Application Note

Food Safety

# Sensitive Quantitation of Cereulide in Infant Formula Following ISO 18465:2017

## Using the Agilent 6495D triple quadrupole LC/MS

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## Abstract

Cereulide, a heat-stable emetic toxin produced by *Bacillus cereus*, poses significant health risks. The recall of multiple batches of infant formula in early 2026 serves as a timely reminder of the importance of vigilance against cereulide contamination. Reinforcing robust preventive measures is essential, especially for products consumed by vulnerable groups such as infants. This study presents a quantitative measurement workflow for cereulide analysis in infant formula following ISO 18465:2017. The workflow uses a simple acetonitrile extraction and filtration process, with analysis performed on an Agilent 1290 Infinity III UHPLC system coupled to an Agilent 6495D triple quadrupole LC/MS system. The method achieves an outstanding limit of quantification (LOQ) of 0.0021 µg/L (0.025 µg/kg sample equivalent) with a signal-to-noise ratio (S/N) greater than 12.9. This sensitivity supports quantification at levels substantially lower than those specified in European Food Safety Authority (EFSA) and European Union (EU) guidelines. Dynamic linearity is demonstrated from 0.0021 to 0.84 µg/L (0.025 to 10 µg/kg) with $R^2$ greater than 0.999. Extraction recovery exceeds 80% across all concentration levels, and exceptional repeatability is observed with %RSD values less than or equal to 3.06% at the LOQ level. Application to a contaminated infant formula sample successfully quantifies cereulide at 0.0036 µg/L, demonstrating the method's robust capability for sensitive detection of a naturally contaminated product. This straightforward, cost-effective workflow enables early and routine detection of cereulide contamination, helping reduce product recall risk and protect brand reputation.

## Introduction

Cereulide is a heat-stable emetic toxin produced by certain strains of *Bacillus cereus*, a ubiquitous bacterium found in environmental reservoirs, including soil and dust. This lipophilic, cyclic dodecadepsipeptide is particularly insidious as a food contaminant because it is odorless and tasteless, making detection impossible through sensory evaluation. Under permissive growth conditions, *Bacillus cereus* can proliferate and synthesize cereulide, which poses a significant public health risk when contaminated food products are consumed. The toxin has been associated with starchy, carbohydrate-rich foods such as cooked rice, pasta, and noodles, as well as infant formula. Exposure to cereulide results in acute gastrointestinal symptoms, including nausea, vomiting, diarrhea, abdominal pain, and cramping.

The vulnerability of infant formula to cereulide contamination represents a critical concern for vulnerable populations. Recent recalls of multiple infant formula product batches underscore the necessity for robust preventive measures and sensitive detection methodologies. Despite the recognized hazard, analytical methods capable of reliably quantifying cereulide at trace levels in complex food matrices have been limited. The European Food Safety Authority (EFSA) and European Commission regulatory guidelines establish an action threshold of 0.054 µg/L of cereulide in liquid infant formula and 0.43 µg/kg of cereulide in infant formula powder; however, achieving the analytical sensitivity required to enforce these standards has presented a technical challenge in routine laboratory operations.[1,2] ISO 18465:2017 provides the definitive international standard for analyzing emetic toxins by tandem liquid chromatography/mass spectrometry and establishes sample preparation protocols and validation parameters for cereulide quantification in various matrices, including infant formula.

This study describes the development of a quantitative workflow for cereulide measurement in infant formula in accordance with ISO 18465:2017 guidelines using a UHPLC system coupled to a triple quadrupole mass spectrometer. The objective is to establish a sensitive, robust, and validated analytical method capable of reliably detecting and quantifying cereulide at concentrations below regulatory thresholds, thereby supporting food safety surveillance and protecting vulnerable consumer populations. Furthermore, the established quantitation workflow enables early detection to minimize product recall risks and safeguard brand image.

## Experimental

### Reagents and chemicals

Cereulide certified reference material (CRM, 20 µg/g in acetonitrile) was obtained from Sigma-Aldrich (Buchs, Switzerland, part number 38708-0.5ML). Two infant formula samples were procured from the regional market. The first infant formula served as the matrix blank and the second as the naturally contaminated product. Agilent InfinityLab Acetonitrile for LC/MS (part number 5191-5101) was used. LC/MS-grade formic acid was obtained from Honeywell Fluka (Michigan, USA). Ammonium formate was purchased from Sigma-Aldrich (Missouri, USA).

### Equipment and materials

**Table 1.** Instrumentation.

| Liquid Chromatography System | |
|---|---|
| Module | Part Number |
| Agilent 1290 Infinity III High-Speed Pump | G7120A |
| Agilent 1290 Infinity III Multisampler | G7167B |
| Agilent 1290 Infinity III Multicolumn Thermostat | G7116B |
| Agilent InfinityLab Assist Hub | G7180A |
| **Mass Spectrometry System** | |
| Module | Part Number |
| Agilent 6495D Triple Quadrupole LC/MS | G6495D |
| Agilent Jet Stream Technology Ion Source (AJS) | G1958B |

**Other equipment and consumables used were the following:**
Agilent Captiva Econofilter PTFE (25 mm 0.2 µm, part number 5190-5267), VWR DVX-2500 Multi-Tube Vortexer (Massachusetts, USA), and Eppendorf Centrifuge 5430R (Leipzig, Germany).

### Standard and sample preparation

A cereulide intermediate stock solution was prepared at a concentration of 2 µg/mL by diluting the CRM with 100% acetonitrile and then stored at −20 °C in an amber vial. A matrix-matched calibration set was made by spiking matrix blank with cereulide standard to the following concentrations: 0.0021, 0.0042, 0.0084, 0.0167, 0.084, 0.167, and 0.84 µg/L.

Figure 1 shows infant formula sample preparation. Extraction recovery was measured at three spiking levels: low (0.0042 µg/L), mid (0.0167 µg/L), and high (0.167 µg/L). Spiking was carried out in the matrix blank (pre-extraction) and after filtration (post-extraction). Recovery was calculated from the analyte response ratios obtained by comparing pre-extraction spiked samples with post-extraction spiked samples. Evaluation of matrix effects was performed by comparing a post-extraction spiked sample at 0.0167 µg/L with cereulide at the same concentration in neat solvent (acetonitrile). Naturally contaminated infant formula was prepared using the same workflow shown in Figure 1.

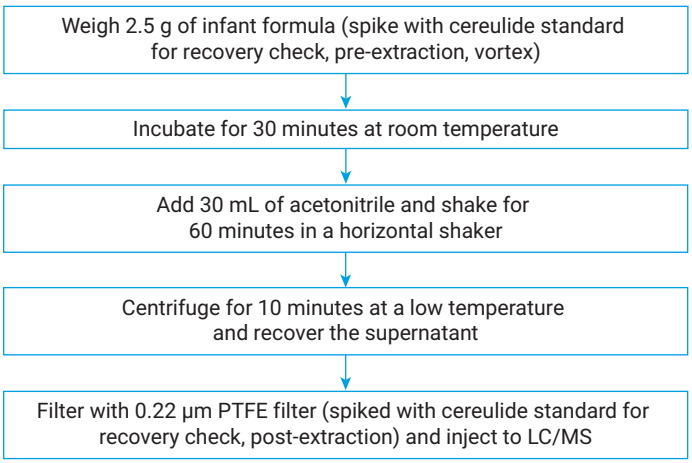



**Figure 1.** Sample preparation protocol.

## Data acquisition and processing

Multiple reaction monitoring (MRM) optimization was performed using the automated Agilent MRM Optimizer software embedded in Agilent MassHunter acquisition software for LC/MS systems version 12.2. This fully automated optimization helps to speed up the development of new methods or fine-tune existing methods. Data were processed using the Agilent MassHunter Quantitative Analysis software version 12.1. Tables 2—4 list the LC/MS conditions.

**Table 2.** Agilent 1290 Infinity III LC parameters.

| Parameter | Description |
|---|---|
| Analytical Column | Agilent Altura ZORBAX Eclipse Plus C18, 2.1 × 50 mm, 1.8 µm (part number 204205-308) |
| Guard Column | Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 5 mm, 1.8 µm (part number 821725-901) |
| Column Temperature | 40 °C |
| Multisampler Temperature | 4 °C |
| Mobile Phase | A: 10 mM ammonium formate + 0.1% formic acid<br>B: 0.1% formic acid in acetonitrile |
| Flow Rate | 0.4 mL/min |
| Gradient Program | Time (min) %B<br>0 80<br>1 99<br>7 99<br>7.1 80 |
| Stop Time | 4.4 minutes |
| Diverter Program | Start time (min) Flow<br>0 Waste<br>4.5 MS<br>6 Waste |
| Injection Volume | 5 µL |

**Table 3.** Agilent 6495D triple quadrupole LC/MS and AJS ion source parameters.

| Parameter | Description |
|---|---|
| Ion Source and Analysis Mode | Agilent Jet Stream (AJS), MRM |
| Drying Gas Temperature | 290 °C |
| Drying Gas Flow | 11 L/min |
| Sheath Gas Temperature | 400 °C |
| Sheath Gas Flow | 11 L/min |
| Nebulizer | 35 psi |
| Capillary Voltage | 5,000 V |
| Nozzle Voltage | 0 V |
| Detector Gain Factor | 10 |

**Table 4.** MRM transitions.

| Compound | Precursor *m/z* | Product *m/z* | Collision Energy (V) | Agilent iFunnel Mode | Cell Accelerator Voltage (V) | Polarity |
|---|---|---|---|---|---|---|
| Cereulide | 1,170.7 | 172.1* | 100 | Standard | 5 | Positive |
| | | 314.2 | 72 | | | |
| | | 1,125.7 | 40 | | | |

*Quantifier ion

## Results and discussion

A sensitive and robust analytical workflow for quantitative measurement of cereulide in infant formula was developed following ISO 18465:2017 guidelines using a 1290 Infinity III UHPLC system coupled to a 6495D triple quadrupole LC/MS. The method employed a simple acetonitrile extraction with filtration cleanup, minimizing sample preparation complexity while maintaining analytical performance.

### Chromatographic performance

Cereulide elutes at a retention time of approximately 5.6–5.8 minutes under the optimized gradient conditions (Figure 2). The chromatographic separation is clean and free of interference, with the diverter program effectively protecting the mass spectrometer from early eluting matrix components. The ammonium adduct $[M + NH_4]^+$ is the most abundant ion identified during the precursor ion search; thus, *m/z* 1,170.7 was selected as the precursor ion for cereulide. The three MRM transitions (*m/z* 1,170.7 → 172.1, 314.2, and 1,125.7) provide robust quantification and confirmation capability, with the transition to *m/z* 172.1 designated as the quantifier ion. The use of multiple transitions enhances method specificity by providing ion ratio confirmation, reducing the risk of false positives from isobaric interferences. A high desolvation temperature and fragmentor voltage are required to enhance cereulide ion abundance.

Optimization of gradient elution was carried out as cereulide elutes late in the gradient under highly organic conditions (> 90%). The Altura ZORBAX Eclipse Plus C18 Ultra Inert column was selected because it produced better peak shape, relatively higher abundance, and more stable retention times. Combining optimized gradient elution and proper column selection is essential to achieving a rapid analysis time. The minimal matrix interference observed in infant formula confirms the workflow's readiness for routine analysis, reducing the need for rework or further re-optimization. The relatively short total run time of 11.5 minutes, combined with the efficient sample preparation workflow, enables high-throughput analysis suitable for routine food safety testing laboratories processing large sample volumes.

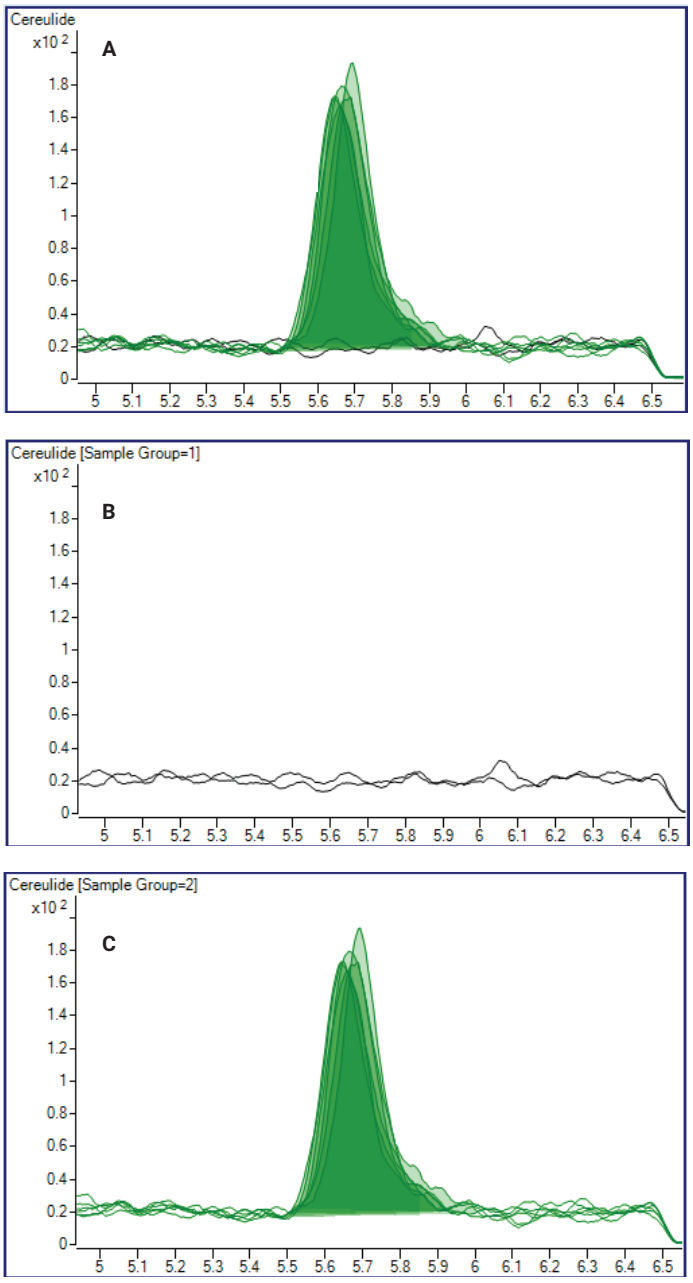



**Figure 2.** (A) Overlaid MRM chromatograms showing cereulide detection at the LOQ (0.0021 µg/L) in infant formula matrix (n = 6) compared with matrix blank. Individual MRM chromatograms of the (B) matrix blank and (C) LOQ. A retention time of approximately 5.6–5.8 minutes demonstrates excellent signal-to-noise ratio (> 12.9) and peak consistency across replicate measurements.

## Sensitivity and limit of quantification

This method achieves an outstanding limit of quantification (LOQ) of 0.0021 µg/L in infant formula, equivalent to 0.025 µg/kg of sample. This LOQ was determined at a signal-to-noise ratio of 6 or greater, as specified in ISO 18465:2017, with observed signal-to-noise ratios exceeding 12.9 (n = 6). This sensitivity substantially surpasses levels specified in current EFSA (0.054 µg/L in liquid infant formula), European Commission (0.43 µg/kg in powder infant formula), and EU guidelines (0.1 µg/kg in general foods)[3], providing enhanced detection capability for this heat-stable toxin in vulnerable populations. The exceptional sensitivity enables reliable, early detection of cereulide contamination at concentrations well below regulatory thresholds. This capability supports proactive food safety monitoring and risk assessment before any escalation occurs.

## Calibration performance and linearity

Dynamic linearity was established across a seven-point calibration range from 0.0021 to 0.84 µg/L (equivalent to 0.025 to 10 µg/kg in matrix) using matrix-matched standards prepared in infant formula extract (Figure 3). The calibration curve demonstrates excellent linearity with $R^2 > 0.9995$, exceeding the ISO 18465:2017 requirement of $R^2$ greater than or equal to 0.995. Good accuracy is maintained across the entire range, with recovery values consistently within the acceptable 80–120% window, even at the lowest concentration levels. No carryover is observed between consecutive injections, confirming the effectiveness of the diverter program, which directs matrix flow to waste during most of the chromatographic run. The broad dynamic range supports confident quantification of cereulide across diverse contamination scenarios, from trace-level detection in routine surveillance to higher concentrations in outbreak investigations.

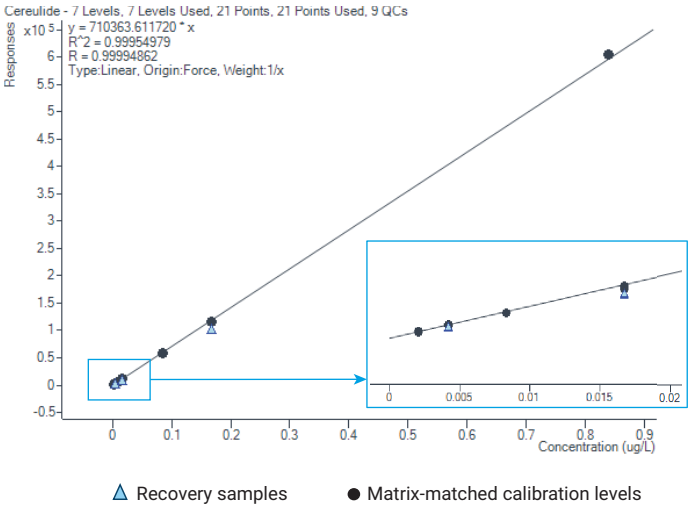



**Figure 3.** Seven-point matrix-matched calibration curve for cereulide from 0.0021 to 0.84 µg/L showing a linear response ($R^2$ = 0.99954979) with inset magnification of the low-concentration range. Recovery samples at three spiking levels (0.0042, 0.0167, and 0.167 µg/L) overlay calibration points, confirming accuracy within 80–120%.

## Repeatability and precision

Exceptional repeatability was demonstrated at the LOQ level (0.0021 µg/L), with a relative standard deviation (%RSD) of peak area and concentration of 3.06% across six replicate measurements (Figure 2). This high precision at trace levels is critical for reliable detection of cereulide contamination in infant formula, particularly when quantifying concentrations near regulatory limits where measurement uncertainty can significantly impact compliance decisions. The low variability confirms the robustness of both the sample preparation workflow and the instrumental analysis, supporting high-throughput laboratory operations where consistent performance across multiple batches and analysts is essential.

**Table 5.** Recovery evaluation.

| Level | Concentration (µg/L) | % Recovery | %RSD of Recovery |
|---|---|---|---|
| Low | 0.0042 | 82.9 | 1.31 |
| Mid | 0.0167 | 82.7 | 2.21 |
| High | 0.167 | 82.3 | 0.40 |

## Extraction recovery

Extraction recovery was evaluated at three spiking levels: low (0.0042 µg/L), mid (0.0167 µg/L), and high (0.167 µg/L), with three replicates per level (Figure 4). Effective extraction recovery exceeds 80% across all concentration levels, with recoveries of 82.9%, 82.7%, and 82.3% at the low, mid, and high levels, respectively (Table 5). The %RSD of recovery measurements is ≤ 2.21%, demonstrating consistent and reliable extraction efficiency independent of cereulide concentration. The simple acetonitrile extraction protocol effectively solubilizes the lipophilic cereulide molecule from the complex infant formula matrix, while the subsequent filtration step removes particulates and high-molecular-weight matrix components that could interfere with chromatographic separation or ionization efficiency. The consistent recovery across the tested concentration range indicates that the extraction procedure does not exhibit concentration-dependent losses or saturation effects, ensuring accurate and robust quantification across diverse contamination scenarios. More importantly, this straightforward extraction method cuts costs and preparation time.

## Matrix effects

Matrix effects were assessed by comparing cereulide response in infant formula matrix versus neat acetonitrile solvent at a concentration level of 0.0167 µg/L across three replicates (n = 3). A matrix effect of 108.0% is observed, indicating minimal matrix suppression or enhancement. The value falls within the acceptable 80–120% range, with %RSD value ≤ 2.51%, confirming that the infant formula matrix does not significantly compromise ionization efficiency or analyte detection. This minimal matrix effect can be attributed to the effective cleanup provided by the acetonitrile extraction and filtration steps, which remove proteins, lipids, and other matrix components that commonly cause ion suppression in electrospray ionization. Additionally, the diverter program prevents early eluting matrix components from entering the mass spectrometer, further reducing potential troubleshooting and minimizing the need for revalidation. The use of matrix-matched calibration standards compensates for any residual matrix effects, ensuring accurate quantification in real samples.

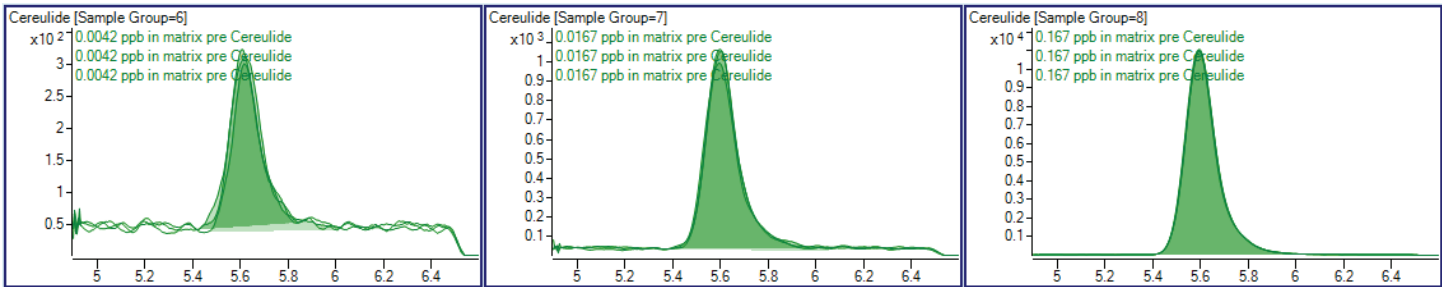
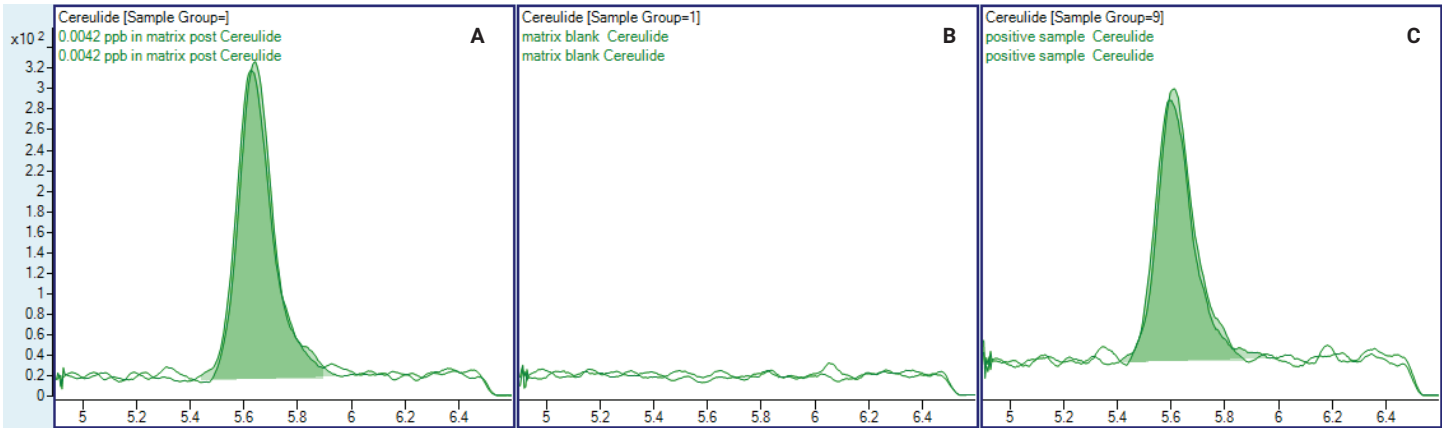



**Figure 4.** MRM chromatograms of recovery samples at low (0.0042 µg/L), mid (0.0167 µg/L), and high (0.167 µg/L) spiking levels (n = 3 per level), demonstrating consistent recovery greater than 80% with %RSD ≤ 2.21% across all concentration ranges.

## Real sample analysis

The developed workflow was applied to a contaminated infant formula sample procured from the regional market (Figure 5). Cereulide was quantitated at 0.0036 µg/L, equivalent to 0.043 µg/kg of sample, demonstrating the practical applicability of the method for detecting naturally occurring contamination at low concentrations. The successful quantification of cereulide in this real-world sample validates the method's performance in authentic matrices and confirms its suitability for food safety surveillance programs. The detected concentration, while low, underscores the importance of sensitive analytical methods capable of detecting cereulide contamination before it reaches levels that pose acute health risks to vulnerable infant populations. This application demonstrates the method's capability to support regulatory compliance monitoring, outbreak investigations, and quality control programs in infant formula manufacturing.

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| Sample | | | | | | | Cereulide... | Cereulide Results | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Name | Acq. Date-Time | Data File | Type | Pos. | Level | Sample Group | Exp. Conc. | RT | Area | Final Conc. | S/N |
| positive sample | 6/6/2026 8:35 PM | positive sample 01.d | Sample | P1-C4 | | 9 | | 5.591 | 2549 | 0.0036 | 12.78 |
| positive sample | 6/6/2026 8:47 PM | positive sample 02.d | Sample | P1-C4 | | 9 | | 5.611 | 2573 | 0.0036 | 12.92 |

**Figure 5.** Representative MRM chromatograms of (A) cereulide at 0.0042 µg/L, (B) infant formula matrix blank, and (C) naturally contaminated infant formula sample. (D) Agilent MassHunter Quantitative Analysis results show cereulide in the contaminated sample at 0.0036 µg/L, illustrating method specificity and real-world applicability.

## Conclusion

A quantitative workflow for cereulide measurement in infant formula was successfully developed using an Agilent 1290 Infinity III UHPLC coupled to an Agilent 6495D triple quadrupole LC/MS system, following ISO 18465:2017 guidelines. The method demonstrates exceptional analytical performance with a limit of quantification of 0.0021 µg/L (0.025 µg/kg sample equivalent), significantly surpassing EFSA and EU requirements. The robust workflow exhibited excellent repeatability, extraction recovery, and matrix effect performance across different contamination scenarios. Successful detection and quantification of cereulide in a naturally contaminated infant formula sample (0.0036 µg/L) validates the method's practical applicability for food safety monitoring. The streamlined sample preparation, combined with sensitive LC/MS/MS detection, provides a reliable analytical solution for protecting vulnerable populations from cereulide contamination in infant formula. This simplified and rapid workflow offers a cost-efficient solution for the early detection of cereulide contamination to mitigate product recall risks and protect brand equity.

## References


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