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ARTICLE

Development of a Stable Isotope Dilution Analysis for the Quantification of the *Bacillus cereus* Toxin Cereulide in Foods

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An increasing number of severe food borne intoxications are caused by a highly stable depsipeptide, named cereulide, which is produced by emetic *Bacillus cereus* strains. As cereulide poses a health risk to humans, the development of an appropriate method for the analysis of this toxin is mandatory. Therefore, the reference material of cereulide as well as its $^{13}C_6$-isotopologue was prepared by means of a biosynthetic approach using a *B. cereus* culture, followed by a rapid but efficient downstream purification. After structure confirmation by means of liquid chromatography (LC)−time-of-flight mass spectrometry, LC−tandem mass spectrometry, and one-/two-dimensional NMR spectroscopy, a stable isotope dilution analysis (SIDA) was developed for the quantification of cereulide in foods using the $^{13}C_6$-cereulide as the internal standard. Validation experiments were performed, and the data were compared to the quantitative analysis using the structurally related valinomycin instead of the $^{13}C_6$-cereulide as an internal standard. Trueness, repeatability, and reproducibility expressed as relative standard deviation showed values <10 or <8% for valinomycin or <8% for $^{13}C_6$-cereulide, respectively. Furthermore, the MS response of the valinomycin was found to be significantly influenced by the food matrix, thus leading to rather low recovery rates of 91% from boiled rice and 80% from boiled rice supplemented with 10% sunflower oil. In comparison, the use of $^{13}C_6$-cereulide as an internal standard gave good recovery rates of 104 and 111% from both matrices, thus demonstrating the robustness and accuracy of the developed SIDA.

KEYWORDS: Cereulide; stable isotope dilution analysis; $^{13}C_6$-cereulide; valinomycin; *Bacillus cereus*

INTRODUCTION

Bacillus cereus is well-known to cause two different types of gastrointestinal diseases arising from food contaminations: diarrhea and emesis. While the diarrheal syndrome is elicited by heat-labile enterotoxins, the emetic syndrome was found to be induced by a small highly heat resistant toxin named cereulide (*1*, *2*). Severe food borne intoxications have been increasingly reported during the past few years, occasionally even involving hospitalization or death (*3*, *4*).

The toxin cereulide belongs to the class of depsipeptides found in several organisms including fungi, bacteria, and marine sponges (*5*, *6*) and was recently demonstrated to be produced by a specific subgroup of the endospore-forming bacterium *B. cereus* (*7*). Cyclic depsipeptide are usually produced enzymatically by large multifunctional protein complexes, so-called nonribosomal peptide synthetase (NRPS) (*8*). Most notably, cyclic depsipeptides show a wide range of biological activities such as, for example, antiviral, insecticidal, cytotoxic, and antitumoral activities (*9*, *10*).

The chemical structure of cereulide (**1**, **Figure 1**) is composed of a cyclic trimer consisting of repeating tetrapeptide units (L-*O*-Val--L-Val--D-*O*-Leu--D-Ala) (*11*, *12*), thus resembling the macrolide antibiotic valinomycin (**2**) produced by *Streptomyces fulvissimus* (**Figure 1**). Reminiscent of the ionophoretic properties of valinomycin (**2**), cereulide (**1**) was found to be able to form complexes with monovalent cations, preferentially with potassium, in a highly ordered intramolecularly hydrogen-bonded structure (*13*). Because of the alternating ester and amide bonds, its cyclic structure, and various intramolecular hydrogen bondings, cereulide is highly resistant to heat, proteolysis, and acid and basic conditions (*14*−*16*). In consequence, cereulide present as a contamination in food products will not be destroyed through either thermal processing or the passage in the intestinal tract after food consumption. Moreover, its hydrophobic character will facilitate the cereulide to be rapidly absorbed from the gut and, potentially, to enter the bloodstream. Furthermore, cereulide was found to disrupt the mitochondrial membrane potential, to

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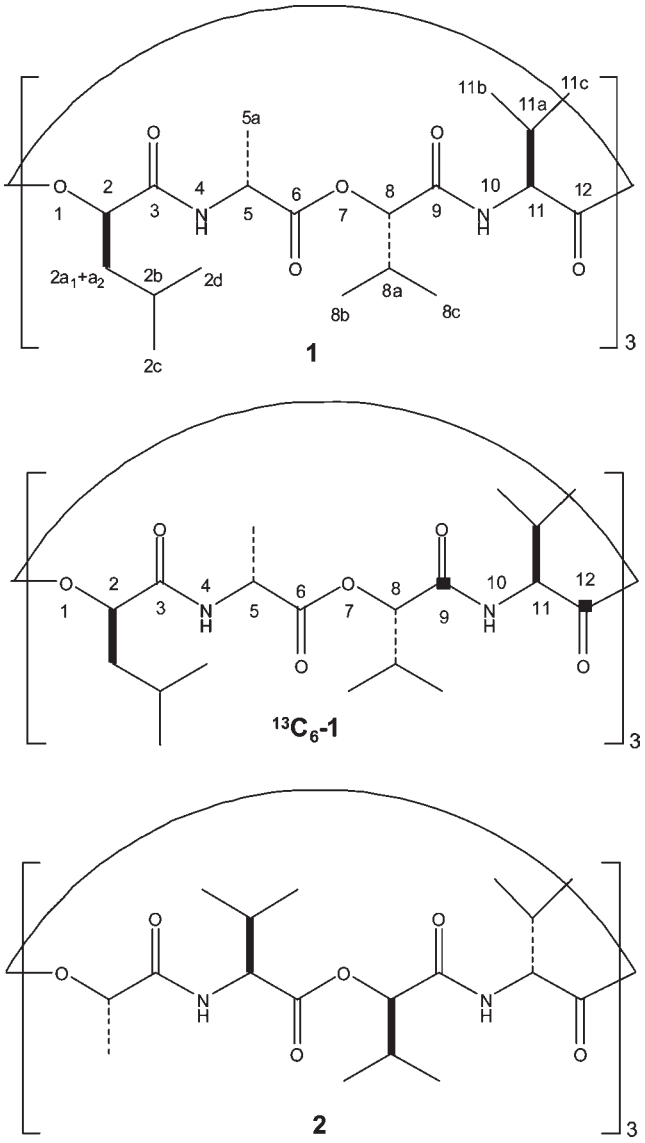

Figure 1. Chemical structures of cereulide (**1**), $^{13}C_6$-cereulide ($^{13}C_6$-**1**), and valinomycin (**2**).

deenergize mitochondria, and to inhibit mitochondrial fatty acid metabolism (*16*).

On the basis of the recently deciphered genetic background for cereulide production (*17*−*19*), a sensitive real-time polymerase chain reaction assay as well as a toxin gene profiling system were developed that allow rapid detection of emetic toxin producing *B. cereus* strains in foods and other complex matrices (*20*, *21*). Although this method is quite helpful for the identification of the pathogenic bacteria itself, it is not able to detect the toxin cereulide released into the food. As cereulide poses a health risk to humans, the development of an appropriate method for the analysis of this toxin is mandatory. To meet the demand for quantitative analysis of cereulide in foods and, in particular, in reheated food products, several cytotoxicity assays employing human larynx carcinoma cells or rat liver cells (*22*, *23*), as well as a boar sperm motility assay (*24*), have been reported in recent years. However, as the in vitro assays are based only on the toxic action of cereulide to mitochondria of eukaryotic cells and do not allow a direct measurement of the toxin, cytotoxic effects of other food ingredients might influence the results of the assays when applied to real food samples. Furthermore, bioassays are time-consuming and critically depend on the viability of cells or the motility of boar sperm, which derogates reproducibility.

To overcome these problems, high-performance liquid chromatography−mass spectrometry (HPLC-MS) procedures using an ion trap mass spectrometer (*25*−*27*) or a single quadrupole mass spectrometer (*28*) were developed to analyze the target toxin directly. As reference materials for cereulide are yet not available, the antibiotic valinomycin was used as a surrogate standard compound for quantification.

However, valinomycin is not to be considered as the ideal internal standard as this compound does not coelute with the cereulide and, therefore, is not suitable to overcome matrix effects during LC−tandem mass spectrometry (MS/MS) analysis of complex food samples. As such matrix effects can be counterbalanced by the use of a stable isotope-labeled internal standard, there is the urgent need for the development of a stable isotope dilution analysis (SIDA) (*29*).

As the SIDA requires the availability of a stable isotope-labeled internal standard, there might be the possibility to chemically synthesize ^{13}C-labeled cereulide using a complex sequence of reactions starting from suitable hydroxyl and amino acid derivatives as reported for nonlabeled cereulide (*11*). As this total synthesis is rather time-consuming and expensive, the biosynthetic production of ^{13}C-labeled cereulide using *B. cereus* strains and ^{13}C-labeled precursor amino acids might be a fast and cost-saving alternative. Therefore, the objectives of the present investigation were to biotechnologically produce and chromatographically purify reference material of cereulide and ^{13}C-labeled cereulide, to confirm their structure and purity by means of LC-MS/MS and one- (1D)/two-dimensional (2D) NMR spectroscopy, and to develop a highly sensitive, accurate, and robust SIDA for the quantitative analysis of cereulide in foods using LC-MS/MS detection. In addition, the SIDA developed should be cross-validated against the use of valinomycin as the internal standard.

MATERIALS AND METHODS

Chemicals. Water for chromatographic separations was purified with an integral 5 system (Millipore, Schwalbach, Germany), and solvents used were of HPLC grade (Merck, Darmstadt, Germany); ethanol was from Mallinckrodt Baker B.V. (Deventer, Holland). Deuterated solvents were obtained from Euriso-Top (Gif-Sur-Yvette, France). $^{13}C_1$-L-valine (99%) was from Cambridge Isotope Laboratories, Inc. (Andover, MA), valinomycin was from Fluka (Sigma Aldrich, Steinheim, Germany), and amino acids and trace elements for MOD broth were from Carl Roth (Karlsruhe, Germany). Sunflower oil, parboiled rice, milk pudding, and liver sausage were purchased from a local supermarket (Freising, Germany).

Bacterial Strain and Growth Conditions. The emetic strain *B. cereus* F4810/72, originally isolated from vomit by the Public Health Laboratory Service (London, United Kingdom), was routinely grown on Luria−Bertani (LB) agar plates or in LB broth at 30 °C.

Biosynthetic Production of Cereulide (1) and $^{13}C_6$-Cereulide ($^{13}C_6$-1). Experiment A: For the production of cereulide, LB broth (100 mL) supplemented with 0.2% glucose (11 mmol) was inoculated with nearly 10^3 cfu/mL from an overnight preculture, and cultures were incubated in baffled flasks (500 mL) while rotary shaking (150 rpm) at 24 °C for 24h. Experiment B: For the production of the $^{13}C_6$-labeled cereulide, the MOD broth (*30*) (550 mL) without added valine and an additional C-source was supplemented with $^{13}C_1$-L-valine (0.5 g) and was inoculated with a 10^3 cfu/mL amount from an overnight preculture. The cultures were incubated with shaking (150 rpm) at 24 °C for 48 h.

The cultures of experiments A and B, respectively, were autoclaved (20 min, 120 °C) to denature heat labile substances and centrifuged (8600*g*, 20 °C, 10 min). The pelletized cells were frozen in liquid nitrogen and stored at −18 °C until used. An aliquot (1 g) of the *B. cereus* biomass was extracted with ethanol (10 mL) by shaking at 20 °C for 15 h, the extract was centrifuged twice at 8.600*g* for 15 min, and the supernatant obtained was

centrifuged at 14000*g* for 5 min and then membrane filtered (0.2 μm; PTFE membrane, Phenomenex, Aschaffenburg, Germany) to remove the remaining spores and cell debris. The solvent was evaporated under reduced pressure, the residue was dissolved in a methanol/water mixture (100 mL; 10/90, v/v), and aliquots (10 mL) were applied onto the top of a C18-SPE cartridge (60 mL, 10 g, Strata C18-E, Phenomenex) conditioned with methanol, followed by water. The cartridge was rinsed with water (40 mL), the target compound was eluted with ethanol (100 mL), and after the effluent was concentrated under reduced pressure to 10 mL, it was isolated by means of RP-HPLC (Jasco, Gross-Umstadt, Germany).

HPLC. The HPLC system consisted of a HPLC pump system PU 2087, a high-pressure gradient unit, and a PU-2075 UV detector using a preparative RP-18 column, Microsorb 100-5 C18, 21.2 mm × 250 mm, 5 μm (Varian, Darmstadt, Germany), as the stationary phase. Monitoring the effluent at 210 nm, chromatography was performed with a mixture (90/10, v/v) of methanol and water for 5 min, increasing the methanol content to 100% over 5 min and, thereafter, eluting with methanol for 10 min at a flow rate of 20.0 mL/min. After the solvent was removed in vacuum, the target compound was suspended in water (10 mL) and freeze-dried twice to afford cereulide (**1**) and $^{13}C_6$-cereulide ($^{13}C_6$-**1**, **Figure 1**), respectively, as a white, amorphous powder in high purities of more than 98%.

Cereulide (***1***) (***Figure* 1**). UV−vis (MeOH): λ_{max} = 204 nm. LC-TOF-MS: *m/z* 1175.6669 ($[M + Na]^+$, measured), *m/z* 1175.6673 ($[C_{57}H_{96}N_6O_{18}Na]^+$, calculated). MS ($ESI^+$): *m/z* 1170.9 (100, $[M + NH_4]^+$), 596.6 (70, $[M + H + K]^{2+}$), 1175.9 (40, $[M + Na]^+$), 1191.7 (25, $[M + K]^+$), 1153.8 (10, $[M + H]^+$), 588.6 (10, $[M + Na + H]^{2+}$). MS (ESI^-): *m/z* 1198.3 (100, $[M + HCOOH - H^+]^-$), 1188.3 (71, $[M + Cl - H^+]^-$), 1152.2 (12, $[M - H^+]^-$. MS/MS of *m/z* 1153.9 (ESI^+, CE = +52 V; intensities > 20%): *m/z* (%) 1153.8 (100), 172.2 (59), 357.2 (47), 314.2 (36), 158.2 (28), 154.2 (27), 186.2 (26), 499.4 (26), 200.2 (25), 385.2 (24), 72.0 (21). MS/MS of *m/z* 1151.8 (ESI^-, CE = −54 V; intensities > 15%): *m/z* (%) 216.2 (100), 202.2 (90), 1151.8 (57), 970.6 (41), 785.6 (26), 984.6 (25), 131.0 (17), 116.0 (16), 401.2 (15). ^{1}H NMR [500 MHz, DMSO-d_6, correlation spectroscopy (COSY), 27 °C]: δ 0.80 [d, 9H, *J* = 7.15 Hz, H−C(8b)], 0.82 [d, 9H, *J* = 6.20 Hz, H−C(2c)], 0.84 [d, 9H, *J* = 6.20 Hz, H−C(2d)], 0.86 [d, 9H, *J* = 6.65 Hz, H−C(8c)], 0.88 [d, 9H, *J* = 6.70 Hz, H−C(11b)], 0.90 [d, 9H, *J* = 6.70 Hz, H−C(11c)], 1.35 [d, 9H, *J* = 7.30 Hz, H−C(5a)], 1.54 [m, 3H, *J* = 12.50 Hz, H−C($2a_1$)], 1.61 [m, 3H, *J* = 6.30 Hz, H−C(2b)], 1.65 [m, 3H, *J* = 12.15 Hz, H−C($2a_2$)], 2.11 [m, 3H, *J* = 4.80, 6.65 Hz, H−C(8a)], 2.18 [m, 3H, *J* = 6.70 Hz, H−C(11a)], 4.29 [dd, 3H, *J* = 7.35, 7.45 Hz, H−C(11)], 4.36 [qd, 3H, *J* = 7.10, 7.30 Hz, H−C(5)], 4.83 [d, 3H, *J* = 4.47 Hz, H−C(8)], 5.00 [dd, 3H, *J* = 3.73, 9.58 Hz, H−C(2)], 7.99 [d, 3H, *J* = 7.66 Hz, H−N(10)], 8.31 [d, 3H, *J* = 6.70 Hz, H−N(4)]. ^{13}C NMR [125 MHz, DMSO-d_6, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), 27 °C]: δ 16.59 [C(8b)], 16.69 [C(5a)], 18.08 [C(11c)], 18.49 [C(8c)], 18.85 [C(11b)], 20.97 [C(2c)], 22.93 [C(2d)], 23.81 [C(2b)], 29.46 [C(11a)], 30.07 [C(8a)], 40.18 [C(2a)], 48.00 [C(5)], 57.60 [C(11)], 71.65 [C(2)], 77.09 [C(8)], 168.83 [C(9)], 169.33 [C(3)], 170.49 [C(12)], 171.32 [C(6)].

$^{13}C_6$-Cereulide (*$^{13}C_6$-**1***) (***Figure* 1**). UV−vis (MeOH): λ_{max} = 204 nm. LC-TOF-MS: *m/z* 1181.6987 ($[M + Na]^+$, measured). MS (ESI^+): *m/z* 1177.0 (100%, $[M + NH_4]^+$), 1182.0 (44%, $[M + Na]^+$), 1197.9 (22%, $[M + K]^+$), 1159.8 (12%, $[M + H]^+$), 591.7 (10%, $[M + Na + H]^{2+}$). MS/MS of *m/z* 1160.0 (ESI^+, CE = +52 V; intensities > 10%): *m/z* (%) 1160.0 (100), 173.2 (36), 358.2 (28), 316.4 (36), 1131.0 (23), 387.2 (16), 186.2 (15), 155.2 (14), 202.2 (14), 298.4 (12), 501.4 (12). ^{1}H NMR (500 MHz, DMSO-d_6, COSY, 27 °C): δ 0.84 [d, 9H, *J* = 6.80 Hz, H−C(8b)], 0.86 [d, 9H, *J* = 6.07 Hz, H−C(2c)], 0.88 [d, 9H, *J* = 6.33 Hz, H−C(2d)], 0.90 [d, 9H, *J* = 6.87 Hz, H−C(8c)], 0.92 [d, 9H, *J* = 6.80 Hz, H−C(11b)], 0.94 [d, 9H, *J* = 6.63 Hz, H−C(11c)], 1.39 [d, 9H, *J* = 7.31 Hz, H−C(5a)], 1.58 [m, 3H, *J* = 12.77 Hz, H−C($2a_1$)], 1.65 [m, 3H, *J* = 6.20 Hz, H−C(2b)], 1.69 [m, 3H, *J* = 12.77 Hz, H−C($2a_2$)], 2.14 [m, 3H, *J* = 1.80, 4.95, 6.69 Hz, H−C(8a)], 2.22 [m, 3H, *J* = 2.22, 6.70 Hz, H−C(11a)], 4.34 [ddd, 3H, *J* = 2.20, 2.40, 6.32, 7.60 Hz, H−C(11)], 4.40 [qd, 3H, *J* = 7.27, 7.39 Hz, H−C(5)], 4.85 [dd, 3H, *J* = 3.80, 4.00 Hz, H−C(8)], 5.03 [m, 3H, *J* = 3.13, 9.72 Hz, H−C(2)], 8.06 [dd, 3H, *J* = 3.50, 7.34 Hz, H−N(10)], 8.37 [d, 3H, *J* = 6.72 Hz, H−N(4)]. ^{13}C NMR (125 MHz, DMSO-d_6, HSQC, HMBC, 27 °C): δ 16.49 [C(8b)], 16.63 [C(5a)], 17.98 [C(11c)], 18.39 [C(8c)], 18.75 [C(11b)], 20.87 [C(2c)], 22.84 [C(2d)], 23.68 [C(2b)], 29.35 [C(11a)], 30.96 [C(8a)], 40.07 [C(2a)], 47.97 [C(5)], 57.55 [d, *J* = 61.91 Hz, C(11)], 71.54 [C(2)], 76.96 [d, *J* = 55.67 Hz, C(8)], 168.78 [C(9)], 169.27 [C(3)], 170.38 [C(12)], 171.21 [C(6)].

Sample Cleanup and HPLC-MS/MS Analysis of Cereulide in Rice Samples. A sample of rice (10 g), prepared by boiling parboiled rice (Le Gusto, Van Sillevoldt Rijst B.V., Netherlands) for 18 min, was spiked with different amounts of purified cereulide (0, 10, 20, and 50 μg), homogenized, and then spiked either with $^{13}C_6$-cereulide (20 μg) or valinomycin (20 μg) as the internal standard. To simulate the matrix of an oil-rich food, a sample of rice (10 g) was supplemented with sunflower oil (1.1 g; Thomy, Nestle) and intimately mixed prior to spiking with cereulide and the internal standard. After equilibration on a rocking table for 2.5 h at 20 °C, each sample was extracted by shaking with ethanol (10 mL) on a rocking table for 15 h at 20 °C, and after centrifugation and membrane filtration as described above, the supernatant was 1:10 diluted with water. An aliquot (1.0 mL) of the diluted extract was applied onto the top of a C18-SPE cartridge (6 mL; 1000 mg; Strata C18-E, Phenomenex), and after they were rinsed with water (4 mL) and methanol/water (2 mL; 70/30, v/v), the target compounds were eluted with ethanol (1 mL), and aliquots (5 μL) were analyzed by means of LC-MS/MS on a Synergi Fusion-RP, 2 mm × 150 mm, 4 μm, column (Phenomenex, Germany) operating at a flow rate of 0.25 mL/min. Chromatography was performed using a solvent gradient starting with methanol/water (92/8, v/v) for 3 min; thereafter, the methanol content was increased to 100% within 7 min and finally held at 100% for 10 min.

LC-MS/MS. LC-MS/MS analysis was performed using an Agilent 1200 HPLC system connected to the API 4000QTrap LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the positive electrospray ionization (ESI^+) mode. Zero grade air served as the nebulizer gas (45 psi) and turbo gas (425 °C) for solvent drying (55 psi); nitrogen was used as the curtain (20 psi) and collision gas (8.7×10^{-7} psi), respectively. Both quadrapoles were set at unit resolution. By means of the multiple reaction monitoring (MRM) mode, cereulide (**1**, *m/z* 1170.9→172.3), $^{13}C_6$-cereulide ($^{13}C_6$-**1**; *m/z* 1176.9→173.3), and valinomycin (**2**, *m/z* 1128.8→172.2) were analyzed using the mass transitions (given in brackets) monitored for a duration of 55 ms. In addition, three further mass transitions of the singly charged pseudomolecular ions ($[M + H]^+$) to the corresponding daughter fragment ions and of the corresponding ammonium ions ($[M + NH_4]^+$) to the same daughter fragment ions were recorded (see the Supporting Information, Table S1). ESI^+ mass and product ion spectra were acquired with direct flow infusion. For ESI^+, the ion spray voltage was set at +5500 V in the positive mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the $[M + H]^+$ and $[M + NH_4]^+$ molecular ions into specific product ions after collision with nitrogen (8.7×10^{-7} psi). The declustering potential (DP), the collision energy (CE), and the cell exit potential (CXP) were set as given in Table S1 of the Supporting Information.

Quantitative Analysis of Cereulide (1) in Food Samples after Incubation with *B. cereus*. To investigate the cereulide production by *B. cereus* in a real food system, boiled rice samples (10 g) were inoculated with 150 cfu/g rice from an overnight preculture diluted in saline buffer (0.9% NaCl) and incubated at 24 °C. After 0, 6, 12, 24, 36, 48, 60, 72, and 96 h, the samples were spiked with the internal standard $^{13}C_6$-cereulide (10 μg), equilibrated for 2 h while shaking at 4 °C, and then extracted by ethanol (10 mL) on a rocking table for 24 h. The samples were centrifuged, the supernatants were membrane filtered and concentrated to a volume of about 3 mL, and after C18-SPE cartridge cleanup as detailed above, aliquots (5 μL) were analyzed by means of LC-MS/MS as described above.

In addition, a sample of milk pudding and liver sausage were artificially contaminated and analyzed as follows: 15 g of milk pudding or liver sausage was inoculated with 150 cfu/g food of a *B. cereus* overnight culture. After incubation at 24 °C for 24 h, the food samples were spiked with $^{13}C_6$-cereulide (10 μg), equlibrated while shaking for 2 h at 4 °C, and extracted with ethanol (15 mL). Sample cleanup and analysis were performed as shown above for rice samples.

Calibration. For quantitation, the analyte cereulide (**1**) and either $^{13}C_6$-cereulide ($^{13}C_6$-**1**), or valinomycin (**2**) as an internal standard was mixed in five molar ratios from 0.1 to 10 (1−100 ng/mL in EtOH) and analyzed by means of HPLC-MS/MS in the MRM mode in triplicates.

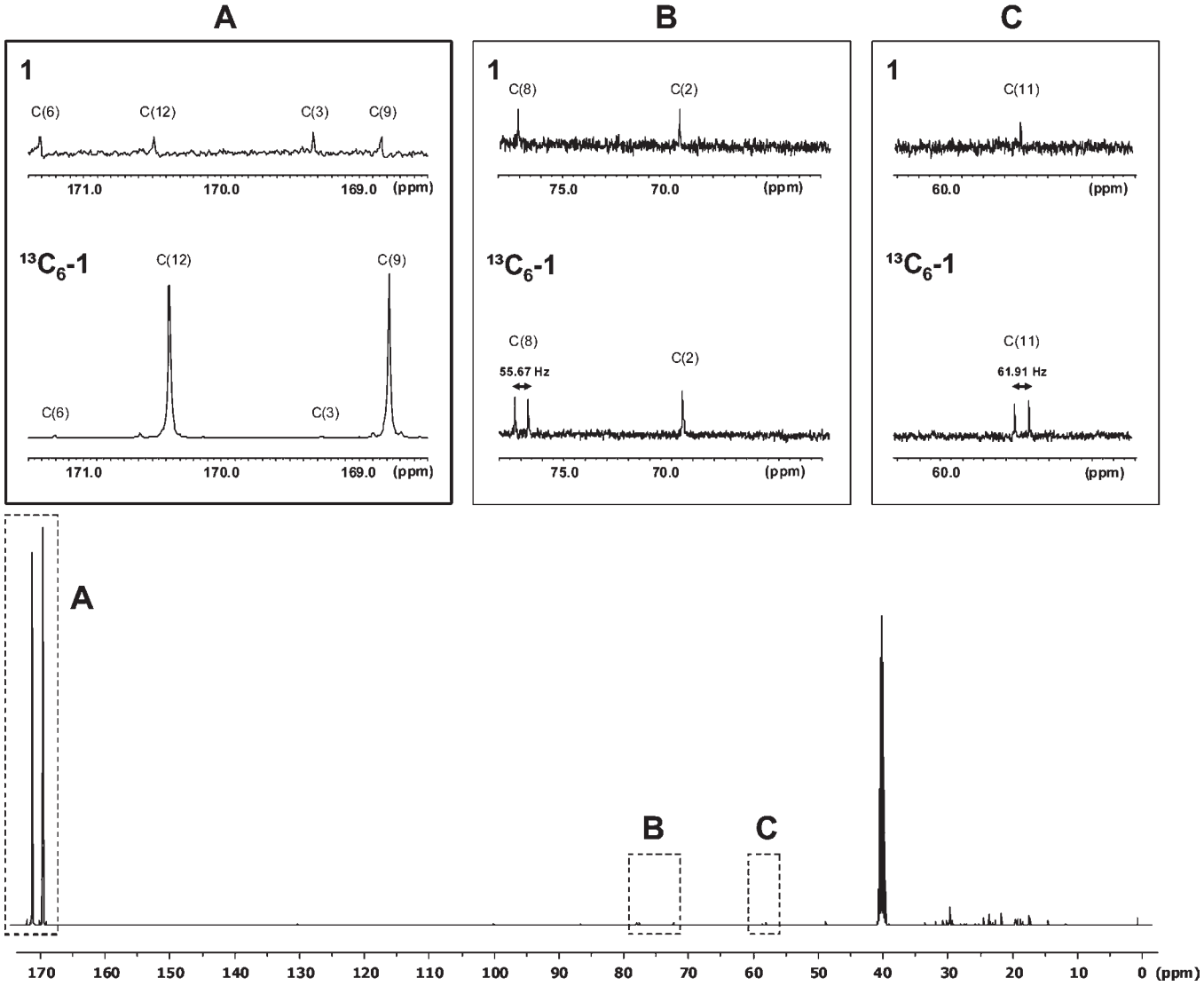

Figure 2. ^{13}C NMR spectrum (500 MHz, DMSO-d_6) of $^{13}C_6$-**1** and enlarged spectrum excerpts (**A**−**C**) representing selected carbon resonances of $^{13}C_6$-**1** and **1**, respectively.

Calibration solutions were stored in the dark at 4 °C, and the stability was checked via HPLC and LC-MS before use. Calibration curves were prepared by plotting peak area ratios of analyte to internal standard against concentration ratios of each analyte to the internal standard using linear regression. The equations obtained were $y = 0.485 \times$ (**1**/$^{13}C_6$-**1**, R = 0.997) and $y = 0.137 \times$ (**1**/**2**, R = 0.998), respectively.

Method Validation. Linearity was evaluated using mixtures of internal standards and analyte in five molar ratios from 0.1 to 10. To study the repeatability (intraday precision) of the method, a sample of boiled rice (10 g) and a sample of boiled rice supplemented with 10% sunflower oil were spiked with two different concentrations of cereulide (1 and 5 μg/g) using three replicates for each concentration in 1 day. Reproducibility (interday precision) was studied with a sample of rice (10 g) and a sample of rice supplemented with 10% sunflower oil, respectively, both spiked with cereulide (2 μg/g) and analyzed at four different days. The recovery determination was performed with a sample of rice (10 g) and a sample of rice supplemented with 10% sunflower oil, respectively, both spiked with cereulide (1, 2, and 5 μg/g) each in three replicates. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the concentrations for which signal-to-noise ratios were 3 and 10, respectively.

Investigation of Matrix Effects. To study possible matrix effects during the LC-MS/MS analysis of the ethanolic rice extracts, a sample of boiled rice (10 g) inoculated with 1×10^7 cfu of *B. cereus* was analyzed using the same LC-MS/MS parameters as listed above. A constant flow of 10 μL/min of a solution of valinomycin (**2**, 0.6 μmol/L) was introduced to the solvent flow via a three-way valve by means of a PHD 4400 Hpsi type syringe pump (Harvard Apparatus).

LC/Time-of-Flight Mass Spectrometry (LC/ESI-TOF-MS). Mass spectra of the target compounds were measured on a Bruker Micro-TOF-Q (Bruker Daltronics, Bremen, Germany) mass spectrometer with flow injection referenced on sodium formate (5 mmol). The compounds were dissolved in MeOH, and 10 μL of a saturated solution of Naformiat in MeOH was added to measure the exact mass of the sodium adducts. Data processing was performed by using Daltonics DataAnalysis software (version 3.4; Bruker).

NMR Spectroscopy. ^1H, gs-COSY, gs-HSQC, gs-HMBC, ^{13}C, and DEPT-135 NMR measurements were performed on an Avance 3 DRX 500 MHz spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts were referenced to tetramethylsilane or the solvent signal. Data processing was performed by using Topspin Version 1.3 (Bruker, Rheinstetten) and MestReNova version 5.2.3 software (Mestrelab Research, Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

To develop a highly selective and sensitive LC-MS/MS method for the accurate quantitation of cereulide, first, reference material of the cereulide (**1**; **Figure 1**) needed to be prepared in high purity. As previously reported procedures for cereulide preparation are rather laborious, time-consuming, and costly (*25*, *26*, *28*, *31*, *32*),

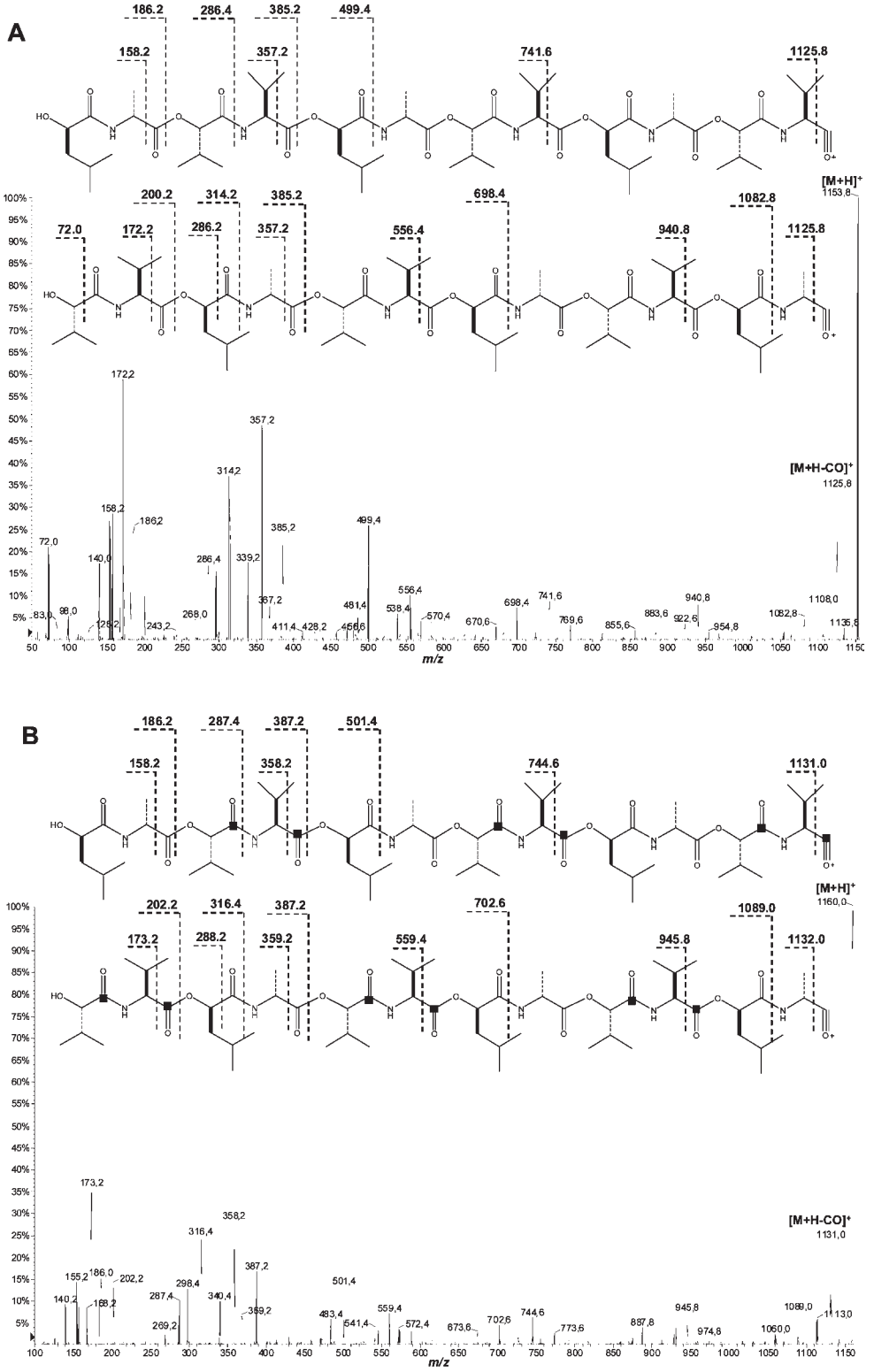

Figure 3. LC-MS/MS spectrum (ESI^+) of (**A**) **1**) and (**B**) $^{13}C_6$-**1** (■ indicates the presence of a ^{13}C atom).

this toxin should be prepared using a biotechnological approach, followed by rapid downstream purification. To achieve this, cereulide was prepared biosynthetically by means of a *B. cereus* F4810/72 culture in LB broth. After incubation for 24 h, the culture was centrifuged, and the cereulide produced was extracted with ethanol and purified by means of RP18 solid-phase extraction and RP-HPLC, thus affording the toxin as a white, amorphous powder in a yield of 4 mg/L culture and a purity of more than 98%.

To confirm the identity of cereulide, UV−vis, LC-MS/MS, and 1D/2D NMR experiments were performed. LC-MS (ESI^+) revealed an intense $[M + NH_4]^+$ ion with m/z 1170.9 as well as sodium, potassium, and proton adducts with m/z 1175.9, 1191.7, and 1153.8, respectively. LC-TOF-MS analysis of the sodium adduct m/z 1175.6669 confirmed the target compound to have the molecular formula $C_{57}H_{96}N_6O_{18}$. 1D/2D NMR data were in accordance with literature reported earlier (*12*, *32*).

Taking all of these spectroscopic data into consideration, the structure of cereulide (**1**; **Figure 1**) could be unequivocally identified as the 36-membered cyclic depsipeptide with the sequence *cyclo*-(D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$, thus being well in line with previously published data (*12*, *26*, *31*−*33*).

Biosynthetic Production of $^{13}C_6$-1. To develop a SIDA for cereulide quantitation in foods, a stable isotope-labeled internal standard was produced by inoculating a valine-depleted, $^{13}C_1$-L-valine enriched MOD broth without an additional carbon source with an overnight preculture for 48 h at 24 °C while shaking.

Thereafter, the culture was centrifuged, and the target compound was extracted with ethanol and purified by means of RP18 solid-phase extraction and RP-HPLC, thus affording the ^{13}C-labeled toxin as a white, amorphous powder in a purity of more than 98%. To confirm the chemical structure of the ^{13}C-labeled cereulide and to identify the position of the ^{13}C atoms in the molecule, LC-MS/MS as well as NMR spectroscopic experiments were performed. When compared to cereulide (**1**), the MS spectrum of ^{13}C-cereulide ($^{13}C_6$-**1**; **Figure 1**) measured in the ESI$^+$ mode showed an increase of the pseudo molecular ions *m*/*z* 1176.9 ([M + NH$_4$]$^+$), 1181.9 ([M + Na]$^+$), and 1197.9 ([M + K]$^+$) by six units, thus demonstrating $^{13}C_6$-cereulide as the predominant isotopologue. LC-MS analysis using an enhanced resolution scan confirmed the 6-fold ^{13}C-labeled cereulide with its pseudo molecular ion *m*/*z* 1159.6 ([M + H]$^+$) as the major isotopologue, whereas the less frequent ion *m*/*z* 1158.6 indicated an incorporation of five ^{13}C carbon atoms into the molecule. Considering the natural ^{13}C abundance in these pseudo molecular ions, the sample was found to consist of 94.2% 6-fold and 5.8% 5-fold ^{13}C-labeled cereulide.

To identify the positions of the incorporated ^{13}C atoms in the target molecule, the cereulide (**1**) as well as a sample of ^{13}C-labeled cereulide were analyzed by means of ^{1}H broadband-decoupled ^{13}C NMR spectroscopy (**Figure 2**). Comparison of the ^{13}C NMR spectrum (**Figure 2A**) obtained from cereulide (**1**), exhibiting four carbonyl carbon signals resonating as singlets with similar relative intensities, with that of the isotopologue $^{13}C_6$-**1** revealed two highly intense ^{13}C signals for the carbonyl atoms C(12) and C(9) of L-Val and L-*O*-Val, respectively. In comparison, the signals of the natural ^{13}C-abundant carbonyl atoms C(3) and C(6) were hardly detectable in the NMR spectrum of $^{13}C_6$-**1**, thus demonstrating the incorporation of the carboxy group of $^{13}C_1$-labeled L-valine into the molecule. Furthermore, the spectra excerpts **B** and **C** (**Figure 2**) between 57.0 and 77.5 ppm in the ^{13}C NMR spectrum of cereulide (**1**) showed three carbon signals resonating as singlets with comparable signal intensities, whereas the same spectrum segments obtained from $^{13}C_6$-**1** exhibited a signal duplet for carbon atoms C(11) and C(8), respectively, thus indicating a $^1J_{c,c}$-coupling to the ^{13}C-labeled carbonyl atoms C(12) and C(9).

LC-MS/MS analysis of cereulide (**1**) revealed that compound fragmentation starts by ring opening of the cyclodepsipeptide by cleavage of an ester bond, followed by the formation of straight chain fragments preferably from the C terminus as reported earlier (*31*, *33*) (**Figure 3A**). The most predominant fragments were found to be *m*/*z* 172.2, 314.2, 357.2, 499.4, and 1125.8. In comparison to **1**, fragmentation of the isotopologue $^{13}C_6$-**1** gives the corresponding fragment ions *m*/*z* 173.2, 316.4, 358.2, 501.4, and 1132.0 (**Figure 3B**). By comparison, the fragments of natural ^{13}C-abundant with $^{13}C_6$-labeled cereulide, it was possible to reconstitute the straight chain and assign the fragments originating from one or the other possible straight chain. For example, the fragment ion *m*/*z* 1131.0 formed from $^{13}C_6$-**1** was found to contain five ^{13}C-labeled carbonyls and, therefore, derived from the upper chain in **Figure 3B**. In contrast, the fragment ion *m*/*z* 1132.0 indicated the presence of six ^{13}C carbons and, therefore, was deduced to originate from the lower chain. The relative frequencies of the fragment ions *m*/*z* 1131.0 and 1132.0 were 24 and 12%, respectively. In addition, the isotopic shift of the fragment ion *m*/*z* 357.2 derived from **1** to *m*/*z* 358.2 or *m*/*z* 359.4 generated from $^{13}C_6$-**1** demonstrated the presence of one or two ^{13}C carbonyl atoms, respectively. Interestingly, the fragment ion *m*/*z* 358.2 originates from the upper chain, whereas the fragment ion *m*/*z* 359.4 is derived from the lower chain (**Figure 3B**). By comparing the intensities of both fragments, again, the fragment of the upper chain (*m*/*z* 358.2) is favored with a frequency of 28% when compared to *m*/*z* 359.4 with 8%. These observations suggest that upon fragmentation the ring opening of cereulide starts by cleavage of the ester bond between D-*O*-Leu and L-Val, which is 2-fold favored in contrast to the ester bond between L-*O*-Val and D-Ala.

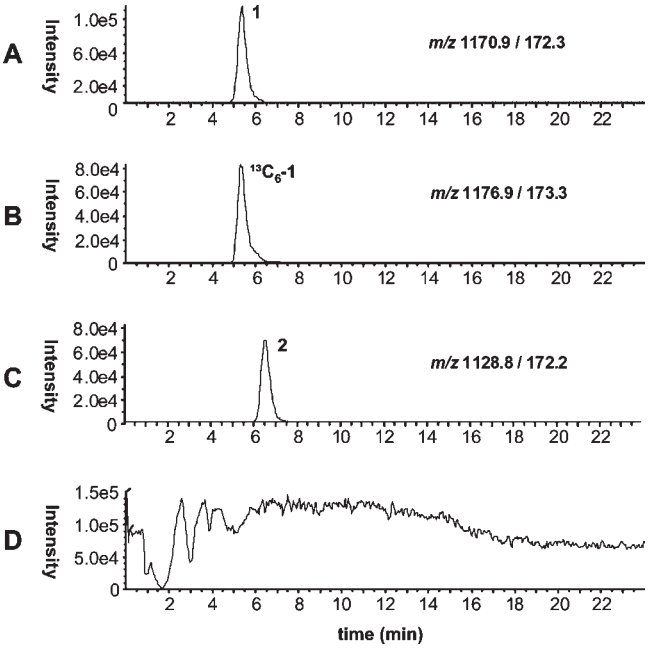

Figure 4. HPLC-MS/MS (ESI$^+$) analysis of a *B. cereus*-infected boiled rice sample and MRM traces of (**A**) **1** and the internal standards (**B**) $^{13}C_6$-**1** and (**C**) **2**. (**D**) HPLC-MS/MS (MRM) chromatogram recorded for a rice sample, while a continuous flow of **2** was introduced into the LC-MS/MS system by means of a syringe pump.

In conclusion, LC-MS/MS as well as NMR experiments confirmed the position of the ^{13}C-labeled carbons in the structure of $^{13}C_6$-cereulide and demonstrated that *B. cereus* incorporated the carbon skeleton of six $^{13}C_1$-L-valine molecules into the toxin. These findings do confirm previous studies on the incorporation of the ^{13}C-labeled L-amino acids in cereulide upon cultivation of *B. cereus* in synthetic media (*34*).

Development of a SIDA. To compare the suitability of $^{13}C_6$-cereulide as an internal standard with that of valinomycin (**2**), both compounds as well as the analyte cereulide (**1**) were individually infused into the mass spectrometer using a syringe pump. Optimum intensities of pseudomolecular ions and respective fragments were obtained by software-assisted ramping of the DP, the cell entrance potential, the CE, and the CXP, respectively (see the Supporting Information, Table S1). As the ion [M + NH$_4$]$^+$ of cereulide and valinomycin was the most predominant pseudomolecular ion, these were selected for the optimization. Tuning of the ammonium adducts and the pseudomolecular ions [M + H]$^+$ revealed similar fragment patterns and fragment intensities. To convert the measured ion intensities into the mass

Table 1. Validation Criteria for the Quantitation of **1** Using $^{13}C_6$-**1** or **2** as an Internal Standard

		food matrix used for experiments			
		boiled rice		boiled rice supplemented with 10% oil	
		recovery rate[a]			
internal standard	amount added (μg/g)	amount recovered (μg/g)	recovery rate (%)	amount recovered (μg/g)	recovery rate (%)
$^{13}C_6$-**1**	0	ND	ND	ND	ND
	1	1.00 ($\pm$0.09)	100.4 $\pm$ 8.7	1.05 ($\pm$0.03)	104.9 $\pm$ 2.8
	2	1.97 ($\pm$0.09)	98.7 $\pm$ 4.5	2.23 ($\pm$0.11)	111.5 $\pm$ 5.0
	5	5.71 ($\pm$0.47)	114.2 $\pm$ 8.3	5.78 ($\pm$0.47)	115.6 $\pm$ 8.2
			mean 104.4 $\pm$ 7.1		mean 110.7 $\pm$ 5.3
2	0	ND	ND	ND	ND
	1	0.81 ($\pm$0.32)	80.5 $\pm$ 4.0	0.92 ($\pm$0.07)	91.6 $\pm$ 8.2
	2	2.21 ($\pm$0.26)	110.5 $\pm$ 11.9	1.66 ($\pm$0.06)	83.1 $\pm$ 3.8
	5	4.08 ($\pm$0.39)	81.5 $\pm$ 9.6	3.26 ($\pm$0.23)	65.1 $\pm$ 7.0
			mean 90.7 $\pm$ 8.5		mean 79.9 $\pm$ 6.3
		repeatability (intraday precision)[b]			
$^{13}C_6$-**1**		<8%			<8%
2		<8%			<10%
		reproducibility (interday precision)[c]			
$^{13}C_6$-**1**		<8%			<8%
2		<8%			<10%
		LOD[d]			
$^{13}C_6$-**1**		3 ng/g			5 ng/g
		LOQ[d]			
$^{13}C_6$-**1**		30 ng/g			50 ng/g

[a] Concentrations are given as the mean of three independent cleanups with triple injection. [b] Repeatability (intraday precision as relative standard deviation in %) was determined by spiking blank rice and rice containing 10% sunflower oil at 1 and 5 μg/g, using three replicates for each concentration in 1 day. [c] Reproducibility (interday precision as relative standard deviation in %) was evaluated at 2 μg/g for both matrices, and spiked samples were analyzed at four consecutive days. [d] Limits of detection (LOD) and quantification (LOQ) were calculated analyzing blank samples and were determined as the lowest concentrations of analyte for which signal-to-noise ratios were 3 and 10.

ratios of the internal standards $^{13}C_6$-cereulide and valinomycin (**2**), respectively, and the analyte cereulide (**1**), a graph was calculated from calibration mixtures of known mass ratios and the corresponding peak area ratios in HPLC-MS/MS. To achieve this, fixed amounts of the internal standards were mixed with the analytes in ratios of 0.1−10 on a molar basis, and the quotients of area internal standard/area analyte obtained by HPLC-MS/MS were plotted against the quotient concentration analyte/concentration internal standard.

To develop and validate the LC-MS/MS method for quantitative analysis of cereulide, samples of cooked rice and cooked rice supplemented with 10% sunflower oil, respectively, were spiked with increasing amounts of cereulide (**1**) and then homogenized. For the quantitative analysis of cereulide, the internal standards $^{13}C_6$-**1** and **2**, respectively, were added, and each sample was equilibrated and then extracted with ethanol at 20 °C. After 15 h, nonsolubles were separated by centrifugation, the supernatant was membrane filtered, and after cleanup using a C18-SPE cartridge with ethanol as the eluent, the target compounds were analyzed by means of HPLC-MS/MS on a RP-18 phase. The mass chromatograms showing the mass transitions for the analyte **1** (**A**) as well as both of the internal standards $^{13}C_6$-**1** (**B**) and **2** (**C**) looked rather clean as no major peaks appeared within the respective traces of the compounds under investigation (**Figure 4**). As the analyte **1** and the isotope-labeled standard $^{13}C_6$-**1** coelute, a similar influence of matrix compounds on the ionization of both molecules is to be expected. However, as valinomycin elutes somewhat later in the chromatogram, the question arises as to whether coeluting matrix components do affect the ionization of the internal standard valinomycin **2**. To visualize such matrix effects (*35*, *36*), a constant flow of a solution of valinomycin (**2**) was introduced into the LC-MS/MS system via a syringe pump during the analysis of rice extract spiked with sun flower oil. As shown in **Figure 4D**, severe influence of eluting matrix compounds on the ionization of valinomycin could be observed depending on the retention time, thus indicating that the use of valinomycin as an internal standard might be not suitable to overcome matrix effects during LC-MS/MS analysis.

Performance of the SIDA. To check the performance of the developed LC-MS/MS method linearity, trueness, intraday and interday precision, sensitivity, and selectivity were investigated for both internal standards $^{13}C_6$-**1** and **2**, respectively. Calibration curves, obtained by linear regression analysis of the peak area versus concentration, showed a linear response with correlation coefficients of >0.99 for both internal standards.

To check the trueness of the analytical method, recovery experiments were performed as follows. Purified reference material of cereulide was added to samples of cooked rice (blank) and, in addition, to rice containing 10% sunflower oil to mimic an oil-rich food product at three concentrations (1, 2, and 5 μg/g), each prior to quantitative analysis, and the amounts determined after workup were compared to those found in the blank rice samples. The recovery rates, calculated on the basis of the content of cereulide added to the rice samples prior to workup, were found to be 104.4 $\pm$ 7.1% for the internal standard $^{13}C_6$-**1**, whereas a lower value of 90.7 $\pm$ 8.5% was found when valinomycin (**2**) was used as an internal standard (**Table 1**). Interestingly, even lower recovery rates of only 79.9 $\pm$ 6.3% were found when the rice sample supplemented with 10% sunflower oil was analyzed by using valinomycin as the internal standard, whereas the use of the ^{13}C-labeled cereulide still gave an excellent recovery rate of 110.7 $\pm$ 5.3% (**Table 1**). These data clearly demonstrate the advantage of using $^{13}C_6$-cereulide rather than valinomycin as the internal standard for quantitative analysis of cereulide and

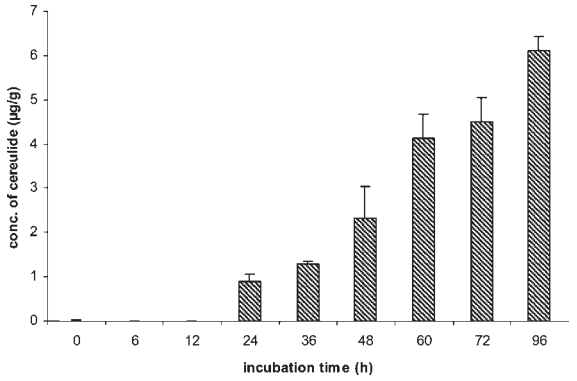

Figure 5. Influence of incubation time on the production of **1** in boiled rice inoculated with *B. cereus* strain F4810/72. Concentrations were calculated as the mean of three independent experiments with double injection.

revealed the developed SIDA as a reliable tool enabling a rapid and accurate quantitative determination of cereulide in low fat as well as high-fat food products.

To study the repeatability (intraday precision) of the analytical method, samples of cooked rice as well as samples of cooked rice supplemented with 10% sunflower oil were spiked with two different concentrations of cereulide (1 and 5 μg/g) and then analyzed using the SIDA developed above using three replicates for each concentration in 1 day. Reproducibility (interday precision) was studied with a sample of cooked rice and a sample of cooked rice supplemented with 10% sunflower oil, both spiked with cereulide (2 μg/g), and they were analyzed by the developed SIDA method at four consecutive days. For repeatability as well as reproducibility, relative standard deviations of less than 8% were found for cereulide quantitatively determined in both food matrices by using $^{13}C_6$-**1** as the internal standard. In comparison, analysis of cereulide by means of valinomycin (**2**) as an internal standard revealed a value of less than 10% for intraday as well as interday precision (**Table 1**).

In addition, LOD and LOQ were determined in cooked rice as well as rice supplemented with 10% sunflower oil as the concentration at which the peak height of the internal standard was at least 3 and 10 times higher than the underground noise (**Table 1**). To achieve this, a reference material of cereulide (**1**) was added to both food matrices in different concentrations prior to LC-MS/MS analysis. The LOD and LOQ were found to be 3 and 30 ng/g for cooked rice and 5 and 50 ng/g for the oil-containing rice sample, respectively.

Quantitative Analysis of Cereulide in Food Samples after Incubation with *B. cereus*. To investigate the cereulide production of the emetic *B. cereus* strain in rice, cooked rice samples were inoculated with an overnight culture of *B. cereus* and incubated at 24 °C for up to 96 h. At different time points, samples were taken, spiked with the internal standard $^{13}C_6$-cereulide, and equilibrated as described in the Materials and Methods. The amount of cereulide was determined using the SIDA developed. The production of cereulide in rice was found to be induced after 24 h (**Figure 5**). With increasing incubation time, the amount of the emetic toxin increased continuously up to 6.12 μg/g at 96 h. This underlines that even weak infestations of emetic *B. cereus* in food products, stored at ambient temperature over a period of 1 day, are able to trigger fatal food poisonings. Hitherto, the poisonous dose of cereulide in humans is unknown, but feeding experiments with *Suncus murinus* and rhesus monkeys (*11*, *32*, *37*) indicate an emesis-inducing dose of about 8−10 μg per kg body weight. This demonstrates that the consumption of even small amounts of rice stored at ambient temperature can induce emesis or other severe food borne intoxications in humans.

Table 2. Influence of *B. cereus* Inoculation on Cereulide Concentration in Selected Food Samples

	concentration of **1** (ng/g)[a]	
food samples	prior to incubation	after 24 h of incubation
cooked rice	ND	896 ± 150
milk pudding	ND	215 ± 20
liver sausage	ND	3 ± 0.9

[a]Concentrations are given as the mean of two independent cleanups with double injection.

In addition to rice, two additional food products obtained from a German retailer, milk pudding and liver sausage, were artificially contaminated with *B. cereus* and incubated for 24 h at 24 °C (cf. inoculation in rice). Prior to inoculation with emetic *B. cereus*, foods were checked for cereulide contamination. Cereulide was not detectable in any of the food samples analyzed (**Table 2**). After an incubation of 24 h, cooked rice exhibited the highest amount of cereulide (896 ng cereulide/g food). A somewhat lower amount of 219 ng/g was detectable in the inoculated milk pudding, whereas a comparatively low amount of 3 ng cereulide/g was found in liver sausage. These data clearly demonstrate the importance of the food matrix for cereulide production in processed foods.

In summary, a rapid biosynthetic procedure for the production and downstream purification of cereulide as well as $^{13}C_6$-labeled cereulide was developed and used for the quantification of cereulide in food matrices by means of a SIDA with HPLC-MS/MS detection. Method validation data confirmed the advantage of $^{13}C_6$-**1** as the internal standard over valinomycin used for calibration in previous investigations (*25*).

Supporting Information Available: Table of optimized mass spectrometric parameters for the LC-MS/MS analysis of **1**, $^{13}C_6$-**1**, and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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