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AMBIENTE
E SALUTE

# ISTITUTO SUPERIORE DI SANITÀ

# Results of the Proficiency Test 2023 on the determination of tropane alkaloids in baby food

Francesca Debegnach (a), Emanuela Gregori (a), Marzia De Giacomo (a), Giuseppina Scialò (a), Martina Enza Grieco (a), Marianna Rizzo (a), Giorgio Fedrizzi (b), Elisabetta Caprai (b), Barbara De Santis (a)

*(a) Dipartimento Sicurezza Alimentare, Nutrizione e Sanità Pubblica Veterinaria*
*Istituto Superiore di Sanità, Roma*
*(b) Reparto Chimico degli Alimenti, "Bruno Ubertini"*
*Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Bologna*

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**Rapporti ISTISAN**
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2024, 40 p. Rapporti ISTISAN 24/31

Every year, the Italian National Reference Laboratory for Mycotoxin and Plant Toxins at the Istituto Superiore di Sanità (the National Institute of Health in Italy) organizes proficiency test trials for the Italian network of the Official Laboratories of the National Health Service. In 2023, a study for the determination of tropane alkaloids, atropine and scopolamine, in baby food was organized in cooperation with the National Reference Laboratory for Plant Toxins at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna. Laboratories invited to participate in this study are Official Control Laboratories and private laboratories. Most participants obtained a satisfactory performance (z-score, ζ-score).

*Keywords*: Atropine; Scopolamine; Baby food; Proficiency test

Istituto Superiore di Sanità
**Risultati del circuito interlaboratorio 2023 sulla determinazione degli alcaloidi del tropano in baby food.**
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Il Laboratorio Nazionale di Riferimento per le Micotossine e per le Tossine Vegetali Naturali presso l'Istituto Superiore di Sanità organizza almeno una prova valutativa ogni anno per il circuito di laboratori ufficiali del Servizio Sanitario Nazionale. Nel 2023, in collaborazione con il Laboratorio Nazionale di Riferimento per le Tossine Vegetali Naturali presso l'Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, è stata organizzata una prova valutativa per la determinazione degli alcaloidi del tropano, atropina e scopolamina, in *baby food* I laboratori invitati sono stati i laboratori del controllo ufficiale e alcuni laboratori privati. La maggioranza dei partecipanti ha ottenuto prestazioni soddisfacenti in termini di *z-score* e *ζ-score*.

*Parole chiave*: Atropina; Scopolamina; Baby food; Prova valutativa

Per informazioni su questo documento scrivere a: lnr-micotossine-tvn@iss.it

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La responsabilità dei dati scientifici e tecnici è dei singoli autori, che dichiarano di non avere conflitti di interesse.


viale Regina Elena, 299 – 00161 Roma

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# INTRODUCTION

In accordance with Article 37 of Regulation (EU) 2017/625, all laboratories involved in official control analyses must provide evidence of their analytical skills, for a specific test or for groups of tests. Furthermore, it is established that the official laboratories designated by the competent authority must be evaluated and accredited according to the international standard ISO 17025:2017. Among the various requirements envisaged by the ISO standard, participation in Proficiency Test (PT) is considered a tool for verifying the performance of the analytical process used by the laboratory and must be guaranteed at least for the tests for which it was requested or already received the accreditation. In fact, as part of the quality control activities, the laboratory shall give evidence of internal and external quality control checks. Internal quality control can be carried out using control charts, reference materials, and also through the evaluation of the recovery factor and the performance of repeatability tests, systematically implemented by the laboratory. The exchange of samples with other laboratories and/or participation in interlaboratory trials are useful tools for external quality control checks.

The use of validated methods and the regular participation of the laboratory in the PTs are fundamental elements for the quality system assurance of the laboratory. Participation in PT exercises allow to independently assess the competence of the laboratory and its compliance with the requirements reported in Regulation (EU) 2017/625.

This PT has been designed according to the IUPAC Technical Report, the international harmonized protocol for the proficiency testing of analytical chemistry laboratories and in compliance with the standard ISO 17043:2010.

The PT has been coded as PT ISS 2023 TAs baby food. Tropane Alkaloids (TAs) are secondary metabolites, which naturally occur in plants of several families including *Brassicaceae*, *Solanaceae* and *Erythroxylaceae*. TAs are found in all parts of the plants and are responsible for the toxic effects of some of these plants. Although more than 200 different TAs were so far identified in various plants, respective data on their occurrence in food and feed and on toxicity are limited. The most studied TAs are (-)-hyoscyamine and (-)-scopolamine, which in contrast to the (+)-enantiomers are formed naturally (Figure 1). The racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine is called atropine.

TAs are the esters of tropine (3α-tropanol) and, to a lesser extent, of pseudotropin (3β-tropanol) with the tropane ring (pyrrolidine ring and one piperidine ring sharing one nitrogen and two carbon atoms) as the common element. The TAs of interest for EU legislation (EU Regulation 2023/915) are (-)-hyoscyamine, which spontaneously racemizes, the resulting racemic mixture is called atropine and (-)-scopolamine.

Some TAs are known as muscarinic receptor antagonists (or antimuscarinics) due to their action at the level of muscarinic-type acetylcholine (ACh) receptors. In mammals, they can induce a variety of distinct toxic syndromes gathered under the definition of anticholinergic poisoning. At therapeutic doses, antimuscarinic TAs have little effect on nicotinic ACh receptors and can be used as anticholinergic drugs in cases of incorrect functioning of voluntary or involuntary muscles.

The main compound of this type of substance with anticholinergic action is atropine, which is able to non-specifically hinder/prevent the action exerted by the muscarinic receptors present in the body.

Another substance with an antimuscarinic action, with a more selective action with respect to the different types of receptors, is scopolamine, which in therapeutic doses is used to relieve nausea and dizziness and to avoid vomiting in case of motion sickness.

It is known that, for atropine and scopolamine, the naturally occurring (-)-L enantiomers show much stronger anticholinergic effects than the (+)-D enantiomers (Figure 1).

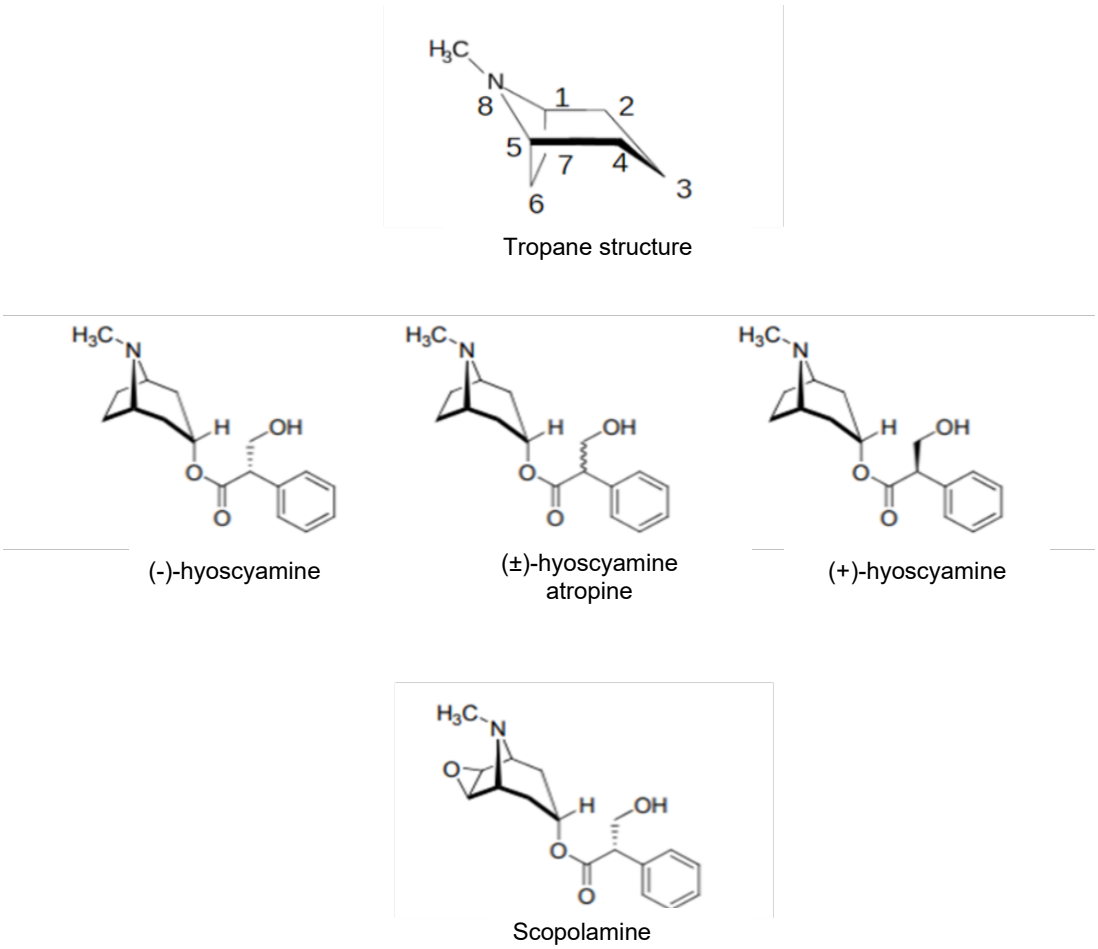



**Figure 1. Tropane structure, (-)-hyoscyamine, atropine e (+)-hyoscyamine, (-)-scopolamine**

For risk characterization purposes, as a basis for the definition of group toxicological threshold (Acute Reference Dose, ARfD), the European Food Safety Authority (EFSA) has identified a No Observed Adverse Effect Level (NOAEL) of 0.16 µg/kg body weight (µg/kg bw), expressed as the sum of (-)-hyoscyamine and (-)-scopolamine, dose defined starting from a study conducted in a group of young healthy male volunteers.

The EFSA Panel noted that the next higher dose in the human volunteer study of 0.48 µg/kg bw resulted in a transient statistically significant lowering of the heart rate, which is not adverse in healthy individuals but could be in more susceptible individuals, such as those with bradycardia. Based on these elements, EFSA, by applying an uncertainty factor of 10 (for interindividual differences that consider the fact that the study derives from information from a group of young healthy male volunteers) to the NOAEL value, has established an ARfD of 0.016 µg/kg bw expressed as the sum of (-)-hyoscyamine and (-)-scopolamine (group ARfD), assuming equivalent potency.

This ARfD value is approximately two orders of magnitude lower than the lowest single doses of (-)-hyoscyamine and (-)-scopolamine used therapeutically. The lowest therapeutic doses identified are 1.4 µg/kg bw for (-)-hyoscyamine, 6 µg/kg bw for atropine (corresponding to approximately 3 µg/kg bw (-)-hyoscyamine) and 2.5 µg/kg bw for (-)-scopolamine, and may be associated with negative side effects, such as heart slowing or dry mouth and should not be associated with people sensitive to these effects.

# ORGANIZATION OF THE PROFICIENCY TEST

## Preparation of the test material

The test material, a cereal-based baby food, was a sample naturally contaminated with atropine and scopolamine.

The material was homogenized by mixing and grinding (0.50 mm sieve) and subsequently packaged in 50 mL conical centrifuge tubes. The material was then tested for homogeneity and stability.

## Homogeneity of the test material

The homogeneity of the material was evaluated in March 2022. To verify the homogeneity, 8 packs of material were randomly sampled. The samples thus taken were analyzed in duplicate. The test material homogeneity was evaluated in accordance with "The international harmonized protocol for proficiency testing of analytical chemistry laboratories".

The material was tested again (end of March 2022) to verify stability of TAs at room temperature. The shipment was carried out at room temperature and the participants were asked, in the instructions sent with the parcel, to store the sample at + 4°C until the analysis.

The one-way ANOVA (ANalysis Of VAriance) test was applied to the obtained data to verify the material stability.

## Shipment of the test material and request to the participants

The PT was nationally announced by e-mail on 29$^{th}$ October 2022 and extended to European participants on 21$^{st}$ December 2022, documentation and the sample were sent on 13 February 2023.

Each participant was free to use his own method according to laboratory routine procedure. Each participant received a randomly assigned laboratory code to ensure confidentiality through anonymity.

The sample was sent to the participants on 13$^{th}$ February 2023. The test material was shipped at room temperature, and it was required to store the sample at + 4°C until the time of analysis.

Each participant received:

- a sample of cereal-based baby food in a 50 mL test tube;
- the PT cover letter;
- the acknowledgement of receipt form;
- the questionnaire on the analytical method used to carry out the PT;
- the results form for sending the results;
- the code assigned to the laboratory.

Copy of the documents sent to the participants is included in Appendix A.

Participants were asked to:

a) send the results no later than 27/03/2023;
b) provide the results for atropine and scopolamine in µg/kg;
c) correct the results for the recovery factor;
d) indicate the value of the recovery factor (expressed in %);
e) associate the measurement uncertainty (expressed in µg/kg) to the analytical result and indicate the coverage factor (k) and the confidence level expressed in %;
f) provide the value of the limit of quantification (LOQ) of the applied method (expressed in µg/kg).

# Statistical evaluation of the results

The statistical evaluation of the results provided by the participants was carried out in accordance with ISO 13528:2022 and the IUPAC Technical Report, The international harmonized protocol for the proficiency testing of analytical chemistry laboratories.

# Assigned value and associated uncertainty

The consensus value based on the results of the participants was used as the assigned value. The consensus value in this PT was obtained by calculating the robust mean, $\widehat{X}$. The $\widehat{X}$ and the robust standard deviation, $\widehat{\sigma}_{rob}$, were calculated using algorithm A (ISO 13528:2022). As uncertainty of the assigned value (u), the standard uncertainty value calculated from $\widehat{\sigma}_{rob}$ was taken.

Dataset of the delivered results shall be unimodal and, approximately, symmetric distributions. The kernel density plot of the data is examined using a normal density and the bandwidth set at $0{,}75\sigma_{PT}$. If there is evidence of unimodality or if the second modality contributes less than about 5% to the kernel density distribution, it is still valid to consider the robust mean and its standard deviation provided that $\widehat{\sigma}_{rob} > 1.2\ \sigma_{PT}$.

The exclusion of results from the data that are clearly invalid (e.g., expressed in the wrong units) or are extreme outliers (for example, outside the range of ±50% of the median) may be necessary to achieve a unimodal distribution.

# PT standard deviation

For the assessment of laboratory performance, a fixed target standard deviation for PT ($\sigma_{PT}$) of 25% was used.

The rationale behind this figure is provided by the European Union Reference Laboratory for Mycotoxins and Plant Toxins (EURL-MP) which states that this generic value is fit for purpose and reflects current analytical capabilities and best practices for the determination of mycotoxins and plant toxins in food and feed.

# z-score and ζ-score calculation

The performance of individual laboratories is evaluated in terms of z-score and, where available the uncertainty value, of ζ-score, in accordance with ISO 13528:2022 and the IUPAC Technical Report, The international harmonized protocol for the proficiency testing of analytical chemistry laboratories.

Participants' z-score values are calculated by applying the following formula:

$$\text{z-score} = \frac{(x_{lab} - \hat{X})}{\sigma_{PT}}$$

where:

- $x_{lab}$ is the result provided by the laboratory, µg/kg
- $\hat{X}$ is the assigned value, µg/kg
- $\sigma_{PT}$ is the target standard deviation for the PT, µg/kg

The target standard deviation for the PT, $\sigma_{PT}$, defines the performance range in the interlaboratory circuit. The EURL-MP has established that, for the determination of mycotoxins and plant toxins in food and feed, it is appropriate to use a target standard deviation of 25% of the concentration value, set regardless of the analyte, matrix or concentration level.

Furthermore, the alarm signal and the action signal are established for the intervals $\hat{X} \pm 2\sigma_{PT}$ and $\hat{X} \pm 3\sigma_{PT}$, respectively. In fact, considering a normal distribution of the results, 95% of the observations fall within the interval $\mu \pm 2\sigma_{PT}$ and 99,7% of the observations fall within the interval $\mu \pm 3\sigma_{PT}$. Thus, there is a 1 in 20 chance that a laboratory result is acceptable but not in the $\mu \pm 2\sigma_{PT}$ range, and only a 1 in 300 chance that the laboratory bias is greater than $3\sigma_p$ by chance.

In conclusion:

- if the participant's z-score is $|z| \leq 2$ the result is considered satisfactory (the z-score value is within the alarm signal range);
- if the z-score score is $2 < |z| < 3$, the result is questionable (the z-score value is outside the alarm signal range, but is still within the action signal range);
- if the z-score score is $|z| \geq 3$, the result is considered unsatisfactory (the z-score value is outside the action signal range).

Participants' ζ-score values are calculated by applying the following formula:

$$\zeta\text{-score} = \frac{(x_{lab} - \hat{X})}{\sqrt{u_{lab}^2 + u_{ref}^2}}$$

where:

- $x_{lab}$ is the result provided by the laboratory, µg/kg
- $\hat{X}$ is the assigned value, µg/kg
- $u_{lab}$ is the standard uncertainty provided by the laboratory, µg/kg
- u is the standard uncertainty associated with the assigned value, µg/kg

The warning and action intervals and the evaluation (satisfactory, questionable and unsatisfactory) are completely similar to those seen for the z-score.

The ζ-score was used as an indication of the consistency of the uncertainty provided by the participant ($u_{lab}$) with the observed deviation from the assigned value (u). Unsatisfactory ζ-score values may indicate an underestimation of the uncertainty or a large deviation of the result from

the assigned value, indicating an uncertainty value that is inconsistent with the uncertainty of the assigned value. Unsatisfactory values of z-score and ζ-score may occur when the uncertainty values are not in agreement with the observed deviation from the assigned value, so it is useful to consider together the values of z and ζ-score.

# Follow-up

In cases where the participating laboratory obtains a result considered questionable or unsatisfactory resulting from a score of $2<|z|<3$ or z-score $|z|\geq 3$, the NRL will contact the laboratory of the Italian network to share the root cause analysis which may have led to the unsatisfactory result and the corresponding planned corrective actions.

The NRL has planned a follow-up questionnaire which requests the planned and implemented actions and the timescales required to verify the adequacy of the corrective action planned or undertaken. If necessary, a new aliquot of the PT test material may be sent upon requested.

# RESULTS AND DISCUSSION

## Assigned value and associated uncertainty

A consensus value, based on the results of the participants, was used as the assigned value.

Before proceeding with the calculation of the z-score and ζ-score values, a verification of the hypothesis of normal distribution of the data was made to check for the presence of outliers.

The visual verification of the kernel density plot and the Quantile-Quantile (Q-Q) plot were carried out, and summary statistics were calculated to confirm the normal trend and exclude any deviation.

While for atropine the plot was apparently unimodal, for scopolamine, the kernel density graphs (with a bandwidth equal to 0.75 $\sigma_{PT}$) indicated non-unimodality, with the second mode contributing more than 5% to the density distribution.

The consensus value, $\hat{X}$, was then obtained after adjusting the data for exclusion of values outside the range of ±50% of the median.

The visual presentation of the remaining results for scopolamine showed a new distribution that was apparently unimodal and roughly symmetric.

The robust standard deviation, $\hat{\sigma}_{rob}$, was below 1.2 $\sigma_{PT}$, indicating no concern about wide distributions. These findings supported the use of robust statistics for data processing, including the calculation of the robust mean and its standard deviation on the entire dataset of results delivered by the laboratories.

As uncertainty of the assigned value (u), the standard uncertainty value calculated from $\hat{\sigma}_{rob}$ was taken. This value compared with the value of $\sigma_{PT}$ satisfied the criterion 0,3 $\sigma_{PT} \geq$ u for atropine and scopolamine.

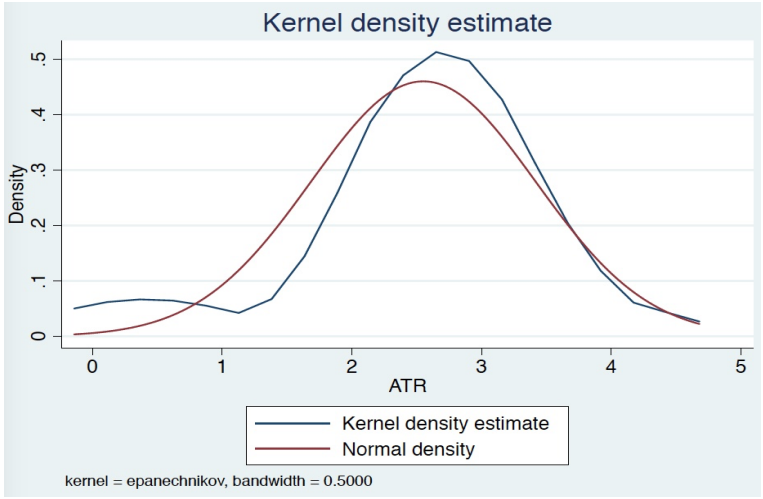
The parameters obtained, assigned value with the robust standard deviation and standard uncertainty, and PT target standard deviation are shown in Table 1.

**Table 1. Assigned value with relative standard deviation and standard uncertainty, obtained with the data of the participants. PT ISS 2023 TAs baby food**

| Parameter | Atropine | Scopolamine |
|---|---|---|
| Assigned value, $\hat{X}$ (μg/kg) (algorithm A) | 2.66 | 0.77 |
| Assigned value robust standard deviation, $\hat{\sigma}_{rob}$ (μg/kg) | 0.49 | 0.19 |
| Assigned value standard uncertainty, u (μg/kg) | 0.14 | 0.06 |
| PT target standard deviation, $\sigma_{PT}$ (μg/kg) | 0.67 | 0.19 |

Figures 2 and 3 show the kernel density plot, box plots and Q-Q plot and obtained for atropine and scopolamine, respectively.

a)

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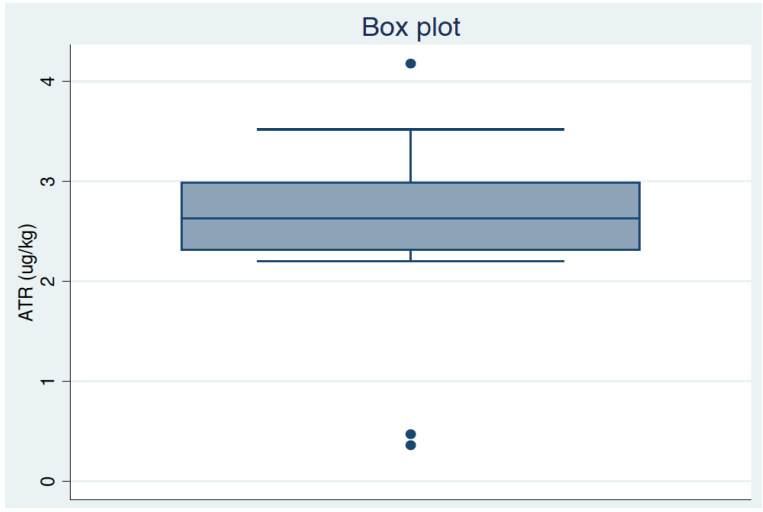


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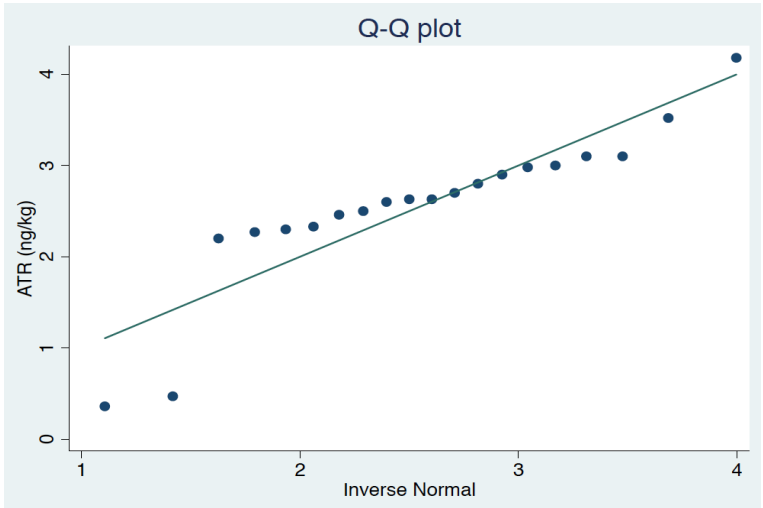


**Figure 2. ATROPINE: a) Kernel density plot, b) Quantile-Quantile plot and c) box plot. PT ISS 2023 TAs baby food**

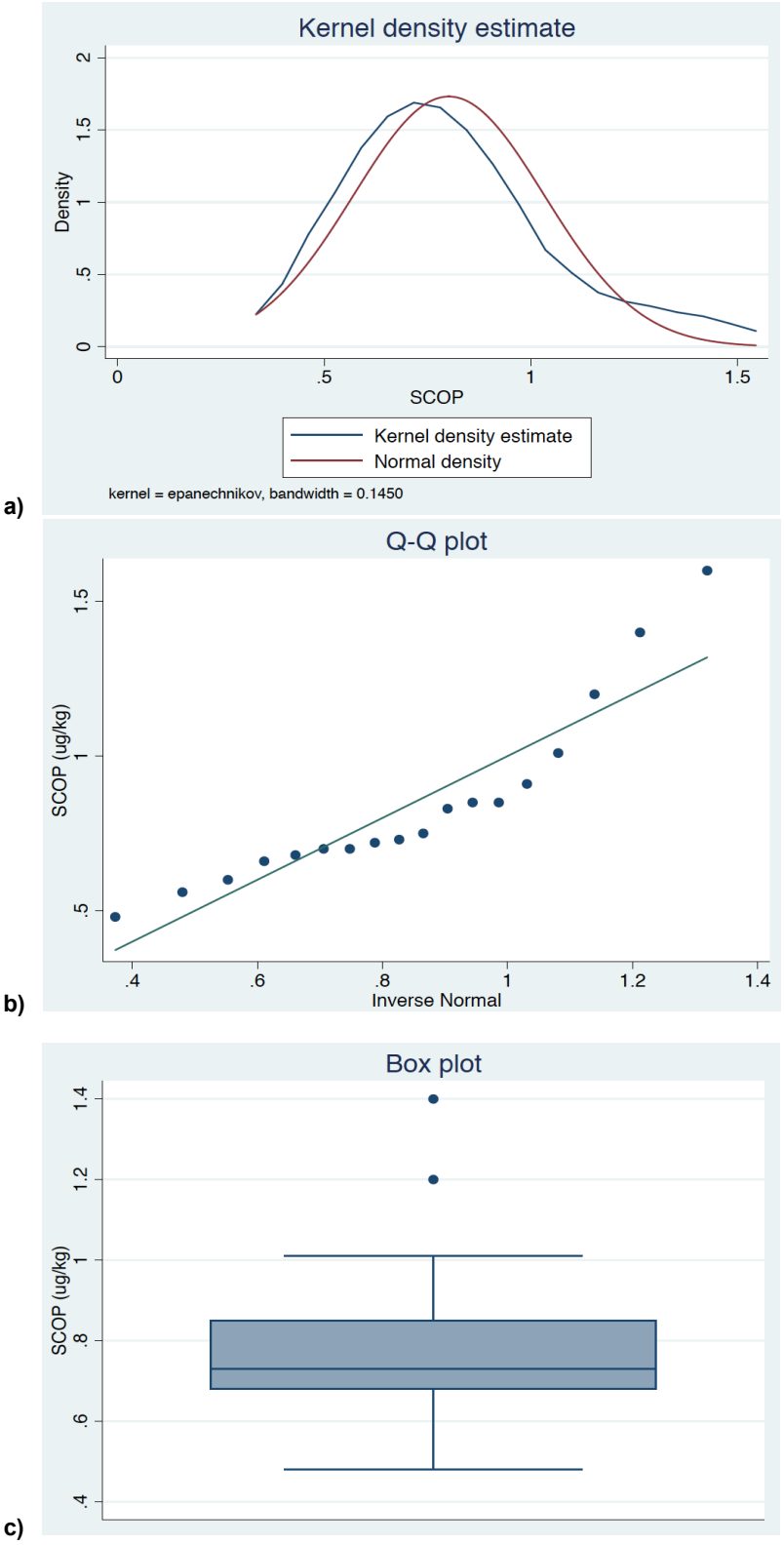


**Figure 3. SCOPOLAMINE: a) Kernel density plot, b) Quantile-Quantile plot and c) box plot. PT ISS 2023 TAs baby food**

# Homogeneity and stability of the test material

After homogeneity tests, the material was found to be sufficiently homogeneous for PT, for both atropine and scopolamine.

The results of the homogeneity study are presented in Table 2, where:

- $x_{hom}$ homogeneity value;
- $\sigma_{PT}$ standard deviation used for PT evaluation (25% of the assigned value);
- $S_{an}$ Analytical standard deviation, ($=\sqrt{(\Sigma Di^2/2m)}$;
- D, difference between the duplicate analysis values; m is the number of duplicates);
- $S^2_{sam}$ Sampling variance (difference between inter and intra packs results. This value is equal to 0 when the difference is negative);
- $\sigma_{all}$ limit standard deviation (= 0.3*$\sigma_{PT}$) for sufficient homogeneity;
- $c = F1\sigma^2_{all} + F2S^2_{an}$ (F1 and F2, factors used for homogeneity test for m samples analyzed in duplicate).

**Table 2. Homogeneity test results. PT ISS 2023 TAs baby food**

| Parameter | Atropine | Scopolamine |
|---|---|---|
| Number of independent replicates (duplicates) | 11 | 11 |
| Homogeneity value, $x_{hom}$ (µg/kg) | 2.32 | 0.75 |
| PT target standard deviation, $\sigma_{PT}$ (µg/kg) (25%) | 0.67 | 0.19 |
| Cochran test | 0.3 | 0.5 |
| Estimated analytical variance, $S^2_{an}$ (µg/kg) | 0.0128 | 0.0012 |
| Estimated sampling variance, $S^2_{sam}$ (µg/kg) | 0.0640 | 0.0064 |
| Limit standard deviation, $\sigma_{all}{}^2$ | 0.0302 | 0.0031 |
| $s_{an} < 0.5\ \sigma_{PT}$ | YES | YES |
| Critical value, c | 0.0670 | 0.0068 |
| $S^2_{sam}$<critical value? | YES | YES |

To test the stability of the test material, 4 packs of material were stored in a hood away from light and at room temperature for two weeks. Analyses were subsequently conducted on these 4 experimental aliquots and on 4 control aliquots stored at +4°C. The experimental and control subsets were analyzed together in a random order within a single run of analysis (under repeatability conditions). To check if any highly significant difference between the mean results of the two subsets can be regarded as evidence of instability, the obtained results were successfully subjected to a two-sample t-test with pooled standard deviation and the differences resulted not significant at the 95% level of confidence (p-values, 0.852 and 0.754, for atropine and scopolamine, respectively).

# Results of the participants

The PT has joined 21 laboratories of which:

– 7 Italian laboratories of the official control network,
– 1 private laboratory,
– 13 European laboratories recruited through the EURL-MP.

The list of laboratories that have joined the PT is reported in Appendix B.

All 21 laboratories had sent the duly completed "Acknowledgement of receipt" form confirming the correct receipt of the material for the execution of the PT. Twenty out of 21 laboratories provided results. Laboratory 14 received a warning about the acceptance deadline but couldn't send the results.

The results obtained were sent by the deadline indicated in the PT cover letter. In addition to the analytical result corrected for recovery, the participants indicated the value of the recovery factor, apart from Laboratory 11. Not all the results provided by the participants were accompanied by the value of the expanded uncertainty ($U_e$), in particular Laboratories 11 and 20 did not report this value.

The results as reported by the participants, the z-score and the ζ-score values obtained are shown in Tables 3 and 4 for atropine and scopolamine, respectively.

**Table 3. ATROPINE: analytical results and participants z-score e ζ-score values. PT ISS 2023 TAs baby food**

| Code LAB | Result (µg/kg)[1] | R (%)2 | $U_e$ (µg/kg) | LOQ (µg/kg)[3] | z-score[4] | ζ-score[4] |
|---|---|---|---|---|---|---|
| 1 | 3.1 | 88.65 | 0.4 | 0.1 | 0.7 | 1.8 |
| 2 | 2.8 | 87 | 1.1 | 0.30 | 0.2 | 0.2 |
| 3 | 2.63 | 95 | 1.05 | 0.33 | 0 | -0.1 |
| 4 | 3.52 | 100 | 0.88 | 0.5 | 1.3 | 1.9 |
| 5 | 2.27 | 82 | 1.00 | 0.3 | -0.6 | -0.8 |
| 6 | 2.63 | 97 | 1.16 | 0.3 | 0 | -0.1 |
| 7 | 4.18 | 120 | 0.94 | 0.5 | **2.3** | **3.1** |
| 8 | 3.1 | 100 | 1.4 | 0.1. | 0.7 | 0.6 |
| 9 | 2.9 | 99 | 0.52 | 0.3 | 0.3 | 0.7 |
| 10 | 2.5 | **55.7** | 1.0 | 0.5 | -0.2 | -0.3 |
| 11 | 2.46 | - | - | **25** | -0.3 | - |
| 12 | 0.47 | 99 | 0.235 | 0.10 | **-3.3** | **-12.0** |
| 13 | 0.36 | 100 | 0.18 | 0.025 | **-3.5** | **-13.8** |
| 14 | - | - | - | - | - | - |
| 15 | 3.0 | 100 | 1.6 | 1 | 0.5 | 0.4 |
| 16 | 2.7 | 77 | 0.5 | 0.3 | 0.1 | 0.1 |
| 17 | 2.33 | 98.6 | 0.58 | 0.125 | -0.5 | -1.0 |
| 18 | 2.3 | 80.0 | 0.9 | 0.3 | -0.5 | -0.8 |
| 19 | 2.60 | 103.4 | 0.91 | 0.20 | -0.1 | -0.1 |
| 20 | 2.2 | 115 | - | 1 | -0.7 | - |
| 21 | 2.98 | 105.3 | 1.49 | 1.0 | 0.5 | 0.4 |

[1] in bold, values considered outliers;
[2] in bold, values out of the range of acceptability;
[3] in bold, inconsistent value of LOQ;
[4] in bold, values $2<|z|<3$ and $2<|\zeta|<3$.

**Table 4. SCOPOLAMINE: analytical results and participants *z-score* e *ζ-score* values. PT ISS 2023 TAs baby food**

| Code LAB | Result (µg/kg)[1] | R (%)[2] | $U_e$ (µg/kg) | LOQ (µg/kg)[3] | z-score[4] | ζ-score[4] |
|---|---|---|---|---|---|---|
| 1 | 1.2 | 84.17 | 0.1 | 0.1 | **2.2** | **5.7** |
| 2 | 0.85 | 98 | 0.37 | 0.30 | 0.4 | 0.4 |
| 3 | 0.68 | 94 | 0.2 | 0.50 | -0.5 | -0.8 |
| 4 | 0.73 | 100 | 0.18 | 0.5 | -0.2 | -0.4 |
| 5 | 0.48 | 80 | 0.21 | 0.3 | -1.5 | **-2.5** |
| 6 | 0.83 | 101 | 0.37 | 0.3 | 0.3 | 0.3 |
| 7 | 0.91 | 100 | 0.25 | 0.5 | 0.7 | 1.0 |
| 8 | 0.7 | 100 | 0.3 | 0.1 | -0.4 | -0.5 |
| 9 | 0.9 | 105.8 | 0.21 | 0.3 | 0.4 | 0.6 |
| 10 | 0.72 | **55.7** | 0.29 | 0.5 | -0.3 | -0.3 |
| 11 | 0.75 | - | - | **25** | -0.1 | - |
| 12 | **0.10** | 99 | 0.05 | 0.10 | **-3.5** | **-10.9** |
| 13 | **0.09** | 100 | 0.045 | 0.05 | **-3.5** | **-11.2** |
| 14 | - | - | - | - | - | - |
| 15 | **1.6** | 100 | 0.8 | 1 | **4.3** | 2.0 |
| 16 | 0.6 | 81 | 0.1 | 0.3 | -0.9 | **-2.3** |
| 17 | 0.66 | 101.4 | 0.17 | 0.125 | -0.6 | -1.1 |
| 18 | 0.7 | 82.7 | 0.3 | 0.3 | -0.4 | -0.5 |
| 19 | 0.56 | 106.7 | 0.20 | 0.20 | -1.1 | -1.9 |
| 20 | 1.4 | 114 | - | 1 | **3.2** | - |
| 21 | 1.01 | **171.2** | 0.51 | 1.0 | 1.2 | 0.9 |

[1] in bold, values considered outliers;

[2] in bold, values out of the range of acceptability;

[3] in bold, inconsistent value of LOQ;

[4] in bold, values $2<|z|<3$ and $2<|\zeta|<3$.

The graphical representation of summarizing the set of z-score and ζ-score, are represented in Figures 4 and 5 for atropine and in Figures 6 and 7 for scopolamine.

In addition, except for Laboratories 4 and 17, the participants completed the questionnaire on the analytical method, in which information on sample preparation and TAs determination was requested. The information gathered from the questionnaires is reported in Appendix C.

According with the results and information provided, the z-score was calculated for 20 laboratories and the ζ-score for 18, in fact two laboratories (11 and 20) did not provide the uncertainty value. Regarding the LOQ, Laboratory 11 reported a value of 25 µg/kg for both atropine and scopolamine. This value is shown in bold in Tables 3 and 4 and was considered inappropriate due to its inconsistency with the expected range of analytical values for TAs determination. Moreover, in terms of recovery, values outside the range 70-120%, highlighted in bold in Tables 3 and 4, were reported by Laboratory 10 for atropine and scopolamine (55.7%) and by Laboratory 21 for scopolamine (171.2%).

Regarding z-score, 85% of the laboratories gave a score with satisfactory values ($|z|\leq 2$) for both atropine and scopolamine. However, for atropine, Laboratory 7 had a questionable score ($2<|z|<3$) and Laboratories 12 and 13 reported unsatisfactory scores ($|z|\geq 3$); while for scopolamine, Laboratory 1 had a questionable score ($2<|z|<3$) and Laboratories 12, 13, 15 and 20 reported unsatisfactory scores ($|z|\geq 3$).

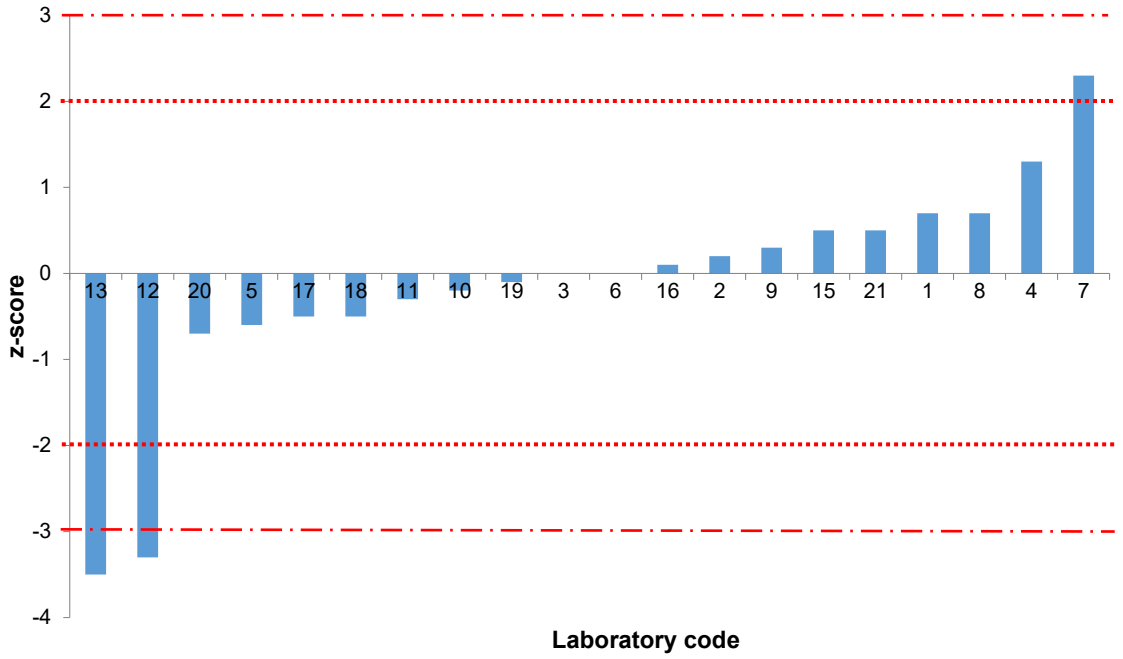


**Figure 4. ATROPINE: z-score values in cereal-based baby food sample (assigned value ± U(x) = 2.66 ± 0.27 µg/kg; $\sigma_{PT}$ = 0.49 µg/kg). PT ISS 2023 TAs baby food**

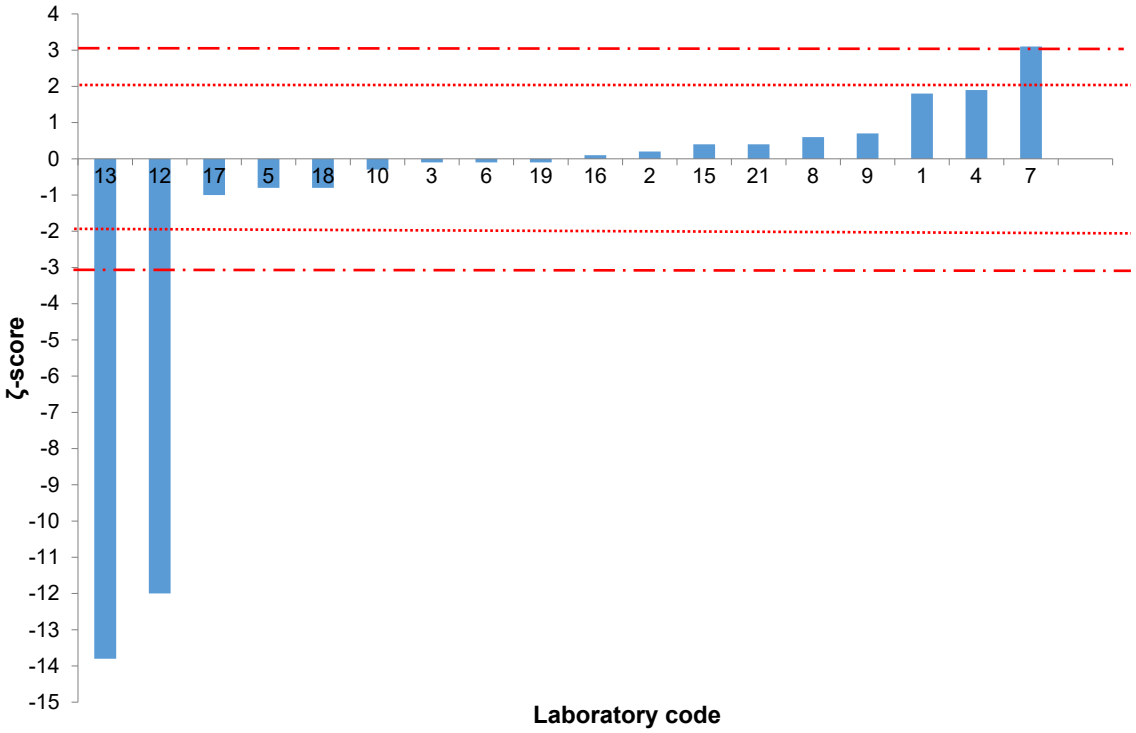


**Figure 5. ATROPINE: ζ-score values in cereal-based baby food sample (assigned value ± U(x) = 2.66 ± 0.27 µg/kg; $\sigma_{PT}$ = 0.49 µg/kg). PT ISS 2023 TAs baby food**

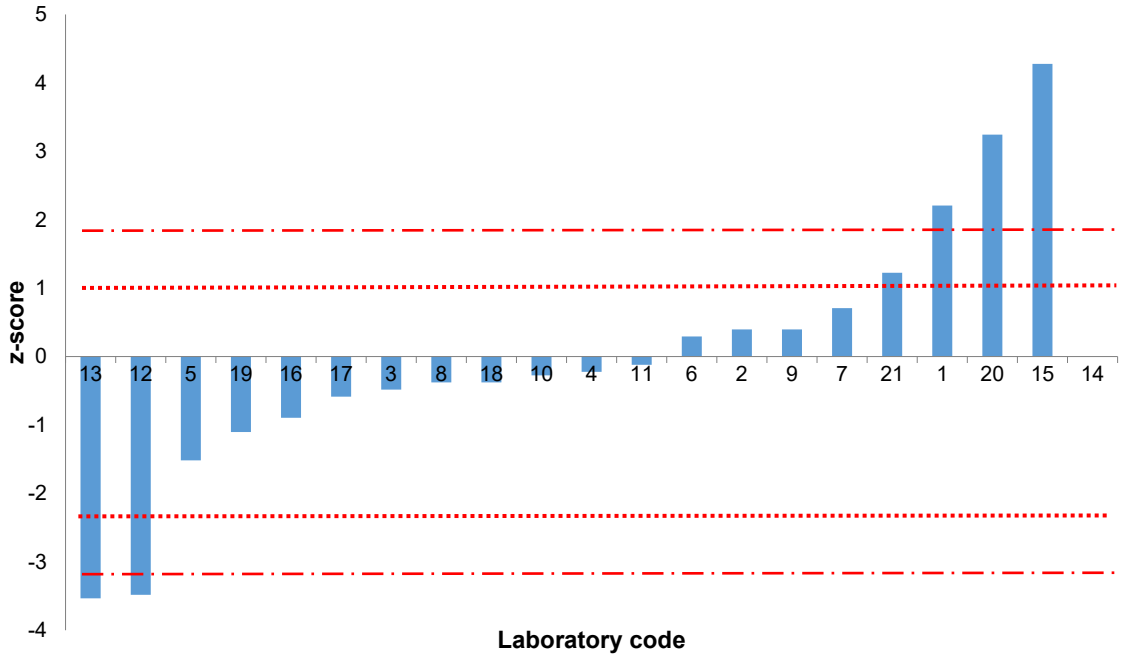


**Figure 6 SCOPOLAMINE: z-score values in cereal-based baby food sample (assigned value ± U(x) = 0.77 ± 0.11 µg/kg; $\sigma_{PT}$ = 0.19 µg/kg). PT ISS 2023 TAs baby food**

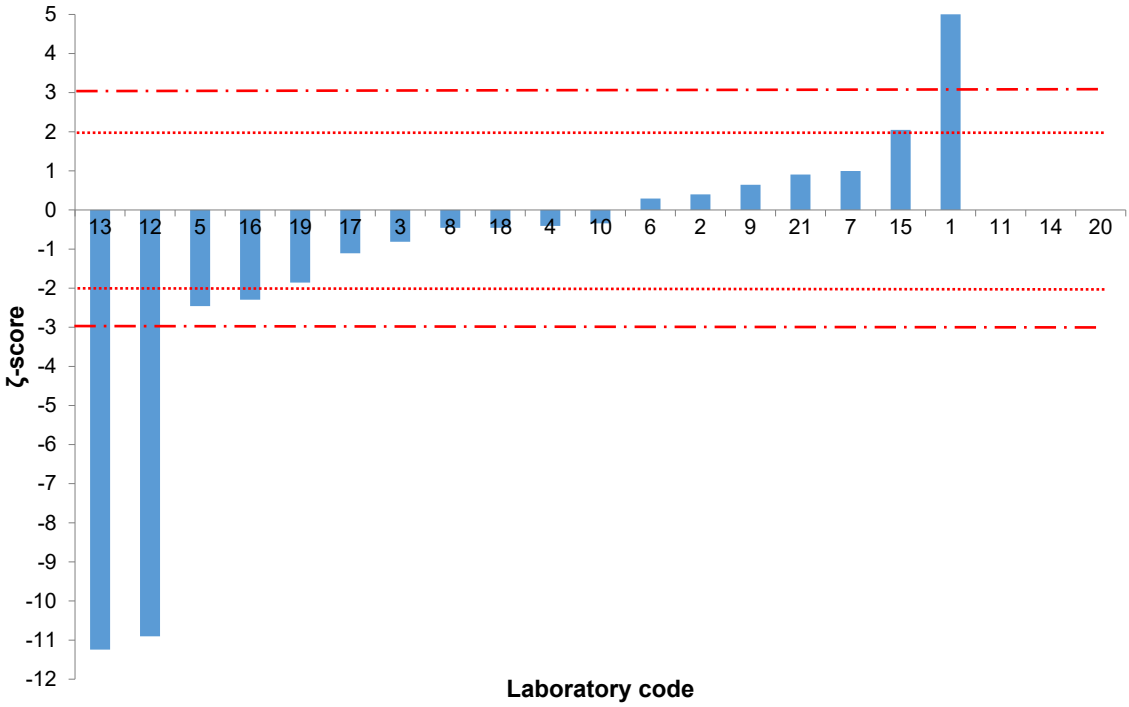


**Figure 7. SCOPOLAMINE: ζ-score values in cereal-based baby food sample (assigned value ± U(x) = 0.77 ± 0.11 µg/kg; $\sigma_{PT}$ = 0.19 µg/kg). PT ISS 2023 TAs baby food**

Analyzing the information provided by the laboratories, no possible causes for the questionable/unsatisfactory performances of the method have been identified.

Nonetheless, for scopolamine, it was noted that Laboratories 15 and 20 reported a LOQ value equal to 1.0 µg/kg, which is above the assigned value (0.76 µg/kg), the determination of this level of concentration is therefore out of the concentration range of the applied method for Laboratories 15 and 20.

The ζ-score was calculated for 18 laboratories, in fact two Laboratories (11 and 20) did not provide the uncertainty value. Eighty-three % and 72% of the laboratories gave a score with satisfactory values ($|\zeta| \leq 2$) for atropine and scopolamine, respectively. As regards the values obtained for atropine, three Laboratories (7, 12 and 13) had unsatisfactory values ($|\zeta| \geq 3$), while for scopolamine, Laboratories 5 and 15 showed questionable results ($2 < |\zeta| < 3$) and Laboratories 1, 12 and 13 showed unsatisfactory ($|\zeta| \geq 3$) scores.

The unsatisfactory scores of some of the laboratories for both z-score and ζ-score for atropine and scopolamine (Laboratories 7, 12 and 13 for atropine and Laboratories 1, 12 and 13 for scopolamine) indicate a significant deviation from the assigned value from one hand and suggests that the expanded uncertainty of the method associated with the sample is not adequate, likely due to the non-inclusion of significant sources of uncertainty. Notably, Laboratories 12, 13, 15 and 21 reported an expanded uncertainty of 50%.

# CONCLUSIONS

In conclusion, the PT was judged positively both in terms of participation and in terms of results. In fact, the number of Italian laboratory participants was considered satisfactory, taking into account that the novelty of the issue and the number of laboratories that carry out analyses on plant toxins, or that are accredited for such testing. are very limited.

In addition, another reason for satisfaction is the large participation of laboratories from other European countries recruited through the EURL. which contributed to the success of the PT.

As the overall results, 17 (85%) and 15 (75%) out of 20 participants obtained a satisfactory z-score (|z|<2) for atropine and scopolamine respectively. while of the 18 ζ-scores that could be calculated. 15 (83%) and 13 (72%) were satisfactory for atropine and scopolamine. respectively.

The summary of the obtained data for obtained data for PT ISS 2023 TAs in baby food is shown in Table 5.

**Table 5. Summary of the obtained data for PT ISS 2023 TAs baby food**

| Analyte | Total number of z-score | Number of \|z\|<2 | Satisfactory results \|z\| ≤2 (%) | Total number of ζ-score | Number of \|ζ\|<2 | Satisfactory results ζ\| ≤2 (%) |
|---|---|---|---|---|---|---|
| Atropine | 20 | 17 | 85 | 18 | 15 | 83 |
| Scopolamine | 20 | 15 | 75 | 18 | 13 | 72 |

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# APPENDIX A
# Documents sent to the participants

# A1. PT cover letter

## PROFICIENCY TESTING ISS

**PT ISS 2023 TAs baby food**

Dear Colleague.

Thank you very much for your participation in the Proficiency Testing for the analysis of tropane alkaloids (atropine and scopolamine) in baby food (PT ISS 2023_TAs baby food).

The parcel shipped contains one baby food sample of approximately 10 grams for the PT exercise. You will receive an e-mail confirming the shipment with attached the acknowledgement of receipt form. the results form and the questionnaire on the applied analytical method.

We ask you to read carefully the following instructions:

- After arrival the sample should be stored at +4°C
- Please fill in the *acknowledgement of receipt form* and return it upon receipt of the sample by e-mail to lnr-micotossine-tvn@iss.it
- Before analysis. homogenise the sample according to your laboratory's procedure
- Process the test sample according to your method as a sample for routine analysis. Please note that the homogenization tests have been performed on 2 grams. Therefore, a test portion of less than 2 grams is not recommended
- Please fill in the *results form* and the *questionnaire on the applied analytical method* and send them back by e-mail to lnr-micotossine-tvn@iss.it **no later than 27/03/2023**
- Please report the analytical results corrected for recovery in **µg/kg**
- Your laboratory code is _____

Please do not hesitate to contact us in case you have any questions or need any assistance

Kind regards,

Italian National Reference Laboratory for Mycotoxins and Plant Toxins

## A2. Acknowledgement of receipt

# PROFICIENCY TESTING ISS

### AKNOWLEDGEMENT OF RECEIPT

**PT ISS 2023 TAs baby food**

Please fill in this form and return it upon receipt of the sample to lnr-micotossine-tvn@iss.it.

Laboratory code _____

I confirm the receipt of the following sample

PT ISS 2023 TAs baby food/_____

☐ The sample was in a good condition

☐ The sample was not in good condition. namely:

| |
|---|
| |

Date of receipt _____

Signature

_____

## A3. Questionnaire on the applied analytical method

| Questionnaire on the applied analytical method PT ISS 2023 TAs baby food | | |
|---|---|---|
| | | |
| General information | Accredited method | YES |
| | Type of method | CONFIRMATION |
| | Method reference | |
| Extraction | Sample weight (g) | |
| | Extraction solvent composition | |
| | Amount of extraction solvent (ml) | |
| | Extraction | SINGLE |
| | Extraction procedure | ULTRASONIC BATH |
| | Sample processing | |
| | Sample clean-up | |
| LC | Injection volume (µl) | |
| | Mobile phase composition | |
| | Flow rate (ml/min) | |
| | Chromatographic column | |
| | Column temperature (°C) | |
| | Detector | |
| MS | Source | ESI |
| | **Atropine** | |
| | Precursor ion | |
| | Product ions, Q/q (m/z) | |
| | **Scopolamine** | |
| | Precursor ion | |
| | Product ions, Q/q (m/z) | |
| Quantification | Calibration | INTERNAL |
| | Certified standard brand | |

## A4. Results form

# PROFICIENCY TESTING ISS

### RESULT FORM

**PT ISS 2023 TAs baby food**

Laboratory ID _____

Institution _____

Reference person _____

| **Sample**<br>PT ISS 2023 TAs baby food/____ | **Result corrected for recovery (µg/kg)** | **Recovery (%)** | **Expanded uncertainty* (µg/kg)** | **LOQ (µg/kg)** |
|---|---|---|---|---|
| **Atropine** | | | | |
| **Scopolamine** | | | | |

*Coverage factor (*k*)_____ Confidence level _____

Please fill in this result form and return it back by email to lnr-micotossine-tvn@iss.it **no later than 27/03/2023**.

Signature _____

## APPENDIX B
## List of the participants

The order of indication shown in the table does not correspond to the assigned ID code.

| Name of the laboratory | Reference person | City/Country |
|---|---|---|
| ARPA Puglia DAP Bari | Ferrieri Francesca | Valenzano (BA)/Italy |
| ARPAL - Lab. Regionale | Ferro Marta | Genova/Italy |
| Chemische und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe | Goldbeck Christophe | Münster/Germany |
| Eurofins WEJ Contaminats GmbH | Reeber Tanja | Hamburg/Germany |
| Federal Institute for Risk Assessment (BfR) - LNR Mycotoxin and Plant Toxins in Food and Feed | These Anja | Berlin/Germany |
| Food Contaminants Laboratory-Ministry of Health | Christou Eftychia | Nicosia/Cyprus |
| General Chemical State Laboratory Division of Piraeus | Sargantanis Ioannis | Piraeus/Greece |
| IZS-Abruzzo e Molise “G. Caporale” | Annunziata Loredana | Teramo/Italy |
| IZS-Lazio e Toscana chimico Firenze | Focardi Claudia | Firenze/Italy |
| IZS-Lombardia ed Emilia Romagna | Prizio Ilaria | Bologna/Italy |
| IZS Mezzogiorno | Duro Ida | Portici (NA)/Italy |
| IZS-Venezie | Gallina Albino | Legnaro (PD)/Italy |
| Laboratoire National de Santé du Luxembourg Surveillance Alimentaire | Pincemaille Justine | Luxembourg |
| LALLF M-V Abt. 5 | Habedank Friederike | Rostock/Germany |
| Landesuntersuchungsamt Institut für Lebensmittelchemie | Simone Kasper | Mainz/Germany |
| National Laboratory of Health. Environment and Food (NLZOH) | Fras Danijela | Maribor/Slovenia |
| NIBIO - Department of Pesticides & Natural Products Chemistry | Galdiga Claus | Aas/Norway |
| Sciensano. Unit Toxins | Malysheva Svetlana | Tervuren/Belgium |
| SCL Laboratoire de Strasbourg | Ledoux Gérald | Illkirch-Graffenstaden/France |
| Service Commun des Laboratoires - Laboratoire de Rennes | Cristol Adelaide | Rennes/France |
| Sir Patrick Dun’s Hospital - Public Analyst’s Laboratory | English Patrick | Dublin/Ireland |

# APPENDIX C
# Technical information on the analytical method applied by the participants

**Table C1. Technical information on the analytical methods used by the participants. Sample preparation**

| Technical information | Laboratory Code ID |
|---|---|
| **Accredited method** | |
| Yes | 1. 3. 7. 8. 10. 12. 16. 18. 21 |
| No | 2. 5. 6. 9. 11. 13. 15. 19. 20 |
| **Type of method** | |
| Screening | - |
| Confirmatory | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20 .21 |
| Not specified | - |
| **Sample weight g** | |
| ≤2 | 1. 2. 5. 6. 8. 9. 10. 11. 12. 16. 19. 20 |
| >2 | 3. 7. 13. 15. 18. 21 |
| **Extraction solvent composition** | |
| **MeOH:H2O:HCOOH (39:60:1)** | **1. 2. 5. 6. 9. 16** |
| MeOH:$H_2O$ 0.4% HCOOH (60:40) | 7. 18 |
| MeOH:$H_2O$ 0.4% HCOOH (75:25) | 10. 19 |
| MeOH:$H_2O$ | 8 |
| $H_2O$ 0.2% HCOOH | 11 |
| MeOH 0.05 M $H_2SO_4$ | 12. 13 |
| ACN:(NH4)2CO3 0.2 g/l (84:16) | 15 |
| ACN (100%) | 20. 21 |
| **Extraction** | |
| Single | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| Multiple | - |
| **Extraction procedure** | |
| Shaker | 1. 2. 3. 5. 6. 7. 8. 9. 11. 15. 16. 18. 19. 20. 21 |
| Ultrasonic bath | 10. 12. 13 |
| **Sample processing** | |
| Dilution | 10. 16. 21 |
| Filtration | 3. 21 |
| Centrifugation | 1. 5. 6. 9. 11. 13. 15. 18. 21 |
| Concentration | 20 |
| No processing | 7 |
| Not specified | 2. 8. 12. 19 |
| **Sample clean-up** | |
| QuEChERS | 20 |
| SPE | 3. 11. 12. 13. 15. 19. 21 |
| None | 1. 2. 5. 6. 7. 8. 9. 10. 16. 18 |

**Table C2. Technical information on the analytical methods used by the participants. Sample determination**

| Technical information | Laboratory Code ID |
|---|---|
| **HPLC** | |
| | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| **Vinj. µL** | |
| ≤ 2 | 3. 5. 12. 15 |
| ≥ 5 | 1. 2. 6. 7. 8. 9. 10. 11. 13. 16. 18. 19. 20. 21 |
| **Mobile phase composition** | |
| H2O HCOOH; ACN HCOOH | 1. 2. 5. 6. 8. 9. 11. 16. 18. 19. 20 |
| H2O CH3COONH4 NH4OH : MeOH CH3COONH4 NH4OH | 7 |
| H2O NH3 : ACN NH3 | 3 |
| H2O (NH4)2CO3 : ACN | 13. 15 |
| H2O NH4COOH : MeOH NH4COOH | 21 |
| H2O NH4COOH HCOOH : MeOH NH4COOH HCOOH | 10. 12 |
| **Flow rate. mL/min** | |
| ≤ 0.35 | 1. 2. 3. 5. 6. 8. 9. 11. 12. 13. 16. 19. |
| ≥ 0.4 | 7. 10. 15. 18. 20. 21 |
| **LC column packing** | |
| C18 | 1. 2. 3. 5. 6. 7. 8. 10. 11. 12. 13. 15. 16. 18. 20. 21 |
| F5 (pentafluorophenyl) | 9. 19 |
| **Column temperature. °C** | |
| TA | 18. 20 |
| 20 | 21 |
| 30 | 2. 10. |
| 40 | 1. 3. 5. 6. 8. 12. 13. 15. 16 |
| 50 | 7. 11. 19 |
| **Detector** | |
| MS/MS | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| **Source** | |
| ESI | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| **ATROPINE** | |
| **Precursor ion** | |
| 290 | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| **Product ion** | |
| 77 | 10. 12. 13 |
| 91 | 6. 10 |
| 93 | 1. 2. 6. 9. 10. 12. 13. 15. 16. 19. 20. 21 |
| 103 | 7. 18. 103 |
| 124 | 1. 2. 3. 5. 6. 7. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |

| **SCOPOLAMINE** | |
|---|---|
| **Precursor ion** | |
| 304 | 1. 2. 3. 5. 6. 7. 8. 9. 10. 11. 12. 13. 15. 16. 18. 19. 20. 21 |
| **Product ion** | |
| 103 | 6. 7. 10. 12. 21 |
| 121 | 13 |
| 138 | 1. 2. 3. 6. 7. 9. 10. 11. 12. 13. 15. 16. 18. 20. 21 |
| 156 | 1. 2. 5. 6. 9. 10. 12. 13. 15. 16. 18. 20. 21 |
| **Calibration** | |
| Internal | 3. 6. 7. 8. 9. 11. 12. 13. 16. 19. 20 |
| External | 1. 2. 4. 5. 10. 15. 18. 21 |

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