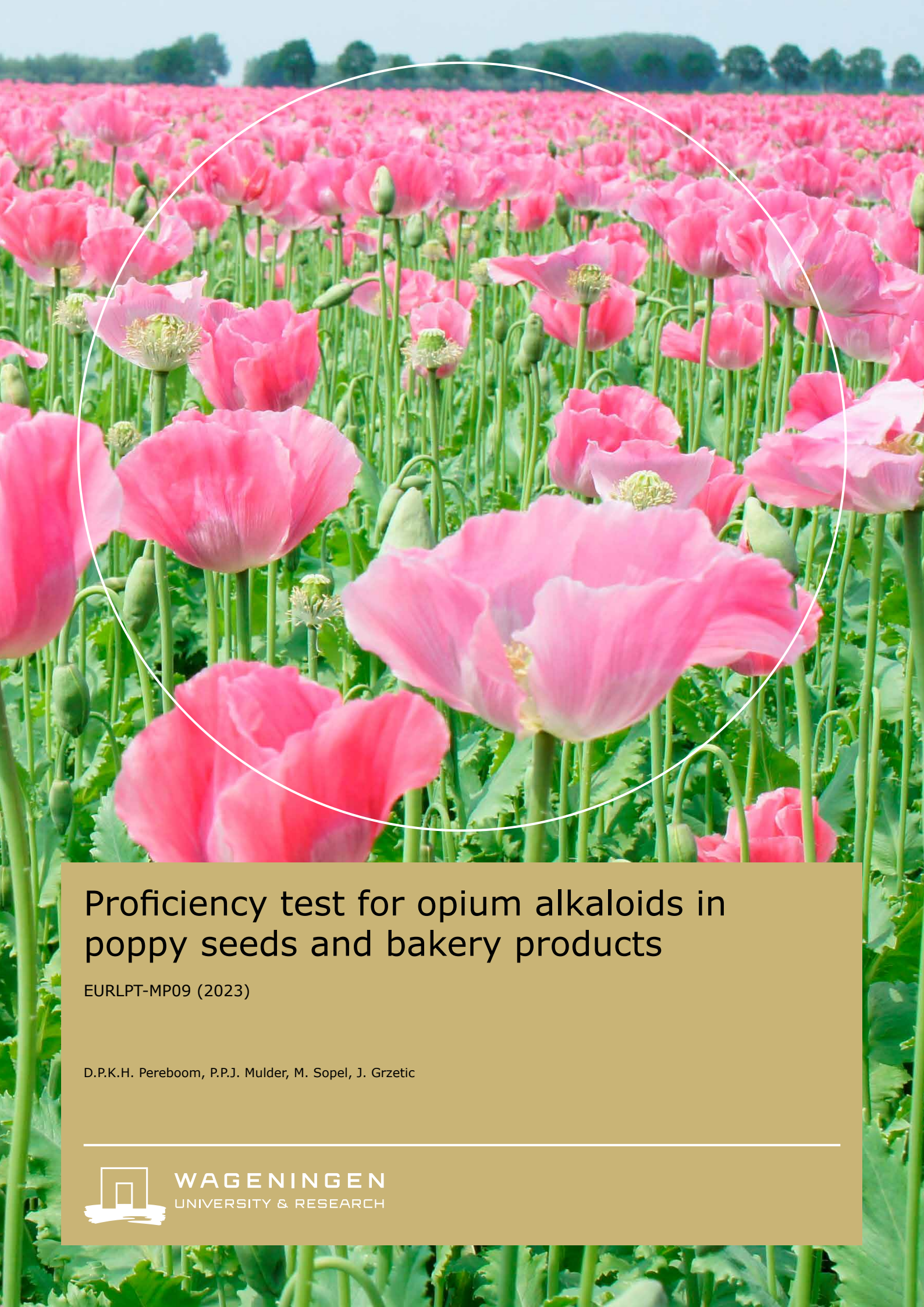

# Proficiency test for opium alkaloids in poppy seeds and bakery products

EURLPT-MP09 (2023)

D.P.K.H. Pereboom, P.P.J. Mulder, M. Sopel, J. Grzetic

WAGENINGEN
UNIVERSITY & RESEARCH

# Proficiency test for opium alkaloids in poppy seeds and bakery products

EURLPT-MP09 (2023)

D.P.K.H. Pereboom, P.P.J. Mulder, M. Sopel, J. Grzetic

Wageningen, November 2023

WFSR Report 2023.013

WAGENINGEN
UNIVERSITY & RESEARCH

PROF. TESTING
RvA R 013

Pereboom, D.P.K.H., P.P.J. Mulder, M. Sopel, J. Grzetic, 2023. *Proficiency test for opium alkaloids in poppy seeds and bakery products; EURLPT-MP09 (2023).* Wageningen, Wageningen Food Safety Research, WFSR Report 2023.013. 44 pp.; 6 fig.; 7 tab.; 14 ref.

Project number: 1237419201-1.3.2 EURLPT-MP09 22 OA afr
Project title: EURL MP 2023/2024 (1.3.2 EURLPT-MP09 22 OA afr)
Coordinator proficiency tests: D.P.K.H. Pereboom (pt.wfsr@wur.nl)
Project leader: J. Grzetic
Scientist: P.P.J. Mulder and M. Sopel
Authorized by: L. Stolker (team leader natural toxins)

This report can be downloaded for free at https://doi.org/10.18174/641239 or at www.wur.eu/food-safety-research (under WFSR publications).



P.O. Box 230, 6700 AE Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.wfsr@wur.nl, www.wur.eu/food-safety-research. WFSR is part of Wageningen University & Research.



WFSR report 2023.013 (final report)

Distribution list:
- Participating laboratories
- Mr. F. Verstraete, European Commission, DG SANTE

# Contents

# Summary

A proficiency test (PT) for the quantitative determination of opium alkaloids (OAs) in poppy seeds and bakery products was organised by the European Union Reference Laboratory for Mycotoxins & Plant toxins in food and feed (EURL-MP) between February and April 2023. This PT was carried out by Wageningen Food Safety Research (WFSR) under accreditation (R013, Dutch Accreditation Council RvA, ISO/IEC 17043:2010). In December 2021 Commission Regulation (EU) 2021/2142 on maximum levels of OAs in certain foodstuffs was published and this has come into effect on July 1, 2022. Recently, CR (EU) 2021/2142 has been incorporated in the new Commission Regulation (EU) 2023/915 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. The primary goal of this PT was to assess the proficiency of the National Reference Laboratories for mycotoxins & plant toxins in food and feed (NRLs) and Official Laboratories (OLs) that participated.

The participants were asked to quantify OAs in 2 materials and to report for each material 3 results, which comprised levels of morphine, codeine and the sum of both OAs expressed as morphine + 0.2 codeine, as stipulated in Regulations (EU) 2021/2142 and (EU) 2023/915. The participants' performance was assessed as z-score in both materials for the individual OAs (maximum score 4 out of 4) and for the sum of the OAs in the samples (maximum score 2 out of 2).

Thirty-one laboratories, of which 24 National Reference Laboratories for mycotoxins and/or plant toxins in food and feed (from 18 EU Member States plus Serbia and the EFTA MS Iceland, Norway and Switzerland) and 7 Official Laboratories (all EU Member States) participated in the PT.

Two materials were prepared. Material A consisted of 5 different batches of poppy seeds that were mixed. For material B, 3 cakes were baked with poppy seeds as an ingredient. The three cakes were mixed afterwards. Both materials were sufficiently homogeneous and stable during the PT. Each participant received one test sample of 50 gram of material A and 30 g of material B. The participants were requested to report their results within 6 weeks after the dispatch of the samples.

From the provided information on the identification and quantification of the OAs almost all participants used LC-MS/MS (27), except 2 participants, who applied LC-HRMS (High Resolution Mass Spectrometry). For material A, 27 participants reported Limit of Quantification (LOQ) values of 1 mg/kg or less for the individual OAs. Three participants reported LOQs in the range of 1.7 to 2 mg/kg. For the bakery product, 26 participants reported LOQ values of 0.4 mg/kg or less. Four participants reported LOQs in the range of 1 to 2 mg/kg. One laboratory did not report LOQs.

In this PT the robust mean was used as consensus value. The consensus value based on the participants' results was used as the assigned value. The proficiency of the participants was assessed as z-scores in both materials, calculated using the assigned values and a relative target standard deviation of 25%. Characteristics of the PT materials and the outcome of this PT are summarised in Table 1a and 1b. Results were calculated for morphine, codeine and the sum of OAs expressed as morphine + 0.2 codeine. For material A, the assigned values of morphine and codeine in material A were, respectively 13.3 and 24.3 mg/kg and in material B, respectively 1.01 and 0.175 mg/kg. For material A and B, none of the $RSD_R$ of the reported results were below the target standard deviation (25%) except for codeine in material A (24%). The $RSD_R$ values for the sum of the OAs (as mentioned in legislation) were 32% and 27% for material A and B, respectively. No false negative results were reported in this PT.

For both materials (A and B) combined, 87% of the results the individual OAs were rated with satisfactory z-scores ($|z| \leq 2$), 7% of the results fell into the questionable range with $2<|z|<3$ and 6% of the results fell into the unsatisfactory range with $|z| \geq 3$. Sixteen participants achieved optimal performance for both materials by detecting morphine and codeine with correct quantification, the absence of false negative results and reporting within the deadline. One participant achieved also satisfactory results for both OAs for

both materials combined but submitted the results after the deadline. With respect to the sum of morphine + 0.2 codeine, 82% of submitted results were satisfactory and 23 participants showed satisfactory performance for both materials.

From the results obtained in this PT on OAs it can be concluded that most participants have an analytical method available with sufficiently low LOQs. Three participants reported relatively high LOQs for codeine in the bakery product. Nevertheless, the results also reveal that for both materials and for the individual OAs as well as for the sum of OAs relatively high robust $RSD_R$ values were obtained, which is caused by a relatively wide variation in the reported results. This in turn may be caused by a reduced effectiveness of the isotopically labelled internal standards that have been incorporated in most of the reported methods. In this respect continued efforts need to be made by the EURL-NRL network to improve the robustness of the implemented methods, in order to produce reliable data.

***Table 1a*** *Summary of proficiency materials parameters and participants' performance – number of laboratories reporting quantitative values, <LOQ and false negative (FN).*

| | | Assigned value | Uncertainty | Robust $RSD_R$[1)] | No of labs out of 31 reporting | |
|---|---|---|---|---|---|---|
| **EA epimer groups** | **Matrix** | **(mg/kg)** | **(mg/kg)** | **(%)** | **Quant. value** | **< LOQ** |
| Morphine | A | 13.3 | 0.966 | 3.31 | 31 | |
| | B | 1.01 | 0.067 | 0.252 | 31 | |
| Codeine | A | 24.3 | 1.29 | 6.07 | 31 | |
| | B | 0.175 | 0.015 | 0.044 | 23 | 8 |
| Sum morphine + 0.2 codeine | A | 18.2 | 1.33 | 4.55 | 31 | |
| | B | 1.06 | 0.064 | 0.264 | 31 | |

Matrix: A= poppy seeds, B= bakery product.

[1)] robust relative standard deviation (interlaboratory RSD based on participants' results).

***Table 1b*** *Summary of proficiency materials parameters and participants' performance – evaluation of results, satisfactory, questionable and unsatisfactory z and z'-scores.*

| | | Assigned | z-scores[1)] | | | Labs out of 31 with | |
|---|---|---|---|---|---|---|---|
| | | Value | Satisfact. | Quest. | Unsatisf. | Accept. z -score | |
| **EA epimer groups** | **Matrix** | **(mg/kg)** | **(% of z-scores)** | **(% of z-scores)** | **(% of z-scores)** | **No[2)]** | **%[2)]** |
| Morphine | A | 13.3 | 87.1 | 6.5 | 6.5 | 27 | 87.1 |
| | B | 1.01 | 80.6 | 9.7 | 9.7 | 25 | 80.6 |
| Codeine | A | 24.3 | 96.8 | 3.2 | | 30 | 96.8 |
| | B | 0.175 | 82.6 | 8.7 | 8.7 | 19 | 61.3 |
| Sum morphine + 0.2 codeine | A | 18.2 | 83.9 | 6.5 | 9.7 | 26 | 83.9 |
| | B | 1.06 | 80.6 | 9.7 | 9.7 | 25 | 80.6 |

Matrix: A= poppy seeds, B= bakery product.

[1)] calculated using a fit-for-purpose target RSD for proficiency of 25%. False negatives were counted here as unsatisfactory z-score.

[2)] the number and percentage here means: analyte determined, method with a sufficiently low LOQ to allow quantification, and obtaining a satisfactory z-score.

# 1 Introduction

Opium alkaloids (OAs) are secondary metabolites that are stored 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. Poppy seeds themselves do not contain OAs, but they can become contaminated with alkaloids from the latex resulting from insect damage, or through poor harvesting practices. Morphine is generally the predominant alkaloid. It is also the most pharmacologically active opiate, having strong narcotic properties. For this reason, OAs such as morphine and codeine have been included in the list of drug abuse substances.

Poppy seeds are used in bakery products, as decoration of dishes, in fillings of cakes and in desserts and to produce edible oil. 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.

The EU has established maximum limits (MLs) for OAs in poppy seeds and bakery products as described in Commission Regulation (EU) 2021/2142 [8], which has recently been incorporated in Commission Regulation (EU) 2023/915 [13]. The maximum limit for poppy seeds is set at 20 mg/kg 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. For bakery products containing poppy seeds, the maximum limit is 1.5 mg/kg morphine + 0.2 x codeine. The MLs have come into effect from July 1$^{st}$, 2022. For bakery products, the limit of quantitation (LOQ) required for individual OAs is specified at 0.5 mg/kg. This requirement will be laid down in the regulation on methods of sampling and analysis for the control of plant toxins in food and repealing Regulation (EU) No 2015/705 [14]. For the poppy seed material, an LOQ is not specified. For this PT an LOQ of 1 mg/kg or lower is recommended for morphine and codeine in poppy seeds.

Proficiency testing is conducted to provide participants with a powerful tool to evaluate and demonstrate the reliability of the data that are produced by the laboratory. Proficiency testing is an important requirement and is demanded by ISO/IEC 17025:2017 [3]. Organisation of proficiency tests (PT) is one of the tasks of European Union Reference Laboratories (EURLs) [4]. Here the primary goal is to assess the proficiency of the National Reference Laboratories (NRLs). To facilitate NRLs in their task, official laboratories (OLs) can also participate, in consultation with their NRL.

# 2 PT material

## 2.1 Scope of the PT

This proficiency test (PT) focused on the OAs in food matrices; using poppy seeds and a bakery product as representative matrices. The scope includes morphine and codeine as mentioned in Commission Regulation (EU) 2021/2142 and 2023/915. The target concentrations aimed for (see Table 2) take the regulatory limits into account.

***Table 2*** *Target concentrations (mg/kg) of opium alkaloids in the PT materials.*

| | Target concentrations (mg/kg) | |
|---|---|---|
| **Opium alkaloid** | **Material A (poppy seeds)** | **Material B (bakery product)** |
| Morphine | 20.2 | - |
| Codeine | 32.1 | - |
| Sum morphine + 0.2 x codeine | 26.6 | 1.5 |

## 2.2 Material preparation

Poppy seeds and a bakery product, respectively, were used for preparation of the two materials A and B. For material A, 5 different poppy seed materials were milled under cryogenic conditions using a centrifugal mill (Retsch ZM 200, Haan, the Netherlands) and the milled materials were cryogenically homogenised using an industrial mixer (Topcraft, Belgium) according to in-house standard operating procedures [6]. For material B, 3 cakes were baked with poppy seeds (15%) as an ingredient. The 3 cakes were crumbled and freeze-dried. The freeze-dried material was first cryogenically homogenised using a centrifugal mill (Retsch ZM 200) and further cryogenically homogenised using an industrial mixer.

## 2.3 Sample identification

After homogenisation, materials A and B were divided into sub-portions of approximately 50 grams and 30 grams, respectively, and stored in polypropylene, airtight closed containers in the freezer until use.

The samples for the participants were randomly selected and coded using a web application designed for proficiency tests. The code used was "2023/EURL PT MP/OAs/xxx", in which the three-digit number of the code was automatically generated by the WFSR Laboratory Quality Services web application. One sample set was prepared for each participant. Each sample set consisted of one randomly selected sample of material A and one of material B. The codes of the samples for each sample set are shown in Annex 2. The samples for homogeneity and stability testing were also randomly selected out of the set of materials A and B.

## 2.4 Homogeneity study

To verify the homogeneity of the PT materials, 10 containers of each of the materials A and B were analysed in duplicate for OAs (EURLMP-method_007 v1) [7].

Method in brief: OAs were extracted from the homogenised sample (10 g of the poppy seeds and 4 g of the bakery product) by addition of, respectively, 100 and 40 mL methanol/water (60/40, v/v) containing 0.4% of

formic acid, followed by agitation in an overhead shaker. An aliquot of the supernatant was diluted with water and spiked with internal standards (morphine-d3 and codeine-d3). Analysis was performed by high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using reversed phase chromatography with alkaline conditions. Quantification was performed on the basis of matrix matched calibration in blank extract with internal standard and recovery correction.

The homogeneity of both materials was evaluated according to the International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [10] and ISO 13528:2015 [2]. Using this procedure the between-sample standard deviation ($s_s$) and the within-sample standard deviation ($s_w$) were compared with the standard deviation for proficiency assessment ($\sigma_P$). The method applied for homogeneity testing is considered suitable if $s_w < 0.5 \times \sigma_P$ and a material is considered adequately homogeneous if $s_s < 0.3 \times \sigma_P$. Both materials proved to be sufficiently homogeneous for this PT.

The results of the homogeneity study (grand means with the corresponding $RSD_r$) are presented in Table 3. The statistical evaluation of materials A and B is presented in Annex 3.

***Table 3*** *Concentrations of OAs in materials A and B obtained during homogeneity testing.*

| Compound | Material A: poppy seeds | | Material B: bakery product | |
|---|---|---|---|---|
| | Conc. (mg/kg) | RSD (%) | Conc. (mg/kg) | RSD (%) |
| Morphine | 17.8 | 6.22 | 0.955 | 6.65 |
| Codeine | 30.2 | 5.42 | 0.148 | 6.19 |
| Morphine + 0.2 codeine | 23.9 | 4.72 | 0.985 | 6.51 |

## 2.5 Stability of the materials

The stability of the OAs in the materials was assessed according to [10, 11]. On March 6th, 2023, the day of distribution of the PT samples, 6 randomly selected containers of material A and B were stored in a freezer. Under these conditions it is assumed that the OAs are stable in the materials. In addition, 6 samples of each material were stored in a refrigerator.

On the 9th of May 2023, 64 days after distribution of the samples, 6 samples of materials A and B, stored in the freezer and refrigerator, were analysed in one batch. For each set of test samples, the average of the results and the standard deviation were calculated.

It was determined whether a consequential instability of the analytes had occurred [10,11] in the materials stored in the refrigerator. A consequential instability is observed when the average value of an analyte in the samples stored in the refrigerator is more than $0.3\sigma_P$ below the average value of the analyte in the samples stored in the freezer. If so, the instability has a significant influence on the calculated z-scores.

The results of the stability of materials A and B are presented in Annex 4. For the analytes in both materials none of the tested storage conditions caused a consequential difference. The OAs in the materials were, therefore, considered stable for the duration of the PT.

# 3 Organisational details

## 3.1 Participants

This PT focused on the determination of OAs in food, using poppy seeds and a bakery product. Invitations to the NRL network were sent out on February 8th, 2023 (Annex 5). Thirty-three laboratories registered for the PT. Thirty-one participants (Annex 1) reported their results of which 2 reported their results after the deadline. Two participants were unable to report results, one due to lack of the analytical standards and one did not have its analytical method operational in time.

Out of the 31 participating laboratories, 24 were NRLs from 18 EU Member States plus Iceland, Norway Serbia, Switzerland, and 7 were Official Laboratories (from various EU Member States). Each participant was free to use their method of choice reflecting their routine procedures. The participants were asked to report the results through a web application designed for proficiency tests as well as to fill in a questionnaire, where it was asked to provide detailed information on the analytical method used for detection and quantification of OAs (extraction solvent/procedure, clean-up, detection technique, limit of detection and limit of quantification).

## 3.2 Material distribution and instructions

Each participant received a randomly assigned laboratory code, generated by the web application. The sample sets with the corresponding numbers, consisting of 2 coded samples (Annex 2) were sent to the participants on March 6th, 2023. The sample sets were dispatched immediately by courier to the participants in insulation boxes containing dry ice. The participants were asked to store the samples in the refrigerator and to analyse the samples according to their routine method. As reported by participants, all parcels were received in good order.

The samples were accompanied by a letter describing the requested analysis (Annex 6) and an acknowledgement of receipt form. In addition, each participant received instructions by e-mail on how to use the web application to report the results. The questionnaire was intended to gather additional information on Limits of Quantification (LOQ), method recovery estimates (%) and other method-related aspects (e.g. extraction and clean-up, chromatographic and detection conditions, calibration strategy) to investigate individual and/or general patterns on the submitted results.

For each material a total of 3 results, comprising levels of morphine, codeine and the sum of morphine + 0.2 codeine was requested. The deadline for submitting the quantitative results was April 17th, 2023, allowing the participants 6 weeks for analysis of the test samples. All results, except from 2 participants, were submitted within the deadline.

# 4 Evaluation of results

The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [10], elaborated by ISO, IUPAC and AOAC and ISO 13528:2015 [2] in combination with the insights published by the Analytical Methods Committee [12, 13] regarding robust statistics.

The evaluation of results was based on assigned values and the standard deviation for proficiency assessment ($\sigma_P$). From this, z-scores were calculated to classify the participants' performance. Detailed information on the methods used for the statistical evaluation can be found in the background document 'EURL-MP-background doc_001 (v1) Performance assessment in proficiency tests organised by the EURL Mycotoxins & Plant toxins in food and feed' available from the EURL mycotoxins & plant toxins website [5].

## 4.1 Calculation of the assigned value

The robust mean was used as consensus value in this PT. The consensus value based on the participants' results (all participants, both NRLs and OLs) was used as the assigned value. The values and their uncertainties are summarised in Table 1 in the Summary section. Assigned values were established for morphine, codeine and for the sum the OAs in both materials.

## 4.2 Standard deviation for proficiency assessment ($\sigma_P$)

A fixed relative target standard deviation for proficiency assessment of 25% was used, irrespective of the plant toxin, matrix or concentration. This generic fit-for-purpose value is considered to reflect current analytical capabilities and best practises for mycotoxin and plant toxin determination in food and feed. The rationale behind this is provided in the background document 'EURL-MP PT performance assessment' on the EURL-MP website [5].

## 4.3 Quantitative performance (z-scores)

For evaluation of numerical results submitted by the participant, z-scores are calculated based on the assigned value, its uncertainty, and the standard deviation for proficiency assessment. When the uncertainty of the assigned value is negligible and no instability of the analytes in the material is observed, z-scores are calculated by:

$$z = \frac{x - C}{\sigma_p} \qquad \text{Equation 1}$$

where:

z = z-score;
x = the result of the laboratory;
C = assigned value, here the consensus value;
$\sigma_P$ = standard deviation for proficiency assessment.

The z-score compares the participants' deviation from the assigned value, taking the target standard deviation accepted for the proficiency test into account, and is interpreted as indicated in Table 4.

***Table 4*** *Classification of z-scores.*

| | |
|---|---|
| $\|z\| \leq 2$ | Satisfactory |
| $2 < \|z\| < 3$ | Questionable |
| $\|z\| \geq 3$ | Unsatisfactory |

If the uncertainty of the assigned value and, if applicable, instability of the analyte in the PT material, is not negligible, this is taken into account in the determination of the z-score. If applicable, this is indicated by assigning a z'-, $z_i$-, or $z_i$'-score. For details see the background document 'EURL-MP PT performance assessment' on the EURL-MP website [5].

In this PT, the uncertainty of the assigned value for codeine in material B was not negligible and taken into account in the assignment of the z-scores (z'). In all the other cases, the uncertainty of the assigned value was negligible. No instability of the analytes in the PT material was observed during the PT period.

## 4.4 Evaluation of non-quantified results

In cases, where participant(s) reported '<[value]', 'detected' or 'not detected' (nd) (i.e. below their LOQ), 'proxy-z-scores' were calculated to assess possible false negatives and to benchmark the LOQ relative to the assigned value and the LOQ of the other participants.

A proxy-z-score was calculated by using equation IV of the background document 'EURL-MP-background doc_001' (for details see the EURL-MP website), using the reported LOQ value as a result [5]. Proxy-z-scores are for information only and indicated as a value between brackets. Proxy-z-scores are not included in the evaluation of the results and do not count as a satisfactory result.

Proxy-z-score values [z<-2] were considered as false negatives (see 4.5). Proxy-z-score values [z>2] indicate that the LOQ is high in relation to the assigned value and high in comparison to other participants.

Reported results, e.g. 'detected' or 'not detected', without specification of LOQ, were excluded from the evaluation. In these cases, the participant was considered to have no quantitative method available for the specific analyte or analyte group/matrix. Non reported results for analytes or analyte groups are to be interpreted as unsatisfactory performance.

## 4.5 False positive and false negative results

A false positive is a quantitative result reported by the participant while the analyte is not detected in the PT material by the organiser, and/or not detected by most of the other participants. A threshold is then applied, above which results are considered false positives, indicated as FP. False positives are to be interpreted as unsatisfactory performance.

When an analyte is present in the material, i.e. an assigned value has been established, and the participant reports the analyte as '<[value]', 'detected' or 'not detected', an assessment is made to judge whether such results should be classified as a false negative. This is the case when the proxy-z-score value (see 4.4) is <-2. False negatives are indicated as 'FN'. False negatives are to be interpreted as unsatisfactory performance.

# 5 Performance assessment

## 5.1 Scope and LOQ

This PT was dedicated to the quantification of OAs in poppy seeds and bakery products. Annex 7 summarises the quantitative scope of each participant, with an indication of the LOQ for each OA. One participant provided no details on the LOQs of the individual OAs.

Thirty-one participants reported for both materials A and B a total of 3 results, comprising levels of morphine, codeine and the sum of morphine + 0.2 codeine, as was requested. Several results for codeine in the bakery product were reported as <LOQ. For these results proxy z-scores were calculated. In regulations (EU) 2021/2142 and (EU) 2023/915 it is mentioned that the maximum levels refer to the sum of morphine and codeine, for which a factor of 0.2 is applied to the level of codeine. Therefore, the maximum level refers to the sum of morphine + 0.2 codeine. Two participants reported the sum of morphine and codeine without considering the factor 0.2.

The LOQs provided by the participants ranged from 0.01 to 2 mg/kg (Annex 7). Some participants reported different LOQs for morphine and codeine for the same material (3) and some participants reported different LOQs for material A and B (5). For poppy seeds, 27 participants reported for both OAs LOQs of 1 mg/kg or less: 7 participants reported LOQs in the range of 0.01 to 0.096 mg/kg, 7 participants reported LOQs of 0.1 mg/kg, 10 participants reported LOQs in the range of 0.15 to 0.5 mg/kg and 3 participants reported an LOQ of 1 mg/kg. Three participants reported LOQs in the range of 1.7 to 2 mg/kg. Most of the participants reported LOQs which are in line with the LOQ of 1 mg/kg recommended for this PT. For the bakery product, 26 participants reported for both OAs LOQs of 0.4 mg/kg or less: 7 participants reported LOQs in the range of 0.01 to 0.096 mg/kg, 11 participants reported an LOQ of 0.1 mg/kg, 5 reported LOQs in the range of 0.15 to 0.3 mg/kg, and 3 participants reported an LOQ of 0.4 mg/kg. All these participants reported LOQs that are in line with the upcoming regulation on methods of sampling and analysis of plant toxins which states that the LOQ for bakery product should be at least 0.5 mg/kg (EC working document SANTE 11494R3/2021 [9]. Four participants reported LOQs in the range of 1 to 2 mg/kg.

It can be concluded that for the bakery product, most participants are able to achieve LOQs of 0.5 mg/kg or lower, which is in line with the (future) requirements of the legislation. This is also the case for the poppy seed material with the recommended LOQ of 1 mg/kg. Some laboratories need to improve the sensitivity of their method for OAs in bakery products, to comply with the upcoming regulation on the methods of sampling and analysis of plant toxins.

## 5.2 Analytical methods

All participating laboratories were asked to fill in a questionnaire addressing their accreditation, conditions used for sample preparation, chromatographic separation, detection, quantification and calibration (Annex 8). Two participants provided no information about method details.

Of the 29 laboratories, 11 participants reported their analytical method covered by ISO 17025 accreditation.

Based on the information provided on the laboratory sample preparation procedure, for poppy seeds the most often reported intake was 5 g (10 participants) and for the bakery product 4 g (9 participants) or 5 g (9 participants). Of the poppy seeds, 11 participants used 4 g or less, while 8 participants used 10 g or more. Of the bakery product 6 participants used 2.5 g or less, while 5 participants used 10 g or more.

The poppy seeds samples were extracted with 50 mL (median volume) of extraction solvent and for the bakery samples this was 40 mL (median volume) for approximately 30 min (median extraction time). For the poppy seed material, the volumes most often used were 100 mL (13) and 20 mL (8). For the bakery product this was 100 mL (10) and 40 mL (8). For both materials, most participants (14) reported an extraction time of 30 min, 3 participants used an extraction time between 10 and 20 min, and the remaining participants used 45 min (2), 60 min (3) and 65 min (1). Five participants used a double extraction with 50 mL in combination with an extraction time of 45 min, 1 participant used a double extraction with 50 mL in combination with an extraction time of 60 min and 1 participant used a triple extraction with 20 mL in combination with an extraction time of 15 min for the bakery product.

For the extraction solvent participants used methanol (27) or acetonitrile (2) as the main organic phase. The reported composition of the extraction solvents was: acidic aqueous/organic (28) or organic (1). The most often used extraction solvent combinations were: methanol in combination with formic acid (20) or acetic acid (5), methanol combined with hydrochloric acid (1), acetonitrile combined with acetic acid (1) or formic acid (1). For sample extract purification one participant used solid phase extraction (SPE) (Oasis MCX), 17 participants diluted the sample extract, 1 participant used another clean-up, without providing details. Ten participants reported that no clean-up or other treatment was applied.

For the identification and quantification of the OAs almost all participants used LC-MS/MS (27). Two participants applied LC-HRMS (High Resolution Mass Spectrometry).

For chromatography participants used either methanol (15) or acetonitrile (13) as an organic modifier in combination with an aqueous buffer while one participant didn't provide information. About half of the participants (15) used alkaline chromatography. For the preparation of the alkaline mobile phase the following buffers were used: ammonium carbonate (11) and ammonium bicarbonate (4). The other participants (14) used acidic chromatography: 8 used ammonium formate with or without addition of formic acid, 1 used ammonium acetate with addition of acetic acid and 5 used formic acid to acidify the mobile phase.

A wide variety of columns from different suppliers was used for chromatography with acidic or alkaline conditions. For methods applying acidic conditions, mostly columns with a C18 based stationary phase were used: Waters: Acquity UPLC BEH (2), Acquity UPLC HSS T3 (1); Phenomenex: Luna (1), Luna Omega Polar (1), Synergi Polar (1); Agilent: Zorbax Eclipse Plus (1); Thermo Scientific: Hypersil Gold (2); MZ anlysetechnik: Perfectsil (1): Restek: Raptor ARC-18 (1). In addition, the following non-C18 stationary phase columns were used: Waters: Acquity UPLC BEH amide (1); Phenomenex: Kinetex pentafluorophenyl (1); Agilent: Pursuit diphenyl (1). For methods applying alkaline conditions all participants used a C18 type stationary phase: Waters: Acquity BEH (6), XBridge (2), XBridge Premier (1) and one participant didn't specify the stationary phase used; Phenomenex: Kinetex EVO (1), Gemini (2), Gemini-NX (1); YMC: Triart (1). The column length mostly used was either 100 mm (10) or 150 mm (12). The total run time reported varied between 4 and 35 min and the median run time was 13 min.

The quantification approach followed by the participants is summarised in Table 5. Two participants did not indicate what they used as quantification approach. Out of 28 participants, 25 used multi-level standard addition: 16 of them performed multi-level calibration with standards in a pure solvent, 4 used multi-level standard addition to the sample, 2 used multi-level standard addition before extraction and 3 after extraction. Two participants used a single-point standard addition approach; 1 of them performed single-point calibration with standards in a pure solvent and one added the standards before extraction. One participant provided no details if the standard addition approach was multi-level or single point. Twelve participants (40%) have corrected their results for recovery, while 60% reported that they didn't.

Twenty-five participants used isotopically labelled morphine and/or codeine as internal standards, while 4 did not use internal standards. Seven participants added the internal standard before extraction and 18 participants added the internal standard to the final extract. Most participants used as internal standards morphine-D3 and codeine-D3 (16), while 3 participants used morphine-D6 and codeine-D6, 3 used a combination of morphine-D3 and codeine-D6 and 3 used only morphine-D3.

***Table 5*** *Analytical strategies followed by the participants.*

| Quantification approach | Calibration/ quantification | No. of participants | Recovery | |
|---|---|---|---|---|
| | | | Corrected | Not corrected |
| standard addition before extraction | single point | 1 | | 1 |
| standards in pure solvent | single point | 1 | 1 | |
| standards in pure solvent | multi-level | 16 | 3 | 13 |
| matrix-matched standards | multi-level | 5 | 4 | 1 |
| standard addition before extraction | multi-level | 3 | 1 | 2 |
| standard addition after extraction | multi-level | 3 | 2 | 1 |
| standards in pure solvent | ? | 1 | | 1 |

## 5.3 Performance

The quantitative performance was assessed through z-scores. The individual z-scores obtained by each participant, including their graphical representation, for the OAs in materials A (poppy seeds) and B (bakery product) are summarised in Annex 9 and 10, respectively. A summary of the performance of the participants in this PT is provided in Annex 11.

A summary of the statistical evaluation of the PT results is presented in Tables 6 and 7. These tables include all relevant parameters: the assigned value (A), the uncertainty of the assigned value (u), the standard deviation for proficiency assessment ($\sigma_p$) and the robust (relative) standard deviation, based on participants' results. In most cases the uncertainty of the assigned value did comply with the criterion $u \leq 0.3\sigma_p$ and was therefore considered as negligible. Uncertainty of the assigned value (u) in the material B exceeded $0.3\sigma_p$ for codeine, and therefore, the uncertainty of the assigned value was taken into account in the evaluation of the z-scores (by calculating the z'-score).

***Table 6*** *Summary of statistical evaluation of the PT results on the individual OAs and the sum of morphine + 0.2 codeine in material A.*

| | Morphine | Codeine | Sum morphine + 0.2 codeine |
|---|---|---|---|
| A (mg/kg) | 13.3 | 24.3 | 18.2 |
| u (mg/kg) | 0.966 | 1.29 | 1.33 |
| $\sigma_p$ (mg/kg) (25%) | 3.31 | 6.07 | 4.55 |
| $u > 0.3\sigma_p$ | No | No | No |
| robust σ (mg/kg) | 4.30 | 5.74 | 5.91 |
| robust σ (%) | 32 | 24 | 32 |
| # reported | 31 | 31 | 31 |
| # quantitative results | 31 | 31 | 31 |
| $\lvert z \rvert \leq 2$ | 27 | 30 | 26 |
| $2 < \lvert z \rvert < 3$ | 2 | 1 | 2 |
| $\lvert z \rvert \geq 3$ | 2 | 0 | 3 |
| S z-scores (%) | 87.1 | 96.8 | 83.9 |

S z-scores = satisfactory z-scores.

***Table 7*** *Summary of statistical evaluation of the PT results on the individual OAs and the sum of morphine + 0.2 codeine in material B.*

| | Morphine | Codeine | Sum morphine + 0.2 codeine |
|---|---|---|---|
| A (mg/kg) | 1.01 | 0.175 | 1.06 |
| u (mg/kg) | 0.067 | 0.015 | 0.064 |
| $\sigma_p$ (µg/kg) (25%) | 0.252 | 0.044 | 0.264 |
| $u>0.3\sigma_p$ | No | Yes | No |
| robust σ (mg/kg) | 0.298 | 0.057 | 0.283 |
| robust σ (%) | 30 | 32 | 27 |
| # reported | 31 | 31 | 31 |
| "<", nd, detected | | 8 | |
| # quantitative results | 31 | 23 | 31 |
| $\|z\| \leq 2$ | 25 | 19 | 25 |
| $2<\|z\|<3$ | 3 | 2 | 3 |
| $\|z\| \geq 3$ | 3 | 2 | 3 |
| S z-scores (%) | 80.6 | 82.6 | 80.6 |

S z-scores = satisfactory z-scores.

nd= not detected.

For the individual OAs in material A, 92% of the results were rated with satisfactory z-scores ($|z| \leq 2$), 5% of the results fell into the questionable range with $2<|z|<3$ and 3% of the results fell into the unsatisfactory range with $|z| \geq 3$ (Table 6). For material B was this respectively 82%, 9% and 9% (Table 7). Overall, 87% of the morphine and codeine results obtained for both materials (A and B) were rated with satisfactory z-scores ($|z| \leq 2$), 7% of the results fell into the questionable range with $2<|z|<3$ and 6% of the results fell into the unsatisfactory range with $|z| \geq 3$.

In case of the sum of morphine + 0.2 codeine, for material A, 84% of the results were rated with satisfactory z-scores ($|z| \leq 2$), 6% of the results fell into the questionable range with $2<|z|<3$ and 10% of the results fell into the unsatisfactory range with $|z| \geq 3$ (Table 6). For the sum of both OAs in material B was this respectively 81%, 10% and 10% (Table 7). Regarding the sum of OAs, for both materials, 82% of submitted results were satisfactory.

In Annex 11 an overview of the overall performance for each participant in this PT is provided. For the 2 materials combined, a maximum of 4 satisfactory z-scores, based on quantitative results for the individual OAs could be obtained, and '4 out of 4' therefore reflects an optimal performance in terms of scope and capability for quantitative determination. Out of 31 participants, 16 participants achieved optimal performance for both materials by detecting morphine and codeine with correct quantification, the absence of false negative results and reporting within the deadline. One participant achieved also satisfactory results for both OAs in both materials but reported the results after the deadline. For the other 14 participants one or more non-satisfactory z-scores were obtained. Seven of these 14 participants could not quantify codeine in material B but achieved otherwise satisfactory results. With respect to the sum of morphine + 0.2 codeine as mentioned in legislation, 23 participants showed satisfactory performance.

## 5.4 Robust relative standard deviation

The robust relative standard deviation ($RSD_R$) was calculated according to ISO 13528:2015 [2] for informative purposes only. In this study it was used as a good estimation of the interlaboratory variability. The $RSD_R$ values are included in for Annex 9, 10, in Tables 6 and 7 (Section 5.3) and in Table 1 (Summary section).

For material A, the $RSD_R$ of codeine (24%) was below the target standard deviation (25%) and for morphine the $RSD_R$ was 32%. For material B, the $RSD_R$ for morphine (30%) and codeine (32%) were above the target standard deviation.

The $RSD_R$ values for the sum of morphine + 0.2 codeine were for material A 32% and for material B it was 27%, both above the target standard deviation (25%).

# 6 Conclusions

Thirty-one laboratories, of which 24 National Reference Laboratories for mycotoxins and/or plant toxins in food or feed (from 18 EU Member States plus Iceland, Norway Serbia, Switzerland), and 7 Official Laboratories (from various EU Member States) participated in the PT on quantitative determination of morphine and codeine, as mentioned in Regulation (EU) 2021/2142 and (EU) 2023/915, in poppy seeds and bakery products.

All laboratories reported a total of six results, comprising of levels of morphine, codeine and the sum of morphine + 0.2 codeine in the two samples, consisting of material A and material B, as was requested. Two participants reported the sum of morphine and codeine without considering the factor 0.2 for codeine.

For the poppy seeds, 27 participants used a method with a reported LOQ of 1 mg/kg or lower. Three participants reported LOQs in the range of 1.7 to 2 mg/kg. For the bakery product, 26 participants used a method with a reported LOQ of 0.4 mg/kg or less. Four participants reported LOQs in the range of 1 to 2 mg/kg. Eight laboratories had problems with reporting quantitative results for codeine in the bakery product, due to the relatively high LOQs of their method. Three of these laboratories had LOQs > 0.5 mg/kg, which is above the regulatory LOQ for bakery products. Since NRLs are expected to have analytical methods in place not only for compliance testing of regulatory limits, but also in the framework of data generation for risk assessment, it is advised to set target LOQs of individual analytes to ≤0.5 mg/kg, at least for bakery products containing poppy seeds and /or derived products thereof.

The large majority of participants used methods based on LC-MS/MS (93%) and two participants applied LC-HRMS (High Resolution Mass Spectrometry). Most of the participants didn't use a sample clean-up (93%), the majority of participants diluted only the sample extract.

For individual OAs in material A, the percentage of satisfactory results for morphine was 87% and for codeine 97%. The $RSD_R$ of the reported results for morphine (32%) was above the target standard deviation (25%) and for codeine (24%) just below the target standard deviation. For material B, the satisfactory results were for morphine 81% and for codeine 83%. The $RSD_R$ were for morphine (30%) and codeine (32%) above the target standard deviation.

With respect to the sum of morphine + 0.2 codeine, for material A and B, respectively, 84% and 81% of the results were satisfactory. The $RSD_R$ for material A and B was 32% and 27%, respectively.

Overall, for individual OAs in both materials combined (4 results), 87% of the results were rated with satisfactory z-scores ($|z| \leq 2$), 7% of the results fell into the questionable range with $2<|z|<3$ and 6% of the results fell into the unsatisfactory range with $|z| \geq 3$. Seventeen participants had a satisfactory performance of which one participant reported results after the deadline. For the sum of morphine and 0.2 codeine in both materials combined (2 results), 82% of the results were satisfactory and 23 participants had a satisfactory performance.

It is noticed that although 81-97% of the results reported for morphine and codeine in materials A and B fell in the satisfactory range, nevertheless the $RSD_R$ values were in most cases higher than the target standard deviation of 25%. Only for codeine (97% satisfactory results) in material A the target $RSD_R$ was met. This outcome a bit surprising because most participants have included isotopically labeled internal standards in their methods to improve quantification. The results seem to suggest that the use of these IS only partly successful. This may be caused by two factors: 1. The retention times of the (deuterated) IS often differ somewhat from the analyte of interest, which may make them less effective in correcting for matrix effects. 2. The IS are often added at the end of the sample preparation procedure, what will limit their use in correction of differences in extraction efficiency and recovery.

From the results obtained in this PT on OAs it can be concluded that most of the participants have an analytical method available with sufficiently low LOQs. For some laboratories lower LOQs would be required for the quantification of OAs in bakery products containing poppy seeds and /or derived products thereof at the level regulated by the Commission Regulation EU 2021/2142 and 2023/915. Furthermore, the room for improvement remains, because the variation in the results is relatively high. For the OAs in both materials, the interlaboratory reproducibility ($RSD_R$) was in most cases at or above 30%.

# References


[1] ISO/IEC 17043:2010. Conformity assessment - General requirements for the proficiency testing.

[2] ISO 13528:2015. Statistical methods for use in proficiency testing by inter-laboratory comparison, 1st edition.

[3] ISO/IEC 17025:2017(E). 2017. General requirements for the competence of testing and calibration laboratories.

[4] Commission Regulation (EU) 2017/625 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, Art. 94.2. Official Journal of the European Union 7.4.2017, L95, 1-142.

[5] EURL mycotoxins & plant toxins, 2019. Performance assessment in proficiency tests organised by the EURL mycotoxins & plant toxins in food and feed. EURL-MP-background doc_001 (version 1). https://www.wur.nl/en/research-results/research-institutes/food-safety-research/reference-laboratory/european-union-reference-laboratory/eurl-mycotoxins-plant-toxins/library-eurl-mp.htm#eurlmp_background_documents.

[6] WFSR SOP-A-0989 – Preparation of PT materials and PT samples.

[7] EURLMP-method_007 v1, Determination of opium alkaloids in poppy seeds and poppy seeds containing bakery products by LC-MS/MS, EURL mycotoxins and plant toxins, RIKILT Wageningen University & Research. https://www.wur.nl/en/research-results/research-institutes/food-safety-research/reference-laboratory/european-union-reference-laboratory/eurl-mycotoxins-plant-toxins/library-eurl-mp.htm#eurlmp_methods.

[8] Commission Regulation (EU) 2021/2142 of 3 December 2021, amending Regulation (EC) No 1881/2006 as regards maximum levels of opium alkaloids in certain foodstuffs.

[9] Working document SANTE 11494R3/2021, Commission implementing Regulation (EU) …/... of XXX laying down the methods of sampling and analysis for the control of plant toxins in food and repealing Regulation (EU) No 2015/705.

[10] Thompson M, Ellison SL, Wood R. 2006. The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories. Pure Applied Chemistry 78(1):145-196.

[11] Analytical Methods Committee. 1989. Robust statistics - How not to reject outliers Part 1. Basic concepts. Analyst 114:1693-1697.

[12] Analytical Methods Committee. 1989. Robust statistics - How not to reject outliers Part 2. Inter-laboratory trials. Analyst. 114:1699-1702.

[13] Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006.

[14] Commission Regulation (EU) No 2015/705 of 30 April 2015 laying down methods of sampling and performance criteria for the methods of analysis for the official control of the levels of erucic acid in foodstuffs and repealing Commission Directive 80/891/EEC.

# Annex 1 List of participants

| Country | Organisation |
|---|---|
| AUSTRIA* | Austrian Agency for Health and Food Safety |
| BELGIUM* | CER Groupe |
| CROATIA* | A. Stampar Teaching Institute of Public Health |
| CYPRUS* | State general laboratory |
| CZECH REPUBLIC* | Czech Agriculture and Food Inspection Authority (CAFIA) |
| CZECH REPUBLIC* | Central Institute for Supervising and Testing in Agriculture |
| DENMARK* | Danish Veterinary and Food Administration |
| FINLAND* | Finnish Customs Laboratory |
| FRANCE* | Laboratoire SCL de Strasbourg |
| GERMANY*** | Chemisches und Veterinaruntersuchungsamt (CVUA) Sigmaringen |
| GERMANY | Federal Institute fur Risk Assessment (BfR) |
| GERMANY*** | CVUA Karlsruhe |
| GERMANY*** | Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit |
| GERMANY*** | Landesuntersuchungsanstalt Sachsen |
| GERMANY** | Eurofins WEJ Contaminants |
| GERMANY*** | LALLF, MV |
| GERMANY*** | Landesamt fur Verbraucherschutz Sachsen-Anhalt |
| GREECE* | General Chemical State Laboratory |
| HUNGARY* | National Food Chain Safety Office |
| IRELAND* | The Public Analyst's Laboratory |
| ITALY* | IZSLER |
| ITALY* | Istituto Superiore di Sanita |
| ITALY*** | Istituto Zooprofilattico delle Venezie |
| LITHUANIA* | National Food and Veterinary Risk Assessment Institute |
| LUXEMBOURG* | Laboratoire National de Sante |
| NORWAY** | Norwegian Institute of Bioeconomy Research (NIBIO) |
| ROMANIA* | Institute for Hygiene and Veterinary Public Health |
| SERBIA | SP LABORATORIJA A.D. |
| SLOVENIA* | National Laboratory of Health, Environment and Food (NLZOH) |
| SPAIN* | Spanish Agency for Consumer Affairs, Food Safety and Nutrition |
| SWITZERLAND** | Kantonales Laboratorium Bern |

* National Reference Laboratory (NRL) of EU Member State.

** National Reference Laboratory (NRL) of the European Free Trade Association (Eurofins WEJ Contaminants = Iceland) and Serbia.

*** Official Laboratory (OL).

# Annex 2 Codification of the samples

| Participant's code | Material A* | Material B* |
|---|---|---|
| PT8258 | 298 | 603 |
| PT8259 | 538 | 282 |
| PT8260 | 953 | 301 |
| PT8261 | 549 | 360 |
| PT8262 | 141 | 984 |
| PT8263 | 574 | 195 |
| PT8264 | 401 | 370 |
| PT8265 | 627 | 742 |
| PT8266 | 801 | 624 |
| PT8267 | 800 | 534 |
| PT8268 | 441 | 581 |
| PT8269 | 686 | 585 |
| PT8270 | 495 | 595 |
| PT8271 | 925 | 806 |
| PT8272 | 963 | 194 |
| PT8273 | 134 | 418 |
| PT8274 | 565 | 489 |
| PT8275 | 608 | 111 |
| PT8276 | 776 | 619 |
| PT8277 | 161 | 375 |
| PT8278 | 874 | 300 |
| PT8279 | 559 | 286 |
| PT8280 | 845 | 917 |
| PT8281 | 974 | 717 |
| PT8282 | 558 | 600 |
| PT8283 | 669 | 877 |
| PT8285 | 428 | 656 |
| PT8286 | 156 | 455 |
| PT8287 | 747 | 198 |
| PT8288 | 487 | 899 |
| PT8289 | 620 | 307 |

* All sample codes start with 2023/EURL PT MP/OAs/.

# Annex 3 Statistical evaluation of homogeneity data

| | Morphine in A (mg/kg) | |
|---|---|---|
| **Sample No.** | **Replicate 1** | **Replicate 2** |
| Hom/B001 | 16.76 | 17.83 |
| Hom/B002 | 18.18 | 18.68 |
| Hom/B003 | 18.46 | 18.22 |
| Hom/B004 | 17.10 | 18.05 |
| Hom/B005 | 16.54 | 17.00 |
| Hom/B006 | 19.42 | 18.09 |
| Hom/B007 | 16.55 | 18.42 |
| Hom/B008 | 19.37 | 16.48 |
| Hom/B009 | 19.83 | 18.79 |
| Hom/B010 | 15.87 | 17.27 |
| Grand mean | 17.8 | |
| Cochran's test | | |
| C | 0.433 | |
| Ccrit | 0.602 | |
| C < Ccrit? | NO OUTLIERS | |
| Target s = $\sigma_P$ | 4.46 | |
| $s_x$ | 0.874 | |
| $s_w$ | 0.981 | |
| $s_s$ | 0.531 | |
| Critical= 0.3 $\sigma_P$ | 1.34 | |
| $s_s$ < critical? | ACCEPTED | |
| $s_w$ < 0.5 $\sigma_P$? | ACCEPTED | |

$s_x$ = Standard deviation of the sample averages.

$s_w$ = Within-sample standard deviation.

$s_s$ = Between-sample standard deviation.

| | Codeine in A (mg/kg) | |
|---|---|---|
| **Sample No.** | **Replicate 1** | **Replicate 2** |
| Hom/B001 | 30.58 | 29.59 |
| Hom/B002 | 29.03 | 28.58 |
| Hom/B003 | 29.37 | 29.62 |
| Hom/B004 | 33.34 | 29.99 |
| Hom/B005 | 34.55 | 31.25 |
| Hom/B006 | 30.15 | 29.10 |
| Hom/B007 | 28.78 | 32.30 |
| Hom/B008 | 30.13 | 28.83 |
| Hom/B009 | 30.06 | 29.94 |
| Hom/B010 | 27.83 | 30.16 |
| Grand mean | 30.2 | |
| Cochran's test | | |
| C | 0.282 | |
| Ccrit | 0.602 | |
| C < Ccrit? | NO OUTLIERS | |
| Target s = $\sigma_P$ | 7.54 | |
| $s_x$ | 1.26 | |
| $s_w$ | 1.48 | |
| $s_s$ | 0.702 | |
| Critical= 0.3 $\sigma_P$ | 1.94 | |
| $s_s$ < critical? | ACCEPTED | |
| $s_w$ < 0.5 $\sigma_P$? | ACCEPTED | |

$s_x$ = Standard deviation of the sample averages.

$s_w$ = Within-sample standard deviation.

$s_s$ = Between-sample standard deviation.

| Sample No. | Morphine in B (mg/kg) | |
|---|---|---|
| | Replicate 1 | Replicate 2 |
| Hom/B001 | 0.988 | 0.907 |
| Hom/B002 | 0.848 | 0.900 |
| Hom/B003 | 0.920 | 1.008 |
| Hom/B004 | 0.974 | 1.059 |
| Hom/B005 | 0.915 | 0.886 |
| Hom/B006 | 0.922 | 0.900 |
| Hom/B007 | 0.935 | 0.993 |
| Hom/B008 | 0.887 | 1.054 |
| Hom/B009 | 0.995 | 0.953 |
| Hom/B010 | 1.072 | 0.993 |
| Grand mean | 0.955 | |
| Cochran's test | | |
| C | 0.432 | |
| Ccrit | 0.602 | |
| C < Ccrit? | NO OUTLIERS | |
| Target s = $\sigma_P$ | 0.239 | |
| $s_x$ | 0.050 | |
| $s_w$ | 0.057 | |
| $s_s$ | 0.029 | |
| Critical= 0.3 $\sigma_P$ | 0.072 | |
| $s_s$ < critical? | ACCEPTED | |
| $s_w$ < 0.5 $\sigma_P$? | ACCEPTED | |

$s_x$ = Standard deviation of the sample averages.
$s_w$ = Within-sample standard deviation.
$s_s$ = Between-sample standard deviation.

| Sample No. | Codeine in B (mg/kg) | |
|---|---|---|
| | Replicate 1 | Replicate 2 |
| Hom/B001 | 0.152 | 0.151 |
| Hom/B002 | 0.149 | 0.160 |
| Hom/B003 | 0.133 | 0.153 |
| Hom/B004 | 0.145 | 0.164 |
| Hom/B005 | 0.141 | 0.148 |
| Hom/B006 | 0.145 | 0.145 |
| Hom/B007 | 0.147 | 0.148 |
| Hom/B008 | 0.146 | 0.139 |
| Hom/B009 | 0.144 | 0.129 |
| Hom/B010 | 0.166 | 0.156 |
| Grand mean | 0.148 | |
| Cochran's test | | |
| C | 0.313 | |
| Ccrit | 0.602 | |
| C < Ccrit? | NO OUTLIERS | |
| Target s = $\sigma_P$ | 0.037 | |
| $s_x$ | 0.007 | |
| $s_w$ | 0.008 | |
| $s_s$ | 0.004 | |
| Critical= 0.3 $\sigma_P$ | 0.011 | |
| $s_s$ < critical? | ACCEPTED | |
| $s_w$ < 0.5 $\sigma_P$? | ACCEPTED | |

$s_x$ = Standard deviation of the sample averages.

$s_w$ = Within-sample standard deviation.

$s_s$ = Between-sample standard deviation.

# Annex 4 Statistical evaluation of stability data

*Stability evaluation for morphine in material A.*

| Storage temperature | freezer | refrigerator |
|---|---|---|
| Time (days) | 0 | 64 |
| Calculated amounts (mg/kg) | 15.5 | 18.2 |
| | 16.6 | 15.0 |
| | 17.7 | 16.4 |
| | 16.2 | 17.3 |
| | 18.5 | 17.6 |
| | 17.1 | 16.9 |
| Average amount (mg/kg) | 16.9 | 16.9 |
| n | 6 | 6 |
| st. dev (mg/kg) | 1.09 | 1.10 |
| Difference | | 0.033 |
| $0.3*\sigma_P$ | | 1.27 |
| Consequential difference? Diff < $0.3*\sigma_P$ | | No |

*Stability evaluation for codeine in material A.*

| Storage temperature | freezer | refrigerator |
|---|---|---|
| Time (days) | 0 | 64 |
| Calculated amounts (mg/kg) | 27.8 | 31.5 |
| | 26.8 | 27.3 |
| | 29.6 | 30.4 |
| | 27.4 | 30.6 |
| | 29.1 | 28.5 |
| | 30.3 | 28.2 |
| Average amount (mg/kg) | 28.5 | 29.4 |
| n | 6 | 6 |
| st. dev (mg/kg) | 1.36 | 1.63 |
| Difference | | -0.904 |
| $0.3*\sigma_P$ | | 2.14 |
| Consequential difference? Diff < $0.3*\sigma_P$ | | No |

*Stability evaluation for morphine in material B.*

| Storage temperature | <-80 °C | <-20 °C |
|---|---|---|
| Time (days) | 0 | 63 |
| Calculated amounts (mg/kg) | 1.10 | 1.01 |
| | 1.09 | 1.07 |
| | 1.18 | 1.12 |
| | 1.19 | 1.09 |
| | 1.14 | 1.13 |
| | 1.14 | 1.15 |
| Average amount (mg/kg) | 1.14 | 1.10 |
| n | 6 | 6 |
| st. dev (mg/kg) | 0.039 | 0.051 |
| Difference | | 0.043 |
| $0.3*\sigma_P$ | | 0.085 |
| Consequential difference? Diff < $0.3*\sigma_P$ | | No |

*Stability evaluation for codeine in material B.*

| Storage temperature | freezer | refrigerator |
|---|---|---|
| Time (days) | 0 | 64 |
| Calculated amounts (mg/kg) | 0.190 | 0.182 |
| | 0.191 | 0.185 |
| | 0.195 | 0.192 |
| | 0.194 | 0.178 |
| | 0.184 | 0.193 |
| | 0.188 | 0.181 |
| Average amount (mg/kg) | 0.190 | 0.185 |
| n | 6 | 6 |
| st. dev (mg/kg) | 0.004 | 0.006 |
| Difference | | 0.005 |
| $0.3*\sigma_P$ | | 0.014 |
| Consequential difference? Diff < $0.3*\sigma_P$ | | No |

# Annex 5 Invitation letter

WAGENINGEN
UNIVERSITY & RESEARCH

European Union Reference Laboratory

P.O. Box 230 | 6700 AE Wageningen | The Netherlands

NRLs mycotoxins & plant toxins

Dear colleague,

The EURL mycotoxins & plant toxins, at Wageningen Food Safety Research (WFSR), will organize a proficiency test regarding opium alkaloids in poppy seeds and bakery products in 2023 (EURLPT-MP09). This proficiency test will focus on the quantification of the opium alkaloids morphine and codeine as mentioned in Regulation (EU) 2021/2142 and will be organised under accreditation according to ISO 17043 (General requirements for proficiency testing - R013).

I would like to invite you to participate in this proficiency test.

Harmonised EU regulation for opium alkaloids in poppy seeds and bakery products is laid down in Regulation (EU) No. 2021/2142 and is applied from July 1, 2022. The primary goal of this proficiency test is to give laboratories the opportunity to evaluate or demonstrate their performance regarding the analysis of morphine and codeine in poppy seeds and bakery products.

According to Regulation (EU) 2017/625, it is mandatory for all EU National Reference Laboratories (NRLs) mycotoxins & plant toxins in food and/or feed to participate.

The following matters are important for participation in this proficiency test:

1. Test materials
   Two test materials, poppy seeds and a bakery product, will be provided.
   The test amount sent for poppy seeds and bakery product will be approximately 50 and 25 gram, respectively.

2. Shipment of the test materials
   Test materials will be sent in the beginning of March 2023. The distribution of the test materials will be announced by e-mail. The deadline for reporting will be six weeks after the shipment of the samples.

3. Scope of the analysis
   Both materials are to be analysed for the opium alkaloids, as defined in regulation (EU) 2021/2142:
   • morphine
   • codeine
   The participants should provide their own analytical standards.

Wageningen Food Safety Research

Natural toxins

DATE
February 8, 2023

SUBJECT
Invitation EURL mycotoxins & plant toxins proficiency test opium alkaloids in poppy seeds and bakery products 2023 (EURLPT-MP09)

POSTAL ADDRESS
P.O. Box 230
6700 AE Wageningen
The Netherlands

VISITORS' ADDRESS
Wageningen Campus
Building 123
Akkermaalsbos 2
6708 WB Wageningen

INTERNET
www.wur.nl

KVK NUMBER
09098104

HANDLED BY
Diana Pinneboom

TELEPHONE
+31(0) 614 323 017

EMAIL
pt.wfsr@wur.nl

4. Questionnaire
A questionnaire will be sent electronically. In this questionnaire the participants will be asked to provide information about the laboratory method(s) used. This information is necessary to conduct a more in depth analysis of the results obtained in this proficiency test.

5. Report
- Preliminary results of this proficiency test will be reported to the participants in July 2023.
- The report is expected to be published in November 2023.
- Results of the proficiency test will be presented anonymously.
- Disclosure of the results of the NRLs to the representative of the European Commission is foreseen after the report is published.
- The follow-up protocol on proficiency tests from DG Santé will be applied.

6. Additional information
- WFSR is allowed to use the anonymous results of the proficiency test in presentations, seminars and publications.
- WFSR will never inform third parties (e.g. accreditation bodies) on specific laboratory results without informing the laboratory first.

7. Costs
- Participation is free of charge for NRLs.
- Official laboratories (OLs) can participate as long as sufficient test material is available, at a first come first serve basis. The participation fee for OLs is €270.- (ex. VAT) as a compensation for the preparation and transportation of the samples.
- If an extra batch of samples is needed after the first shipping, the courier costs will be charged.

If you would like to participate, please fill out the accompanying participation form (preferably digitally) and send it back **before February 24th 2023**: pt.wfsr@wur.nl.

Looking forward to welcome you for this proficiciency test,

D. Pereboom

Diana Pereboom
Proficiency tests

EURL mycotoxins & plant toxins in food and feed
Wageningen Food Safety Research

# Annex 6 Instruction letter

WAGENINGEN
UNIVERSITY & RESEARCH

European Union Reference Laboratory

P.O. Box 230 | 6700 AE WAGENINGEN | The Netherlands

Dear Contactperson,

Thank you very much for your interest in the proficiency test for the analysis of opium alkaloids in poppy seeds and bakery product.

The parcel shipped to you should contain:

- One material consisting of a poppy seed and one material consisting of a bakery product. The poppy seed material contains approximately 50 grams of the homogenised test material and the bakery product approximately 25 grams.

Instructions:

- After arrival the samples should be stored at 4°C.
- Please fill in the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples by e-mail (pt.wfsr@wur.nl).
- Before analysis, homogenise the samples according to your laboratory's procedure.
- Treat the test material as a sample for routine analysis, according to your laboratory procedure. Report one result and not an average of multiple measurements.
- Quantify the opium alkaloids morphine and codeine as defined in Commission Regulation (EU) No. 2021/2142.
- Report for each material a total of 3 results, comprised of morphine, codeine and the weighted sum of these opium alkaloids:
  1. Morphine
  2. Codeine
  3. Sum of morphine and codeine, for which a factor of 0,2 is applied to the level of codeine. The concentration refers to the sum of morphine + 0,2 codeine.


Wageningen Food Safety Research

DATE
March 6, 2023

SUBJECT
Instructions proficiency test opium alkaloids in poppy seeds and bakery products

YOUR REFERENCE

OUR REFERENCE
2303211/WFSR

POSTAL ADDRESS
P.O. Box 230
6700 AE WAGENINGEN
The Netherlands

VISITORS' ADDRESS
Wageningen Campus
Building 123
Akkermaalsbos 2
6708 WB WAGENINGEN

INTERNET
www.wur.nl/rikilt

CoC NUMBER
09098104

HANDLED BY
Diana Pereboom

TELEPHONE
+31 (0) 614323017

EMAIL
pt.wfsr@wur.nl

- Reporting:
  - Report all analytical results in **mg/kg**
  - Report other results as '**<[value mg/kg]**' or '**nt**' as follows:
    - '**<[value in mg/kg]**': When the result for the analyte is below the LOQ of the method, report the result as below the LOQ value in mg/kg, e.g. as '<10 mg/kg'.
    - '**nt**': If an analyte is not included in the scope of the method, report the result as not tested, 'nt'.
- Results reported in any other format (e.g. nd, detected, <LOQ, etc) will be regarded as not tested, 'nt'.
- Please use the web application to submit the results for the test samples (https://crlwebshop.wur.nl/ordsp/f?p=107:LOGIN). Information about the use of this web application was sent to you earlier by e-mail.
- Provide detailed information in the questionnaire on the analysis of the morphine and codeine and the analytical method used and send it back to us by e-mail (pt.wfsr@wur.nl).
- You can download the EURL method "EURL-MP-method-007 Opium alkaloids in food, for the analysis of opium alkaloids using LC-MS/MS", from the EURL mycotoxins & plant toxins website (https://www.wur.nl/en/Research-Results/Research-Institutes/food-safety-research/Reference-laboratory/European-Union-Reference-Laboratory-1/EURL-mycotoxins-plant-toxins/Library-EURL-MP.htm).
- The deadline for submitting test-results for this proficiency test is **17th of April 2023.**

- Your username is:
- Your password is:
- Your lab code to enter this proficiency test is:

Please contact me in case you have any questions or need any assistance.

With kind regards,

D. Peeleboom

# Annex 7 Scope and LOQ

| | LOQ (mg/kg) | |
|---|---|---|
| **Lab code** | **Morphine** | **Codeine** |
| PT8258 | 0.03 | 0.03 |
| PT8259 | 0.01 | 0.01 |
| PT8260 | 1 | 1 |
| PT8261 | 0.01 | 0.01 |
| PT8262 | 0.1 | 0.1 |
| PT8263 | 0.1 | 0.1 |
| PT8264 (A)* | 1.7 | 1.7 |
| PT8264 (B)* | 0.4 | 0.4 |
| PT8265 | 0.1 | 0.1 |
| PT8266 | 0.2 | 0.2 |
| PT8267 (A)* | 0.5 | 0.5 |
| PT8267 (B)* | 0.1 | 0.1 |
| PT8268 | 0.2 | 0.2 |
| PT8269 | | |
| PT8270 | 0.1 | 0.1 |
| PT8271 | 0.1 | 0.1 |
| PT8272 (A)* | 0.5 | 0.5 |
| PT8272 (B)* | 0.1 | 0.1 |
| PT8273 (A)* | 0.5 | 0.5 |
| PT8273 (B)* | 0.1 | 0.1 |
| PT8274 | 1 | 1 |
| PT8275 | 0.21 | 0.1 |
| PT8276 | 0.15 | 0.15 |
| PT8277 | 0.25 | 0.25 |
| PT8278 | 0.1 | 0.1 |
| PT8279 | 0.4 | 0.4 |
| PT8280 | 0.01 | 0.01 |
| PT8281 | 0.1 | 0.3 |
| PT8282 | 0.01 | 0.01 |
| PT8283 | 0.096 | 0.083 |
| PT8285 (A)* | 2 | 2 |
| PT8285 (B)* | 0.4 | 0.4 |
| PT8286 | 0.1 | 0.1 |
| PT8287 | 0.05 | 0.05 |
| PT8288 | 1 | 1 |
| PT8289 | 2 | 2 |

* (A)= material A (poppy seeds) and (B)= material B (the bakery product).

# Annex 8 Analytical method details

| Lab code | Method | Column | Column length (mm) | Total run time (min) | Mobile phase | Detection technique | Morphine RT (min) | Codeine RT (min) |
|---|---|---|---|---|---|---|---|---|
| PT8258 | acid | Phenomenex, Kinetex PFP, 150 x 2.1 mm, 1.7 µm | 150 | 35 | A: 5 mmol ammonium formate + 0.1% formic acid in water<br>B: 5 mmol ammonium formate + 0.1% formic acid in methanol | MS/MS | 2.19 | 3.55 |
| PT8259 | alkaline | Waters, XBridge C18, 75 x 3.0 mm, 2.5 µm | 75 | 20 | A: 20 mM ammonium bicarbonate<br>B: methanol | MS/MS | 4.94 | 5.24 |
| PT8260 | alkaline | Phenomenex, Kinetex EVO C18, 150 x 3 mm, 5 µm | 150 | 12 | A: 1 mmol ammonium bicarbonate in methanol/water 5/95 (V/V)<br>B: 1 mmol ammonium bicarbonate in methanol/water 95/5 (V/V) | MS/MS | 4.7 | 5.0 |
| PT8262 | alkaline | Waters, UPLC BEH C18, 100 x 2.1 mm, 1.7 µm | 100 | 12 | A:10 mM ammonium carbonate in water, pH 9.0<br>B: acetonitrile | MS/MS | 3.4 | 5.1 |
| PT8263 | alkaline | Waters, Acquity BEH C18, 100 x 2.1 mm, 1.7 um | 100 | 16 | A: 10 mM ammonium carbonate in water, pH 9<br>B: acetonitrile | MS/MS | 5.1 | 6.6 |
| PT8264 | alkaline | Waters, XBridge C18, 150 x 3.0 mm, 5 µm | 150 | 20 | A: 17 mM ammonium carbonate in water, pH 9<br>B: methanol | MS/MS | 9.11 | 9.97 |
| PT8265 | alkaline | Waters, BEH C18, 100 x 2.1 mm, 1.7 µm | 100 | 12 | A: 10 mM ammonium carbonate in water pH 9.0<br>B: acetonitrile | MS/MS | 3.0 | 4.6 |
| PT8266 | acid | Thermo Scientific, Hypersil Gold® C18, 150 x 2.1 mm, 1.9 µm | 150 | 15 | A: 5 mmol ammonium formate + 0.1% formic acid in water<br>B: 5 mmol ammonium formate + 0.1% formic acid in methanol | MS/MS | 3.28 | 4.78 |
| PT8267 | alkaline | Waters, Acquity UPLC BEH C18, 100 x 2.1 mm, 1.7 µm | 100 | 6 | A: 10 mM ammonium carbonate<br>B: acetonitrile | MS/MS | 1.32 | 1.96 |
| PT8268 | acid | Phenomenex Luna C18, 150 x 2.0 mm | 150 | 10 | A: 5 mmol ammonium formate + 0.1% formic acid in water<br>B: 5 mmol ammonium formate + 0.1% formic acid in methanol | MS/MS | 3.5 | 5.2 |
| PT8270 | acid | Waters, Acquity UPLC BEH Amide, 100 x 2.1 mm, 1.7 µm | 100 | 10 | A: 50 mM ammonium formate<br>B: acetonitrile | MS/MS | 4.24 | 2.3 |
| PT8271 | acid | Waters, HSS T3, 100 mm x 2.1, 1.8 µm | 100 | 22 | A: water + ammonium acetate, 0.1% acetic acid<br>B: acetonitrile + 0.1% acetic acid | MS/MS | 1.97 | 2.20 |
| PT8272 | alkaline | Phenomenex, Gemini-NX C18, 150 x 2.00 mm, 5 µm | 150 | 13 | A: water + ammonium carbonate buffer pH 9<br>B: acetonitrile | MS/MS | 4.95 | 5.82 |
| PT8273 | alkaline | Waters, Acquity BEH C18, 100 x 2.1 mm, 1.7 µm | 100 | 12 | A: 10mM ammonium carbonate (pH 9)<br>B: acetonitrile | MS/MS | 3.6 | 5.4 |
| PT8274 | alkaline | Phenomenex, Gemini RP18, 150 x 2 mm, 3 µm | 150 | 18 | A: 5 mM ammonium bicarbonate in water + 0.1% ammonium hydroxide | MS/MS | 7.7 | 8.5 |

| Lab code | Method | Column | Column length (mm) | Total run time (min) | Mobile phase | Detection technique | Morphine RT (min) | Codeine RT (min) |
|---|---|---|---|---|---|---|---|---|
| | | | | | B: 5 mM ammonium bicarbonate in methanol/water (50:50, v/v) + 0.1% ammonium hydroxide | | | |
| PT8275 | acid | Phenomenex, Synergi Polar RP, 100 x 2 mm, 4 µm, 80 A | 100 | 20 | A: water with 0.1% formic acid<br>B: acetonitrile with 0.1% formic acid | MS/MS | 2.7 | 7.1 |
| PT8276 | alkaline | Waters, XBridge Premier BEH C18, 50 x 2.1 mm, 2.5 µm | 50 | 12 | A: 10 mM ammonium carbonate in water<br>B: acetonitrile | MS/MS | 3.9 | 5.6 |
| PT8277 | acid | Waters, Aquity BEH C18, 100 x 2.1 mm, 1.7 µm | 100 | 13 | A: water + formic acid 0.1%<br>B: methanol + formic acid 0.1% | HRMS | 1.5 | 2.8 |
| PT8278 | acid | Agilent, Pursuit 3 Diphenyl, 150 x 3.0 mm, 3 µm | 150 | 9 | 5 mmol ammonium formate, 1 mL formic acid in 1 L water | MS/MS | 3.0 | 3.6 |
| PT8279 | alkaline | Waters | | 15 | A: 30 mmol ammonium bicarbonate/L 0.1% ammonia in water<br>B: 30 mmol ammonium bicarbonate/L 0.1% ammonia in methanol-water (95:5) | MS/MS | 6.29 | 7.35 |
| PT8280 | alkaline | YMC, Triart C18, 100 x 2.0 mm, 1.9 µm | 100 | 12 | A: 10 mM ammonium carbonate in water, pH 9.0<br>B: acetonitrile | MS/MS | 4.22 | 5.3 |
| PT8281 | acid | Waters, Acquity BEH C18, 50 x 2.1 mm, 1.7 µm | 50 | 4 | A: water + 0.1% formic acid<br>B: acetonitrile + 0.1% formic acid | MS/MS | 1.45 | 2.33 |
| PT8282 | acid | Phenomenex, Luna Omega Polar C18, 50 x 4.6 mm, 3.0 µm | 50 | 23 | A: water + 0.1% formic acid<br>B: methanol + 0.1% formic acid | HRMS | 3.0 | 6.8 |
| PT8283 | alkaline | Waters, Acquity UPLC BEH C18, 150 x 2.1 mm, 1.7 µm | 150 | 16 | A: 10mM ammonium carbonate in water pH 9<br>B: acetonitrile | MS/MS | 2.87 | 3.87 |
| PT8285 | acid | Thermo Scientific, Hypersil Gold C18, 150 x 2.1 mm; 3 µm | 150 | 20 | A: water + 5 mM ammonium formate + 1% formic acid<br>B: methanol + 5 mM ammonium formate + 1% formic acid | MS/MS | 2.4 | 4.4 |
| PT8286 | acid | Restek, Raptor ARC-18, 150 x 2.1 mm, 1.8 µm | 150 | 12 | A: water + 0.1% formic acid + 5 mM ammonium formate<br>B: acetonitrile + 0.1% formic acid | MS/MS | 0.6 | 0.637 |
| PT8287 | acid | Agilent, Zorbax Eclipse Plus C18, 50 mm x 2.1 mm, 1.8 µm | 50 | 10 | A: 2 mM ammonium formate + 0.1% formic acid in water<br>B: 2 mM ammonium formate + 0.1% formic acid in methanol | MS/MS | 1.02 | 1.56 |
| PT8288 | acid | MZ analysentechnik, Perfectsil 100 ODS-3, 250 x 4.6 mm, 5 µm | 250 | 24 | A: 1% formic acid in water<br>B: 1% formic acid in methanol | MS/MS | 10.34 | 11.86 |
| PT8289 | alkaline | Phenomenex, Gemini C18, 150 x 3 mm, 3 µm | 150 | 15 | A: 10 mM ammonium carbonate in water, pH 9.0<br>B: methanol | MS/MS | 10.8 | 12.3 |

| Lab code | Sample weight (g) | Extraction solvent | Extraction solvent volume (ml) | Extraction conditions | Extraction time (min) | Sample clean-up | Internal standard | Matrix equivalent final extract (g/ml) |
|---|---|---|---|---|---|---|---|---|
| PT8258 | A: 5 g: (wet sample mix: 50 g A/100 g water)<br>B: 10 g: (wet sample mix: 30 g B/60 g water) | methanol with formic acid 2% | A: 50<br>B: 3 x 20 | mechanical shaking | 3 x 15 | SPE | codeine-D3; morphine D3 (only for the bakery product) | A: 0.00666<br>B: 2.5 |
| PT8259 | 2.5 | methanol + 0.1% acetic acid | 20 | mechanical shaking | 20 | none | morphine-D3 | 0.05 |
| PT8260 | A: 5<br>B: 5 | methanol/water/formic acid 60/40/0.4 (v/v/v) | 2 x 50 | mechanical shaking | 2 x 45 | none | morphine-D3; codeine-D3 | |
| PT8262 | A: 2<br>B: 4 | 0.4% formic acid in methanol/water (60/40, v/v) | A: 20<br>B: 40 | mechanical shaking | 30 | dilution | morphine-D3; codeine-D3 | |
| PT8263 | A: 10<br>B: 4 | 0.4% formic acid in methanol/water (60/40, v/v) | 100 | mechanical shaking | 30 | dilution | morphine-D3; codeine-D3 | 0.1 |
| PT8264 | 10 | methanol + 5% acetic acid | 100 | other | 65 | dilution | morphine-D3; codeine-D3 | 0.1 |
| PT8265 | 2 | 0.4% formic acid in methanol/water (6/4 v/v) | 20 | mechanical shaking | 30 | dilution | | 0.1 |
| PT8266 | 5 | methanol/water 60/40 + 0.4% formic acid | 2 x 50 | mechanical shaking | 2 x 45 | dilution | morphine-D6; codeine-D6 | 0.0005 |
| PT8267 | A: 2<br>B: 4 | methanol/water/formic acid (60/40/0.4, v/v/v) | A: 20<br>B: 40 | mechanical shaking | 30 | dilution | morphine-D3 | 0.1 |
| PT8268 | 10 | methanol/water/formic acid | 100 | mechanical shaking | 45 | dilution | morphine-D3; codeine-D3 | 0.0025 |
| PT8270 | 4 | 0.4% formic acid + methanol:water = 60:40 | 40 | mechanical shaking | 30 | dilution | morphine-D6; codeine-D6 | 0.1 |
| PT8271 | 5 | water:acetonitrile+0.5% acetic acid | 20 | mechanical shaking | 30 | dilution | morphine-D3; codeine-D3 | 0.001 |
| PT8272 | A: 10<br>B: 4 | 0.4% formic acid in methanol/water (60/40, v/v) | A: 100<br>B: 40 | mechanical shaking | 30 | dilution | morphine-D3; codeine-D6 | 0.1 |
| PT8273 | A: 10<br>B: 4 | 0.4% formic acid in methanol/water 60/40 | A: 100<br>B: 40 | shaking (hand/vortex) | 30 | none | morphine-D3; codeine-D3 | 0.002 |
| PT8274 | 5 | methanol/water (60:40, v/v) + 0.4% formic acid | 2 x 50 | mechanical shaking | 2 x 45 | none | morphine-D3; codeine-D3 | 0.05 |
| PT8275 | 5 | methanol/water/formic acid (600/400/4) | 2 x 50 | mechanical shaking | 2 x 45 | none | morphine-D3; codeine-D3 | 0.05 |

| Lab code | Sample weight (g) | Extraction solvent | Extraction solvent volume (ml) | Extraction conditions | Extraction time (min) | Sample clean-up | Internal standard | Matrix equivalent final extract (g/ml) |
|---|---|---|---|---|---|---|---|---|
| PT8276 | 5 | methanol/water/formic acid 60/40/0.4 | 2 x 50 | mechanical shaking | 2 x 45 | dilution | morphine-D3; codeine-D3 | 0.01 |
| PT8277 | A: 10<br>B: 4 | methanol + water + formic acid | A: 100<br>B: 40 | mechanical shaking | 30 | none | morphine-D3; codeine-D3 | |
| PT8278 | 18 | methanol + acetic acid | 120 | mechanical shaking | 30 | dilution | morphine-D3 | 0.03 |
| PT8279 | 2.5 | acetic acid 0.1% in methanol | 50 | mechanical shaking | 60 | dilution | morphine-D3; codeine-D3 | 0.0025 |
| PT8280 | 4 | 60:40 methanol:water 0.4% formic acid | 40 | ultra turrax 1 min/shaker | 30 | none | morphine-D3; codeine-D6 | A: 0.002<br>B: 0.005 |
| PT8281 | A: 5<br>B: 10 | 0.4% acetic acid in methanol/water (60:40) | 30 | mechanical shaking | 60 | none | morphine-D3; codeine-D3 | |
| PT8282 | 10 | 80% acetonitrile: 20% water + 1% formic acid | 50 | mechanical shaking | 30 | none | | |
| PT8283 | A: 2<br>B: 4 | 0.4% formic acid in methanol/water (60/40, v/v) | A: 20<br>B: 40 | shaking (hand/vortex) | 30 | dilution | morphine-D3; codeine-D3 | A:0.002<br>B: 0.01 |
| PT8285 | 5 | A: 1% formic acid in methanol (poppy seeds)<br>B: 1% formic acid in methanol/water 80/20 | 2 x 50 | mechanical shaking | 2 x 60 | dilution | morphine-D6; codeine-D6 | A: 0.001<br>B: 0.0025 |
| PT8286 | 0.1 | methanol | 10 | ultrasonic | 10 | none | | |
| PT8287 | 5 | methanol/water/formic acid (10ml/10ml/100µl) | 20 | mechanical shaking | 15 | dilution | morphine-D3; codeine-D6 | 0.025 |
| PT8288 | 5 | 1 ml HCl/1000 ml methanol | 100 | mechanical shaking | 60 | other | | |
| PT8289 | 2 | 0.4% formic acid in methanol/water (60/40, v/v) | 20 | shaking (hand/vortex) | 30 | dilution | morphine-D3; codeine-D3 | 0.005 |

A= material A; B= material B; HCl = hydrochloric acid.

# Annex 9 Results material A (poppy seeds)

| | Morphine<br>A: 13.3 mg/kg<br>u: 0.966 mg/kg<br>$\sigma_p$: 3.31 mg/kg (25%)<br>robust σ: 4.30 mg/kg (32%) | | Codeine<br>A: 24.3 mg/kg<br>u: 1.29 mg/kg<br>$\sigma_p$: 6.07 mg/kg (25%)<br>robust σ: 5.74 mg/kg (24%) | | Sum morphine + 0.2 x codeine<br>A: 18.2 mg/kg<br>u: 1.33 mg/kg<br>$\sigma_p$: 4.55 mg/kg (25%)<br>robust σ: 5.91 mg/kg (32%) | |
|---|---|---|---|---|---|---|
| **Lab code** | **Result (mg/kg)** | **z-score** | **Result (mg/kg)** | **z-score** | **Result (mg/kg)** | **z-score** |
| PT8258 | 11.93 | -0.40 | 19.22 | -0.83 | 15.77 | -0.53 |
| PT8259 | 8.58 | -1.41 | 17.0 | -1.20 | 12.0 | -1.36 |
| PT8260 | 15.8 | 0.77 | 27.03 | 0.45 | 21.2 | 0.66 |
| PT8261 | 15.26 | 0.61 | 31.69 | 1.22 | 21.6 | 0.75 |
| PT8262 | 4.3 | **-2.70** | 8.4 | **-2.62** | 6.0 | **-2.68** |
| PT8263 | 13.982 | 0.22 | 26.746 | 0.41 | 19.331 | 0.25 |
| PT8264 | 17.57 | 1.30 | 33.09 | 1.45 | 24.19 | 1.32 |
| PT8265 | 11.7 | -0.47 | 20.5 | -0.62 | 15.8 | -0.53 |
| PT8266 | 13.1 | -0.05 | 20.5 | -0.62 | 17.2 | -0.22 |
| PT8267 | 10.05 | -0.97 | 21.1 | -0.52 | 14.27 | -0.86 |
| PT8268 | 15.4 | 0.65 | 26.4 | 0.35 | 20.7 | 0.55 |
| PT8269 | 2.3 | **-3.31** | 28.6 | 0.71 | 8.02 | **-2.24** |
| PT8270 | 10.95 | -0.69 | 18.75 | -0.91 | 14.7 | -0.77 |
| PT8271 | 14.5 | 0.38 | 25.3 | 0.17 | 19.6 | 0.31 |
| PT8272 | 16.7 | 1.04 | 28.6 | 0.71 | 22.4 | 0.92 |
| PT8273 | 15.85 | 0.78 | 28.53 | 0.70 | 21.56 | 0.74 |
| PT8274 | 12.7 | -0.17 | 19.3 | -0.82 | 16.6 | -0.35 |
| PT8275 | 19.6 | 1.92 | 31.6 | 1.20 | 25.9 | 1.69 |
| PT8276 | 18.7 | 1.65 | 26.86 | 0.42 | 24.07 | 1.29 |
| PT8277 | 10 | -0.98 | 16 | -1.36 | 13 | -1.14 |
| PT8278 | 16.84 | 1.08 | 31.13 | 1.13 | 47.97 | **6.54** |
| PT8279 | 8.622 | -1.40 | 24.265 | 0.00 | 13.475 | -1.04 |
| PT8280 | 17.29 | 1.22 | 25.62 | 0.22 | 42.91 | **5.43** |
| PT8281 | 12.904 | -0.10 | 23.41 | -0.14 | 17.586 | -0.13 |
| PT8282 | 11 | -0.68 | 17 | -1.20 | 14 | -0.92 |
| PT8283 | 16.1 | 0.86 | 30.2 | 0.97 | 22.2 | 0.88 |
| PT8285 | 14.9 | 0.50 | 27.8 | 0.58 | 20.5 | 0.51 |
| PT8286 | 5.4 | **-2.37** | 22.65 | -0.27 | 9.93 | -1.82 |
| PT8287 | 11.4 | -0.56 | 18.2 | -1.00 | 15 | -0.70 |
| PT8288 | 9.06 | -1.27 | 23.85 | -0.07 | 13.83 | -0.96 |
| PT8289 | 166 | **46.11** | 26 | 0.28 | 192 | **38.20** |

A = consensus value (robust mean).

u = uncertainty of consensus value.

$\sigma_p$ = target standard deviation for proficiency test.

robust σ = robust (relative) standard deviation based on participants' results.

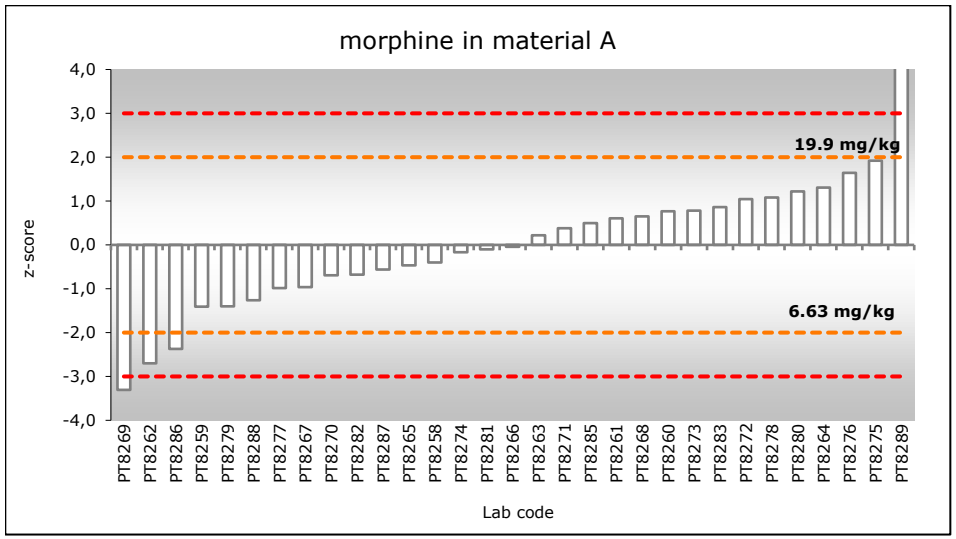


***Figure 1*** *Graphical representation of the z-scores for morphine in material A. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

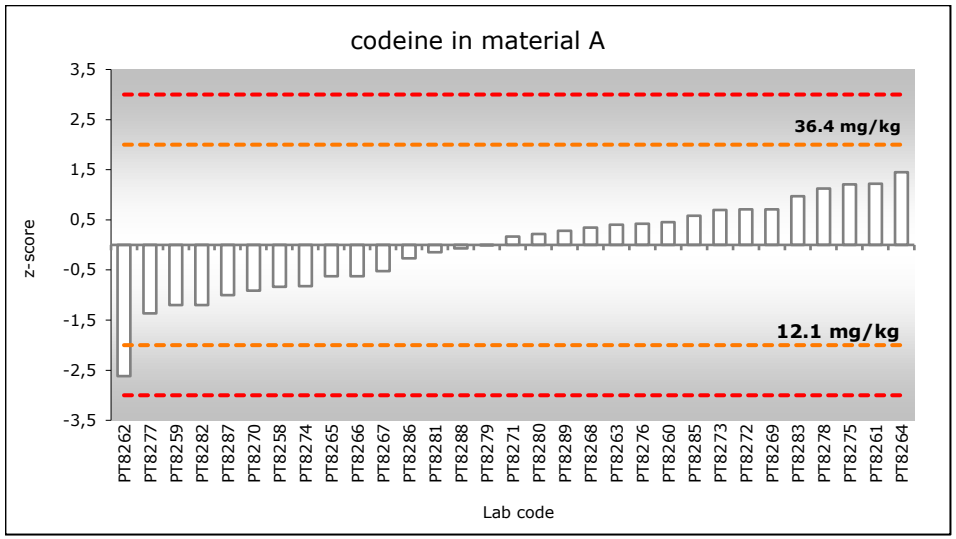


***Figure 2*** *Graphical representation of the z-scores for codeine in material A. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

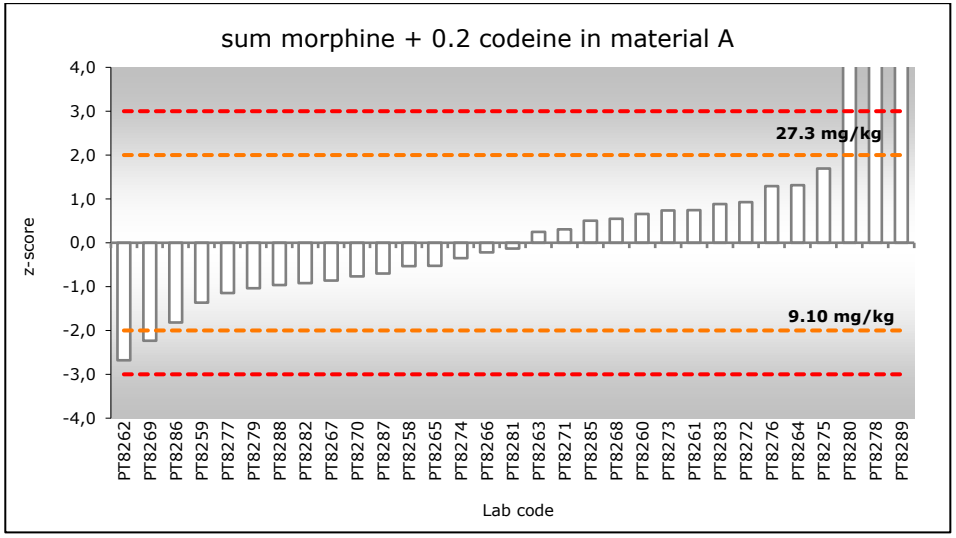


***Figure 3*** *Graphical representation of the z-scores for the sum of morphine + 0.2 codeine in material A. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

# Annex 10 Results material B (bakery product)

| | Morphine<br>A: 1.01 mg/kg<br>u: 0.067 mg/kg<br>$\sigma_p$: 0.252 mg/kg (25%)<br>robust σ: 0.298 mg/kg (30%) | | Codeine<br>A: 0.175 mg/kg<br>u: 0.015 mg/kg<br>$\sigma_p$: 0.044 mg/kg (25%)<br>robust σ: 0.057 mg/kg (32%) | | Sum morphine + 0.2 x codeine<br>A: 1.057 mg/kg<br>u: 0.064 mg/kg<br>$\sigma_p$: 0.264 mg/kg (25%)<br>robust σ: 0.283 mg/kg (27%) | |
|---|---|---|---|---|---|---|
| **Lab code** | **Result (mg/kg)** | **z-score** | **Result (mg/kg)** | **z' -score** | **Result (mg/kg)** | **z-score** |
| PT8258 | 0.983 | -0.09 | 0.161 | -0.30 | 1.015 | -0.16 |
| PT8259 | 0.67 | -1.34 | 0.11 | -1.40 | 0.69 | -1.39 |
| PT8260 | 1.17 | 0.65 | <1 | **[17.90]** | 1.17 | 0.43 |
| PT8261 | 0.33 | **-2.69** | 0.12 | -1.19 | 0.35 | **-2.68** |
| PT8262 | 1.74 | **2.91** | 0.34 | **3.58** | 1.76 | **2.66** |
| PT8263 | 0.751 | -1.02 | <0.1 | [-1.62] | 0.751 | -1.16 |
| PT8264 | 0.88 | -0.50 | <0.4 | **[4.88]** | 0.88 | -0.67 |
| PT8265 | 1.0 | -0.03 | 0.13 | -0.97 | 1.0 | -0.22 |
| PT8266 | 1.4 | 1.56 | 0.2 | 0.55 | 1.5 | 1.68 |
| PT8267 | 1.02 | 0.05 | 0.13 | -0.97 | 1.05 | -0.03 |
| PT8268 | 1.09 | 0.33 | 0.2 | 0.55 | 1.13 | 0.28 |
| PT8269 | 0.22 | **-3.13** | 0.04 | **-2.92** | 0.228 | **-3.14** |
| PT8270 | 1.16 | 0.61 | 0.196 | 0.46 | 1.20 | 0.54 |
| PT8271 | 1.98 | **3.87** | 0.33 | **3.37** | 2.05 | **3.76** |
| PT8272 | 1.22 | 0.85 | 0.289 | **2.48** | 1.28 | 0.84 |
| PT8273 | 1.08 | 0.29 | 0.22 | 0.98 | 1.12 | 0.24 |
| PT8274 | 1.2 | 0.77 | <1 | **[17.90]** | 1.2 | 0.54 |
| PT8275 | 1.11 | 0.41 | 0.21 | 0.76 | 1.15 | 0.35 |
| PT8276 | 1.25 | 0.97 | 0.22 | 0.98 | 1.29 | 0.88 |
| PT8277 | 0.56 | -1.77 | <0.25 | [1.63] | 0.56 | -1.88 |
| PT8278 | 0.94 | -0.26 | 0.15 | -0.54 | 1.09 | 0.13 |
| PT8279 | 0.531 | -1.89 | <0.4 | **[4.88]** | 0.531 | -1.99 |
| PT8280 | 0.88 | -0.50 | 0.17 | -0.10 | 1.05 | -0.03 |
| PT8281 | 1.163 | 0.62 | 0.17 | -0.10 | 1.197 | 0.53 |
| PT8282 | 1.1 | 0.37 | 0.21 | 0.76 | 1.1 | 0.16 |
| PT8283 | 1.23 | 0.89 | 0.16 | -0.32 | 1.26 | 0.77 |
| PT8285 | 1.00 | -0.03 | <0.40 | **[4.88]** | 1.00 | -0.22 |
| PT8286 | 0.45 | **-2.21** | 0.15 | -0.54 | 0.48 | **-2.18** |
| PT8287 | 0.909 | -0.39 | 0.14 | -0.75 | 0.958 | -0.37 |
| PT8288 | 0.54 | -1.85 | 0.11 | -1.40 | 0.56 | -1.88 |
| PT8289 | 11 | **39.71** | <2 | **[39.58]** | 11 | **37.63** |

A = consensus value (robust mean).

u = uncertainty of consensus value.

$\sigma_p$ = target standard deviation for proficiency test.

robust σ = robust (relative) standard deviation based on participants' results.

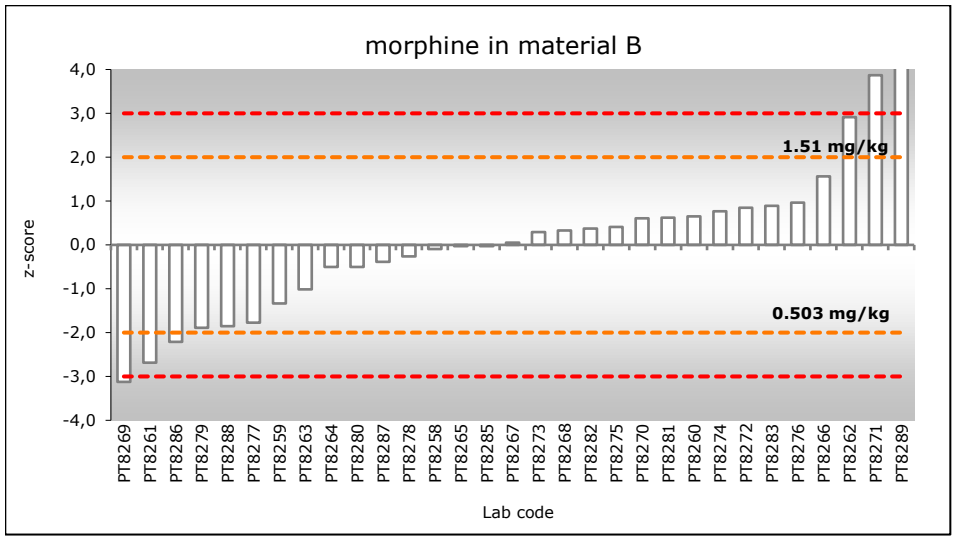


***Figure 4*** *Graphical representation of the z-scores for morphine in material B. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

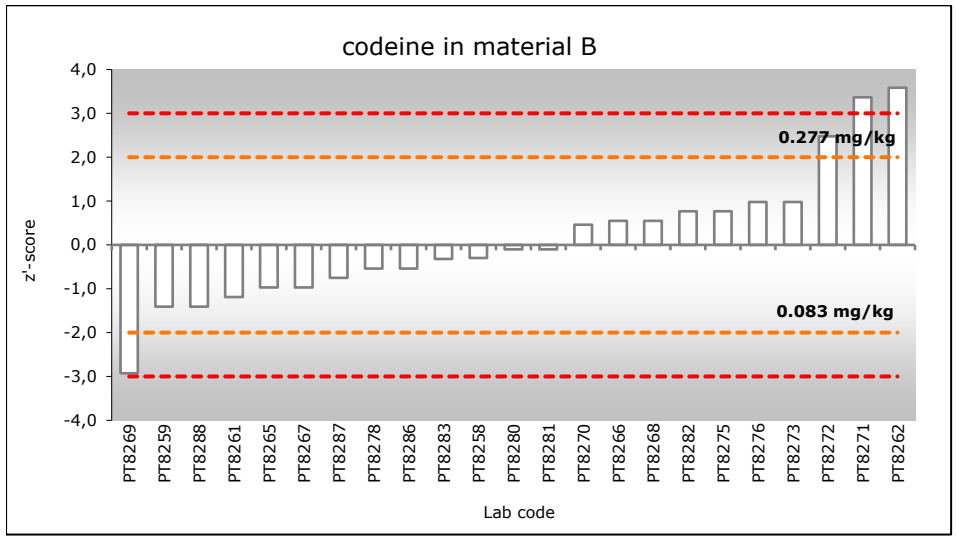


***Figure 5*** *Graphical representation of the z-scores for codeine in material B. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

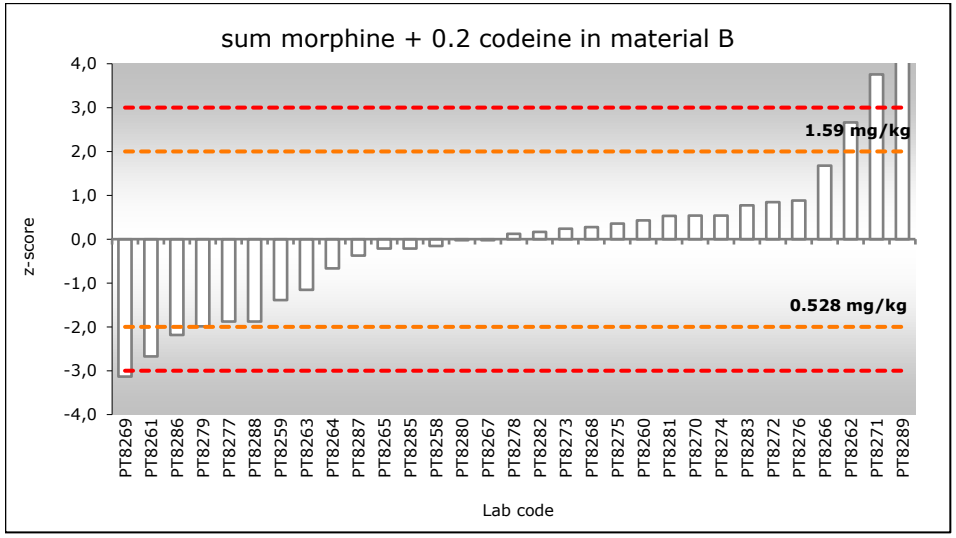


***Figure 6*** *Graphical representation of the z-scores for the sum of morphine + 0.2 codeine in material B. Dotted lines show PT performance boundaries ± 2 (also in mg/kg) and ± 3.*

# Annex 11 Overview performance per laboratory

| Lab code | Individual opium alkaloids<br>Satisfactory performance * | Sum opium alkaloids<br>Satisfactory performance * |
|---|---|---|
| PT8258 | **4 out of 4** | **2 out of 2** |
| PT8259 | **4 out of 4** | **2 out of 2** |
| PT8260 | 3 out of 4 | **2 out of 2** |
| PT8261 | 3 out of 4 | 1 out of 2 |
| PT8262 | 0 out of 4 | 0 out of 2 |
| PT8263 | 3 out of 4 | **2 out of 2** |
| PT8264 | 3 out of 4 | **2 out of 2** |
| PT8265 | **4 out of 4** | **2 out of 2** |
| PT8266 | **4 out of 4** | **2 out of 2** |
| PT8267 | **4 out of 4** | **2 out of 2** |
| PT8268 | **4 out of 4** | **2 out of 2** |
| PT8269 | 1 out of 4** | 0 out of 2** |
| PT8270 | **4 out of 4** | **2 out of 2** |
| PT8271 | 2 out of 4 | 1 out of 2 |
| PT8272 | 3 out of 4 | **2 out of 2** |
| PT8273 | **4 out of 4** | **2 out of 2** |
| PT8274 | 3 out of 4 | **2 out of 2** |
| PT8275 | **4 out of 4** | **2 out of 2** |
| PT8276 | **4 out of 4** | **2 out of 2** |
| PT8277 | 3 out of 4 | **2 out of 2** |
| PT8278 | **4 out of 4** | 1 out of 2 |
| PT8279 | 3 out of 4 | **2 out of 2** |
| PT8280 | **4 out of 4**** | 1 out of 2** |
| PT8281 | **4 out of 4** | **2 out of 2** |
| PT8282 | **4 out of 4** | **2 out of 2** |
| PT8283 | **4 out of 4** | **2 out of 2** |
| PT8285 | 3 out of 4 | **2 out of 2** |
| PT8286 | 2 out of 4 | 1 out of 2 |
| PT8287 | **4 out of 4** | **2 out of 2** |
| PT8288 | **4 out of 4** | **2 out of 2** |
| PT8289 | 1 out of 4 | 0 out of 2 |

* Satisfactory performance here means a quantitative result with a satisfactory z-score was obtained for the individual OAs or the total sum of OAs present in material A and B. Results reported as <LOQ are not considered a satisfactory z-score.

** reported results after the deadline.

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
wur.eu/food-safety-research

WFSR Report 2023.013

The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 7,600 employees (6,700 fte) and 13,100 students and over 150,000 participants to WUR's Life Long Learning, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.

To explore the potential of nature to improve the quality of life

Wageningen Food Safety Research
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0) 317 48 02 56
wur.eu/food-safety-research

WFSR report 2023.013

The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 7,600 employees (6,700 fte) and 13,100 students and over 150,000 participants to WUR's Life Long Learning, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.