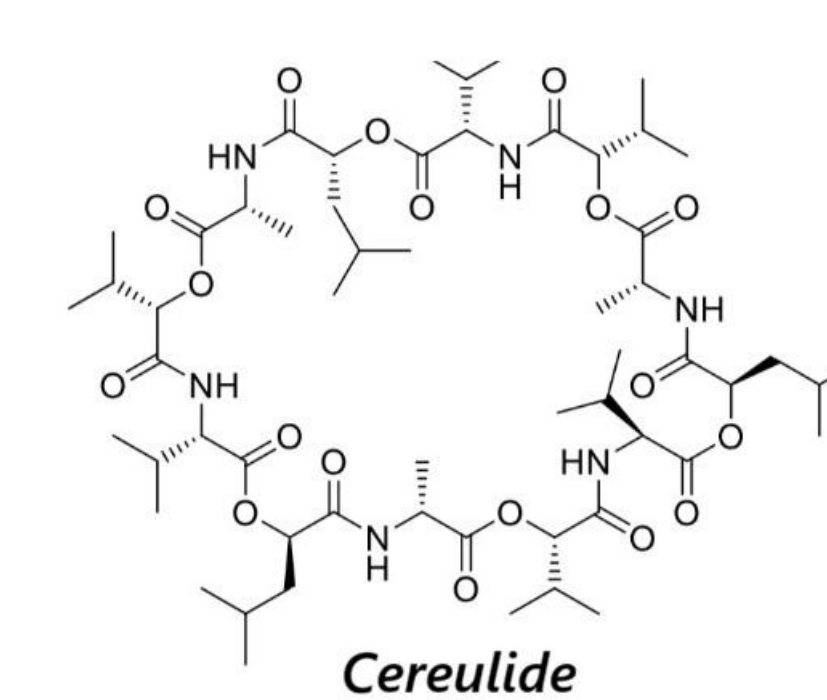
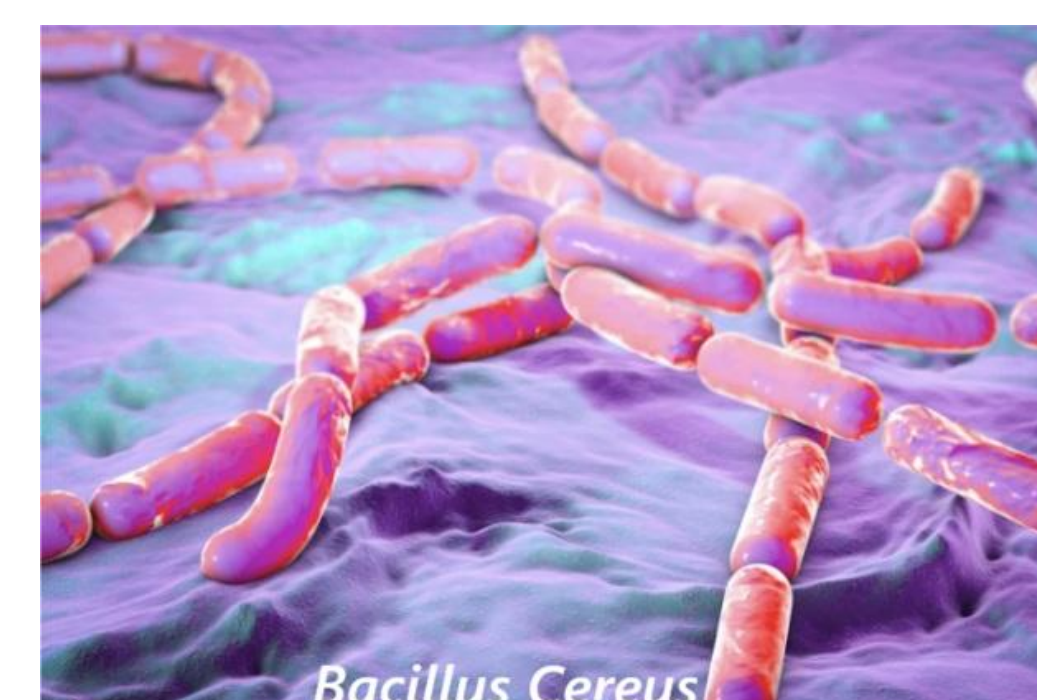




Establishment of proficiency testing for the detection and quantification of cereulide in milk powders

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INTRODUCTION

Cereulide is an extremely stable toxin that is resistant to heat and acidity and causes severe food poisoning characterized by intense vomiting. It is produced by certain strains of the bacterium *Bacillus cereus* (found in the environment – soil, dust, water, etc.). Once formed or already present in an industrial food processing operation, cereulide is extremely difficult to remove.

number of infant formula products worldwide. The addition of an oil rich in arachidonic acid (a compound naturally present in breast milk) to infant formula was the cause of this contamination. These events highlight the importance of rigorous control of products intended for infant feeding, and therefore the need for reliable and efficient analytical methods that meet the regulatory requirements in this field.

It was the cause of a recent health crisis, triggered by the recall in early 2026 of a large

REGULATION AND ANALYTICAL APPROACH

In this context, the European Food Safety Authority (EFSA) has published an evaluation that challenges established practices. A new acute reference dose has been set at 0.014 µg cereulide/kg body weight for infants (a threshold half that previously applied), based on the concept of vomiting as the critical effect. The risk of exceeding this dose arises when the concentration of cereulides in infant formula is as follows (see Fig. 1).

Type of preparation	Age group	Critical threshold (per liter of reconstituted milk)	Critical threshold (per kg of milk powder)*
Infant formula (stage 1)	0-4 months	0.054 µg/l	0.432 µg/kg
Follow-on infant formula	4-12 months	0.100 µg/l	0.800 µg/kg

Fig. 1 - Summary of critical concentration thresholds for cereulides

The analytical method that remains the standard for this measurement is LC-MS/MS, in accordance with the recommendations of ISO 18465 [1]. However, EFSA warns of a major methodological bias, not covered by the standard, namely that a preliminary hydration step for the milk powder is essential for reliable extraction of the toxin. Acetonitrile alone as an extraction solvent is not sufficient and may lead to

an underestimation of the cereulide result.

These new scientific and regulatory data require analytical rigor and performance monitoring of the methods used to check raw materials and finished products relating to infant nutrition. Implementing a proficiency test [2] on this subject therefore perfectly meets this need and the requirements of the ISO 17025 standard [3] for accredited laboratories.

* A factor of 8 is applied when converting between the units µg/l of reconstituted milk and µg/kg of milk powder.

DESIGN AND IMPLEMENTATION

A proficiency test was therefore quickly organized in April 2026 to give participants the opportunity to evaluate the analytical performance of their measurements in this area.

This trial, as conceived and designed, aims to provide laboratories with insights to help them assess their performance and make improvements.



SAMPLE PRODUCTION

The samples produced for the test involve the use of a batch of infant formula naturally contaminated with cereulide (positive commercial batch) and a negative batch. These raw materials were characterized by an external laboratory to confirm the absence or presence of the toxin, as well as its concentration in µg/kg in milk powder.

Thus, three sets of samples randomly contaminated - n°0274 (negative), n°0374 (positive), n°0474 (negative) - are produced. A 10-gram sample from each of these sets is sent to the participants.



HOMOGENEITY CONTROL

The homogeneity of positive samples, prepared by gradually filling the vials by hand, is verified before shipment (in accordance with the requirements of ISO 13528 [4]). These analyses are performed by a subcontracted laboratory that has been selected and evaluated in accordance with our internal procedures.

Ten samples are taken from the production at regular intervals and analyzed in duplicate, in random order. The results are analyzed by calculating the ratio of the inter-sample standard

deviation (Ss) to half the tolerance value (VT/2). Additional statistical tests are also performed (Fisher's test, test for significant inhomogeneity, Cochran's test). In addition, a manufacturing inspection is conducted on the negative series produced to confirm the absence of cereulide and cross-contamination during the production phase.

ANALYSIS

Samples are shipped at room temperature to the laboratories participating in the trial.

The expected results are qualitative (detection/absence of cereulide) and quantitative (cereulide concentration). Participants are asked to provide additional information (detection and quantification limits, details of the method used) in order to take stock

of current practices in this field.

The analysis is mainly performed using LC-MS/MS.

Reply forms for each sample are available on the BIPEA website, allowing laboratories to submit their results for cereulide within 4 or 5 weeks.

STATISTICAL TREATMENT

The statistical analysis of the qualitative data was based on the calculation of relative specificity, sensibility, and accuracy, compared to the median value of the participants' results.

The statistical analysis of the quantitative data was performed in accordance with ISO 13528. The assigned value (x_{pt}) for cereulide content is estimated using the robust

mean according to Algorithm A, based on the participants' results (x_i). Each laboratory's performance is evaluated using a tolerance value (24% of the assigned value; minimum = 0.8 µg/kg), derived from precision data in ISO 18465 and feedback from experts in the field. This value defines the range within which participants' results are considered satisfactory.

Each result (x_i) can thus be evaluated

and classified using a z-score:

- $|z| \leq 2$: satisfactory
- $2 < |z| < 3$: questionable
- $|z| \geq 3$: unsatisfactory.

where: $z = (x_i - x_{pt}) / (VT/2)$

Parameters	Summary	Definition	Interpretation
Specificity rate	r_{sp} (%)	Number of negative results found divided by the total expected negative samples, expressed in percentage.	Global relative overview for each laboratory to find the negative samples of the test.
Sensitivity rate	r_{sc} (%)	Number of positive results found divided by the total expected positive samples, expressed in percentage.	Global relative overview for each laboratory to find the positive samples of the test.
Relative accuracy	r_{ac} (%)	Number of true negative and positive results, found divided by the total analysed samples, expressed in percentage.	Global relative overview for each laboratory to find the right conclusion for the samples of the test.

Fig. 2 – Definition of qualitative criteria

General total	Test	Relative specificity	Relative sensitivity	Relative accuracy
Month	74A-1-04/2026			
LAB.	rSP (%)	rSE (%)	rAC (%)	
G6436	0	100	33	
G7106	0	100	33	
G8464	50	100	67	

Fig. 3 – Review of unsatisfactory qualitative results

Series	Characterization of the raw material	Manufacturing control of the negative series (samples)	Homogeneity control of the positive series (samples)	Participants' results (assigned value)
	Cereulide detected / not detected	Cereulide content (µg/kg milk powder)	Cereulide content (µg/kg milk powder)	Cereulide content (µg/kg milk powder)
0274	-	ND	ND	/
0374	+	12,00	11,59	13,50
0474	-	ND	ND	/

Fig. 4 – Overview of quantitative results

RESULTS

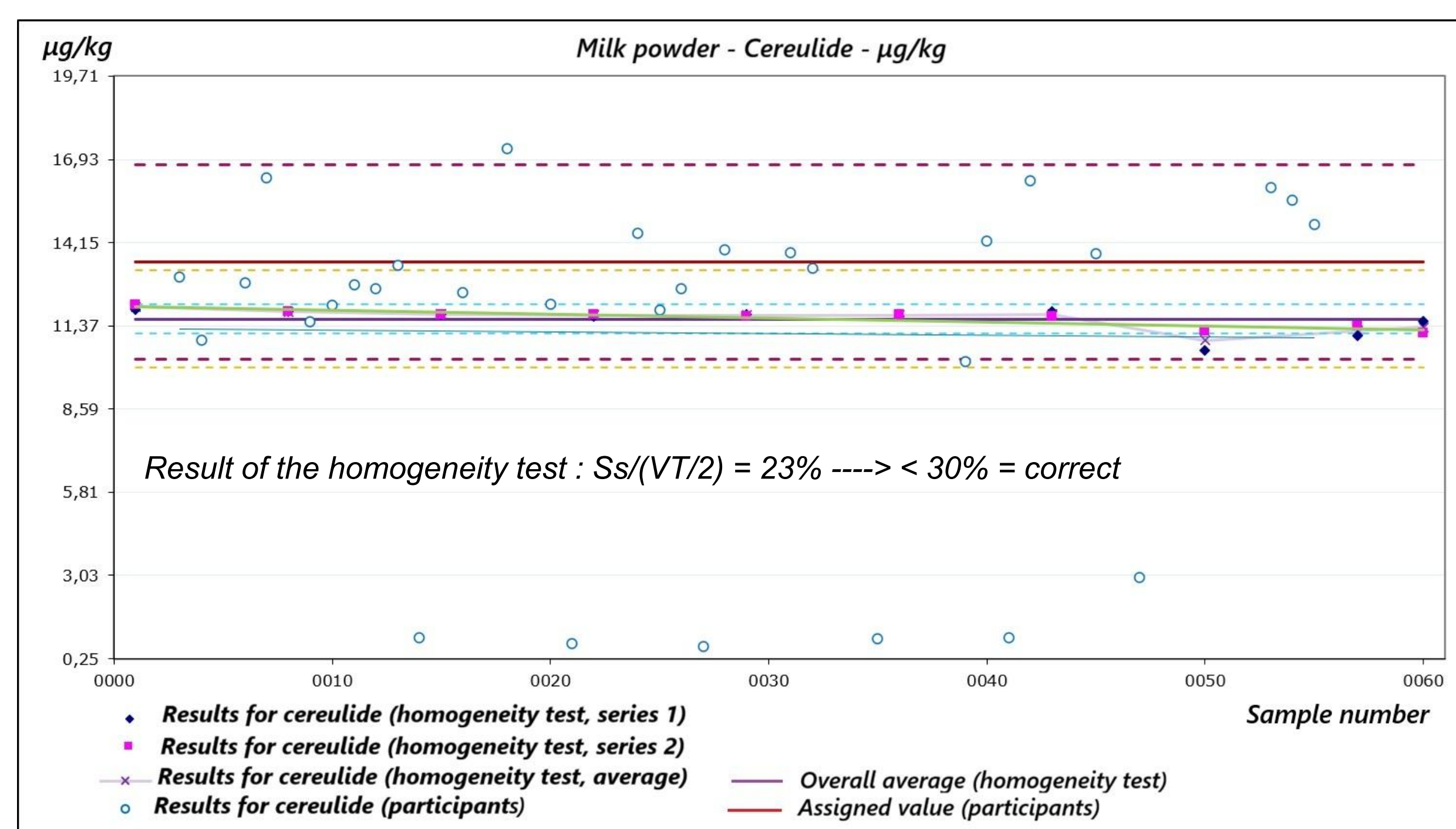


Fig. 5 - Results of the homogeneity test, both a priori (subcontractor) and a posteriori (participants), for the set of positive samples

REFERENCES

[1] ISO 18465:2017 – Food Chain Microbiology — Quantitative determination of emetic toxin (cereulide) by LC-MS/MS.

[2] ISO/IEC 17043:2023 – Conformity assessment - General requirements for the competence of proficiency testing providers.

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

[4] ISO 13528:2022 – Statistical methods for use in proficiency testing by interlaboratory comparisons

DISCUSSION & CONCLUSION

The preparation and conduct of the trial were carried out successfully. The homogeneity of the positive samples was satisfactory (see Fig. 5), and no contamination of the negative samples was detected (see Fig. 4).

42 laboratories registered for the trial, and 34 participated (by submitting their results).

From a qualitative point of view, 3 out of 34 laboratories (~9%) did not achieve satisfactory results (score ≠ 100% for at least one of the qualitative criteria) – see Fig. 2&3. In this case, the issue involved the detection of false positives in series 0274 and 0474.

From a quantitative perspective, the value assigned to series 0374 (theoretically positive) – 13.50 µg/kg milk powder – is

consistent, within the range of variation in the participants' results, with the values obtained during the characterization and homogeneity testing of the contaminated milk powder– see Fig. 4. 9 out of 34 laboratories (~26%) had an unsatisfactory result (outside the tolerance range) – see Fig. 5.

Among these 9 results:

- 2 are close to the expected concentration range (these laboratories used a hydration step prior to cereulide extraction).
- 7 are more than a factor of 10 away from the result (6 of these 7 laboratories did not use a hydration step prior to cereulide extraction), excluding a simple unit conversion error (µg/L of reconstituted milk versus µg/kg of milk powder).

Therefore, these interlaboratory trials are a source of vital knowledge and, through their results, provide laboratories with guidance on how to improve their analytical practices in the context of food safety. This also helps to comply with the increasingly stringent regulatory requirements in this area. In the future, these interlaboratory tests could become more challenging – for example, by diluting the contaminated milk powder to bring the results closer to regulatory limits.