

Mass spectrometric profiling of *Bacillus cereus* strains and quantitation of the emetic toxin cereulide by means of stable isotope dilution analysis and HEp-2 bioassay

Timo Stark · Sandra Marxen · Andrea Rüttschle ·
Genia Lücking · Siegfried Scherer ·
Monika Ehling-Schulz · Thomas Hofmann

Received: 24 July 2012 / Revised: 29 September 2012 / Accepted: 5 October 2012 / Published online: 19 October 2012
© Springer-Verlag Berlin Heidelberg 2012

Abstract A fast and robust high-throughput ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC–TOF MS) profiling method was developed and successfully applied to discriminate a total of 78 *Bacillus cereus* strains into no/low, medium and high producers of the emetic toxin cereulide. The data obtained by UPLC–TOF MS profiling were confirmed by absolute quantitation of cereulide in selected samples by means of high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) and stable isotope dilution assay (SIDA). Interestingly, the *B. cereus* strains isolated from four vomit samples and five faeces samples from patients showing symptoms of intoxication were among the group of medium or high producers. Comparison of HEp-2 bioassay data with those determined by means of mass spectrometry showed differences, most likely because the HEp-2

bioassay is based on the toxic action of cereulide towards mitochondria of eukaryotic cells rather than on a direct measurement of the toxin. In conclusion, the UPLC–electrospray ionization (ESI)–TOF MS and the HPLC–ESI–MS/MS–SIDA analyses seem to be promising tools for the robust high-throughput analysis of cereulide in *B. cereus* cultures, foods and other biological samples.

Keywords Cereulide · Stable isotope dilution analysis · $^{13}\text{C}_6$ cereulide · *Bacillus cereus* · HEp-2 assay

Introduction

Although cases of severe food-borne intoxications caused by *Bacillus* toxins are increasing, quantitative prevalence data are still lacking [1]. *Bacillus cereus* is well known to cause diarrhoea and emesis, two different types of gastrointestinal diseases arising from food contaminations. While the diarrhoeal syndrome is elicited by heat-labile enterotoxins, the emetic syndrome was found to be induced by the highly heat-resistant toxin cereulide, a depsipeptide composed of a cyclic trimer of the repeating tetrapeptide units [L-O-Val-L-Val-D-O-Leu-D-Ala] [2–6].

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 a quantitative analysis of cereulide in foods, cytotoxicity assays employing human larynx carcinoma cells such as, e.g. the HEp-2 bioassay, or rat liver cells [7, 8], as well as a boar sperm motility assay [9] have been reported in recent years. However, as the in vitro assays are based only on the toxic action of cereulide towards eukaryotic cells and do not allow the penultimate quantitation of the toxin, cytotoxic effects of other food ingredients might influence the results of the bioassays when applied to

Sandra Marxen and Andrea Rüttschle contributed equally to this work.

T. Stark · S. Marxen · T. Hofmann (✉)
Food Chemistry and Molecular Sensory Science,
Technische Universität München,
Lise-Meitner-Straße 34,
85354 Freising, Germany
e-mail: thomas.hofmann@tum.de

A. Rüttschle · G. Lücking · S. Scherer
Department of Microbiology, Central Institute for Food and
Nutrition Research, Technische Universität München,
85350 Freising, Germany

M. Ehling-Schulz
Institute of Functional Microbiology, Department of Pathobiology,
University of Veterinary Medicine Vienna,
1210 Vienna, Austria

S. Scherer
Microbial Ecology, Department of Biosciences, WZW,
Technische Universität München,
85350 Freising, Germany

authentic food samples. Therefore, $^{13}\text{C}_6$ -labelled cereulide was recently prepared for the first time by means of a bio-synthetic approach and used to develop a stable isotope dilution analysis (SIDA) enabling the robust and accurate quantitation of cereulide in food samples [10]. This SIDA approach was shown to effectively counterbalance losses during sample workup and to overcome matrix effects during subsequent high-performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) analysis [10].

Although *B. cereus* strains show a rather high variability in their potential for toxin production [11–13], reliable quantitative data are still not available due to the lack of a fast and sensitive high-throughput method enabling unequivocal identification of cereulide and quantitative comparison of the strains' toxin-producing activity. Ultra-performance liquid chromatography (UPLC) in combination with time-of-flight mass spectrometry (TOF MS) was recently demonstrated to fulfill this high-throughput demand and enabled the rapid detection and quantitation of bioactives [14] and urinary dietary biomarkers for coffee consumption [15].

Therefore, the objectives of the present investigation were to develop a fast and robust high-throughput profiling method using a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer and to analyse a series of 78 *B. cereus* strains including 5 environmental isolates, nine food-related clinical isolates, 8 non-food-related clinical isolates, 26 food outbreak isolates, and 31 isolates from food samples without any outbreak history (Table 1). After classifying these *B. cereus* strains into no or low, medium, high and excellent cereulide producers, the UPLC–quan–TOF MS data were validated by means of the recently developed HPLC–tandem mass spectrometry (MS/MS)–SIDA [10] and compared to data obtained via the HEp-2 bioassay [7].

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). The following compounds were obtained commercially: methanol and ethanol (Mallinckrodt Baker B.V., Deventer, Holland), pepton tryptone, yeast extract (Oxoid Hampshire, England), NaCl (Carl Roth, Karlsruhe, Germany) and D-(+)-glucose monohydrate (Fluka, Sigma Aldrich, Steinheim, Germany). Biosynthetic production and purification of cereulide and $^{13}\text{C}_6$ cereulide, followed by spectroscopic validation (LC–MS/MS, NMR) of compound identity were done as reported recently [10].

Bacterial strains and growth conditions

A previously designed Microsoft Access database [12] was used to generate a panel of 78 representative strains out of a total of

500 strains (Table 1), including the emetic reference strain F4810/72 (strain no. 1; [16]) isolated from vomit by the Public Health Laboratory Service (London, UK). Strains were selected on the basis of the following criteria: (1) source: clinical isolates from patients (associated with food poisoning/associated with other clinical infections), isolates from food (associated with food poisoning/not associated with food poisoning), environmental isolates, (2) diversity in geographical origins and (3) diverse toxin gene profiles and representing different subtypes. Strains were routinely grown on Luria Bertani (LB) agar plates or in LB broth (5 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) at 24 °C for 24 h. Strains were labeled with a designation number (no. 1–78) and stored at the Chair Microbial Ecology Group, Department of Biosciences, Technische Universität München. Culturing of the strains was done as detailed below.

Profiling of *B. cereus* strains by means of ultra-performance liquid chromatography–time-of-flight mass spectrometry

For strain profiling, 100 mL of LB broth supplemented with 0.2 % D-glucose was inoculated with an overnight pre-culture (10^3 cfu/mL) and then incubated for 24 h at 24 °C in baffled flasks (500 mL) whilst rotary shaking (120 rpm). At the end of incubation, the optical density of each cell culture was measured at 600 nm (OD_{600}) against LB medium as a reference, using a GeneQuant pro spectrophotometer (Biochrom, UK). After autoclaving (15 min, 121 °C) and centrifugation ($7,800\times g$, 20 °C, 12 min, Sigma 3–18 K) of the culture, the pellet was frozen in liquid nitrogen and stored at -20 °C until use. The pellet was extracted with ethanol (10 mL) by shaking at room temperature for 15 h, and the extract was centrifuged twice ($7,800\times g$) for each 12 min. The obtained supernatant was centrifuged ($18,600\times g$) for 5 min and membrane filtered (0.2 μm ; Phenomenex, Aschaffenburg, Germany) to remove remaining cells and cell debris. Aliquots of the filtered supernatants were analysed by means of UPLC–TOF MS on a Waters Synapt G2 HDMS mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC core system (Waters, Milford, MA, USA) consisting of a binary solvent manager, sample manager and column oven.

Aliquots (3 μL) of ethanolic cell pellet extracts were injected into the UPLC–TOF MS system equipped with a 2×150 mm, 1.8 μm , HSS T3 C18 column (Waters, Manchester, UK). Operated with a flow rate of 0.4 mL/min at 45 °C, the following gradient was used for chromatography: starting with a mixture (10/90, v/v) of water and methanol, the methanol content was increased to 100 % within 8 min and then kept constant for 1 min. Scan time for the MS^E method (centroid) was set to 0.1 s. Analyses were performed in the positive ESI and the resolution mode using the following ion source parameters: capillary voltage +2.0 kV, sampling cone 30 V, extraction cone 4.0 V, source

Table 1 Number, designation and origin of *Bacillus cereus* strains

No.	Strain designation ^a	Source	Country
1	F4810/72	Human vomit	UK
2	MHI 87	Infant food	Germany
3	MHI 1305	Rice dish	Germany
4	NC7401 ^b	Human vomit	Japan
5	F3080B/87	Chicken korma and rice	UK
6	F3351/87	Human faeces	UK
7	F3876/87	Patient with pyrexia and wheezing cough	UK
8	SDA GR177	Raw milk	Sweden
9	IH41385	Dialysis liquid (connected to illness)	Finland
10	RIVM BC 51	Rice dish	Netherlands
11	RIVM BC 62	Human vomit	Netherlands
12	RIVM BC 67	Human faeces	Netherlands
13	SDA JO303	Raw milk	Sweden
14	INRA I9	Flour	France
15	UHDAM B315	Cake	Finland
16	UHDAM 3/pkl	Filler material from hospital	Finland
17	RIVM BC 124	Human faeces	Netherlands
18	RIVM BC 379	Chicken	Netherlands
19	F2938/03	Human faeces	UK
20	WSBC10925	Wate bottle	Germany
21	WSBC 10927	Cauliflower	Germany
22	A529 ^c	Rice dish	Netherlands
23	UHDAM NS-58	Endophytes from spruce tree	Finland
24	F5881/94	Chinese takeaway fried rice	UK
25	F4552/75	Human vomit	UK
26	UHDAM ML127	Pasta	Finland
27	UHDAM B308	Risotto	Finland
28	UHDAM IIFI(3)	Infant food	Finland
29	UHDAM 143/pl	dust	Finland
30	WSBC 10879	Rice	Germany
31	WSBC 10914	Infant food (milk based)	Germany
32	WSBC 10935	Pasta	Switzerland
33	WSBC 10938	Infant food (milk based)	Germany
34	WSBC 10939	Cheese	Germany
35	WSBC 10942	Herb butter	Germany
36	WSBC 10941	Strawberry mush	Germany
37	WSBC 10954	Milk rice pudding	Germany
38	WSBC 10940	Liquid egg	Germany
39	05812 TP2 ^c	Rice with vegetables	Germany
40	07395/2 ^d	Pasta with egg, vegetables and chicken	Germany
41	10262/1 I ^d	Chinese noodles	Germany
42	WSBC 10950	UHT milk	Brasil
43	BC 164	UHT milk	Brasil
44	BC 165	Human faeces	Germany
45	WSBC 10951	Ear	Germany
46	BC 167	Catheter	Germany
47	BC 168	Swab from penis	Germany
48	BC 169	Swab from conjunctiva	Germany
49	BC 170	Mastitis	Germany

Table 1 (continued)

No.	Strain designation ^a	Source	Country
50	BC 171	Dried mushrooms (cep)	Germany
51	BC 172	Taramas	Germany
52	MC 67 ^e	Soil	Denmark
53	MC 118 ^f	Soil	Denmark
54	BC 173	Pasta	Germany
55	WSBC 10952	Mortadella	Germany
56	BC 175	Mozarella	Germany
57	BC 176	Meat balls	Germany
58	BC 177	Pasta	Germany
59	BC 178	Vegetable salad	Germany
60	BC 179	Pressack	Germany
61	BC 180	Beef with potatoes and leek	Germany
62	BC 181	Apple pie	Germany
63	BC 182	Fruit ice cream (lemon)	Germany
64	BC 183	Milk ice cream (vanilla)	Germany
65	BC 184	Sesame	Germany
66	BC 185	Cream cheese	Germany
67	BC 186	Cooked potatoes	Germany
68	BC 187	Watermelon	Germany
69	BC 188	Liver dumpling	Germany
70	BC 189	Pea soup	Germany
71	BC 190	Ice cream (goat)	Germany
72	BC 191	Pasta	Germany
73	BC 192	Cream cheese	Germany
74	BC 193	Turkey with tomato ragout	Germany
75	BC 194	Mashed potatoes	Germany
76	BC 196	Faeces	Germany
77	BC 197	Spices	Germany
78	BC 198	Fillet of beef	Germany

^a Coding of strains from: *INRA* Institut National de la Recherche Agronomique (France); *SDA* Svensk Mjolk, Swedish Dairy Association, Lund (Sweden); *WSBC* Weihenstephan *Bacillus cereus* collection, ZIEL Weihenstephan, TU München (Germany); F: Public Health Laboratory Service, London (UK); *RIVM* Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven (The Netherlands); *UHDAM and IH* University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki (Finland); *MIH* Ludwig Maximilians-Universität, München (Germany); *BC Bacillus cereus* collection, Institute for Functional Microbiology, University of Veterinary Medicine, Vienna (Austria); majority of strains originally derived from U. Messelhäusser, Bavarian Health and Food Safety Authority, Oberschleissheim, Germany

^b Originally from Nagoya City Public Health Institute, Japan [4]

^c M. Salkinoja-Salonen, University of Helsinki, Finland

^e Originally from University of Wageningen (The Netherlands)

^d Originally from D. Mäde, Landesamt für Verbraucherschutz Sachsen-Anhalt, Germany

^f Originally from L. Thorsen, Copenhagen University, Denmark [25]

temperature 150 °C, desolvation temperature 450 °C, cone gas 30 L/h and desolvation gas 850 L/h. Data processing was performed by using MassLynx 4.1 SCN 779 (Waters, Manchester, UK) and the elemental composition tool for determining the exact mass. All data were lock mass corrected on the pentapeptide leucine enkephaline (Tyr-Gly-Gly-Phe-Leu, m/z 556.2771, $[M+H]^+$) in a solution (2 ng/ μ L) of acetonitrile/0.1 % formic acid (1/1, v/v). Scan time for the

lock mass was set to 0.3 s, an interval of 15 s and three scans to average with a mass window of ± 0.3 Da. Calibration of the Synapt G2 in the range from m/z 50 to 1,300 was performed using a solution of sodium formate (5 mmol/L) in 2-propanol/water (9/1, v/v). The UPLC and Synapt G2 systems were operated with MassLynx™ software (Waters, Manchester), data processing and analysis were performed using MarkerLynx XS™ software (Waters, Manchester).

The raw data obtained from UPLC–TOF MS analysis were processed with MarkerLynx XS using ApexTrack peak integration to detect chromatographic peaks. Marker intensity threshold was set to 2000 cps, mass window was 0.02 Da, retention time window was 0.1 s and data were de-isotoped. Pareto scaling was used for principal components analysis (PCA).

Quantitative analysis of cereulide by means of liquid chromatography mass spectrometry and stable isotope dilution analysis

After incubating *B. cereus* strains in three independent cultures as described above, the cultures were autoclaved (15 min, 121 °C). An aliquot (1 mL) was then centrifuged (Eppendorf Centrifuge 5415 D) for 4 min at 13,000 rpm, and the pelletized cells were frozen in liquid nitrogen and kept at –20 °C until use. For ethanolic extraction, the pellets were thawed, suspended in ethanol (1 mL), spiked with a solution of $^{13}\text{C}_6$ cereulide (1 μg in 3.2 μL ethanol) as internal standard and then equilibrated for 15 h whilst rotary shaking. After centrifugation (13,000 rpm, 4 min, Eppendorf Centrifuge 5415), the supernatant was membrane filtered (0.2 μm ; Phenomenex, Aschaffenburg, Germany), and aliquots (5 μL) were analysed in triplicate by means of HPLC–MS/MS.

HPLC–MS/MS analysis was performed using an Agilent 1200 HPLC system equipped with a Synergi Fusion-RP, 2 \times 150 mm, 4 μm , column (Phenomenex, Germany) connected to the API 4000 QTrap LC-MS/MS (Applied Biosystems, Darmstadt, Germany) running in the positive electrospray ionization (ESI⁺) mode. 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.

For mass spectrometry, zero grade air served as nebulizer gas (45 psi) and turbo gas (425 °C) for solvent drying (55 psi), nitrogen was used as curtain (20 psi) and collision gas (8.7×10^{-7} psi), respectively. Both quadrupoles were set at unit resolution. By means of the multiple reaction monitoring (MRM) mode, the pseudomolecular ion $[\text{M}+\text{NH}_4]^+$ of cereulide (**1**, m/z 1,170.9 \rightarrow 172.3) and $^{13}\text{C}_6$ -cereulide ($^{13}\text{C}_6$ -**1**; m/z 1,176.9 \rightarrow 173.3), respectively, were analysed using the mass transitions (given in brackets) monitored for a duration of 55 ms and using the declustering potential (201 V for **1**, 176 V for $^{13}\text{C}_6$ -**1**), the entrance potential (10 V for **1** and $^{13}\text{C}_6$ -**1**), collision energy (123 for **1**, 119 V for $^{13}\text{C}_6$ -**1**) and the cell exit potential (6 V for **1**, 10 V for $^{13}\text{C}_6$ -**1**). ESI⁺ mass and product ion spectra were acquired with direct flow infusion. For ESI⁺, the ion spray voltage was set at +5,500 V in the positive mode. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{NH}_4]^+$ molecular

ions into specific product ions after collision with nitrogen (8.7×10^{-7} psi).

For quantitation, ethanolic solutions of the analyte cereulide and the internal standard $^{13}\text{C}_6$ cereulide were mixed in seven molar ratios from 0.02 to 50 (0.1, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 $\mu\text{g}/\text{mL}$) and analysed in triplicates by means of HPLC–MS/MS using the MRM. 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 equation obtained was $y=0.429x+0.148$ ($1/^{13}\text{C}_6$ -**1**, $R=0.9999$).

HEp-2 cell culture assay

The cytotoxicity of the strains was measured with a cell culture assay employing HEp-2 cells [7]. Therefore, cell samples of the strains cultivated in LB broth supplemented with 0.2 % D-glucose and cultivated in LB broth containing 1 % NaCl instead of 0.5 % were autoclaved (15 min, 121 °C) and kept chilled (6 °C) until used. Samples were serially diluted in 96-well microtiter plates with Eagle minimum essential medium with Earle salts (Biochrom AG, Germany) supplemented with sodium pyruvate (1 %), foetal calf serum (1 %), penicillin–streptomycin (0.4 %, Roche Applied Science, Germany) and ethanol (2 %). Purified cereulide (50 ng/mL in ethanol) served as a positive control and standard. To each well, 150 μL of 6×10^4 HEp-2 cells was added, followed by an incubation of the plate for 48 h at 37 °C under 5 % CO_2 . For analysis, an aliquot of medium (100 μL) was removed from each well, and viability of cells was tested by the addition of 10 μL tetrazolium salt WST-1 (Roche, ready-to-use). After incubation (25 min, 37 °C, 5 % CO_2), the absorbance was measured at 450/620 nm by means of a microtiter plate reader (Tecan, Switzerland). For determination of the toxin concentrations, the resulting dose–response curve and the known cereulide standard concentration were used [17]. Ethanolic extracts obtained from the pellets of the *B. cereus* biomass were analysed in the same way as the aliquots of autoclaved cells.

Quantitative nuclear magnetic resonance spectroscopy

Quantitative ^1H NMR (qNMR) spectroscopy of solutions of cereulide and its isotopologue was performed on an Avance III 500 MHz spectrometer with a CTCI probe (Bruker, Rheinstetten, Germany) following the protocol reported recently [18].

Results and discussion

All *Bacillus cereus* strains carrying the *ces* genes, which encode the enzymatic complex responsible for non-ribosomal

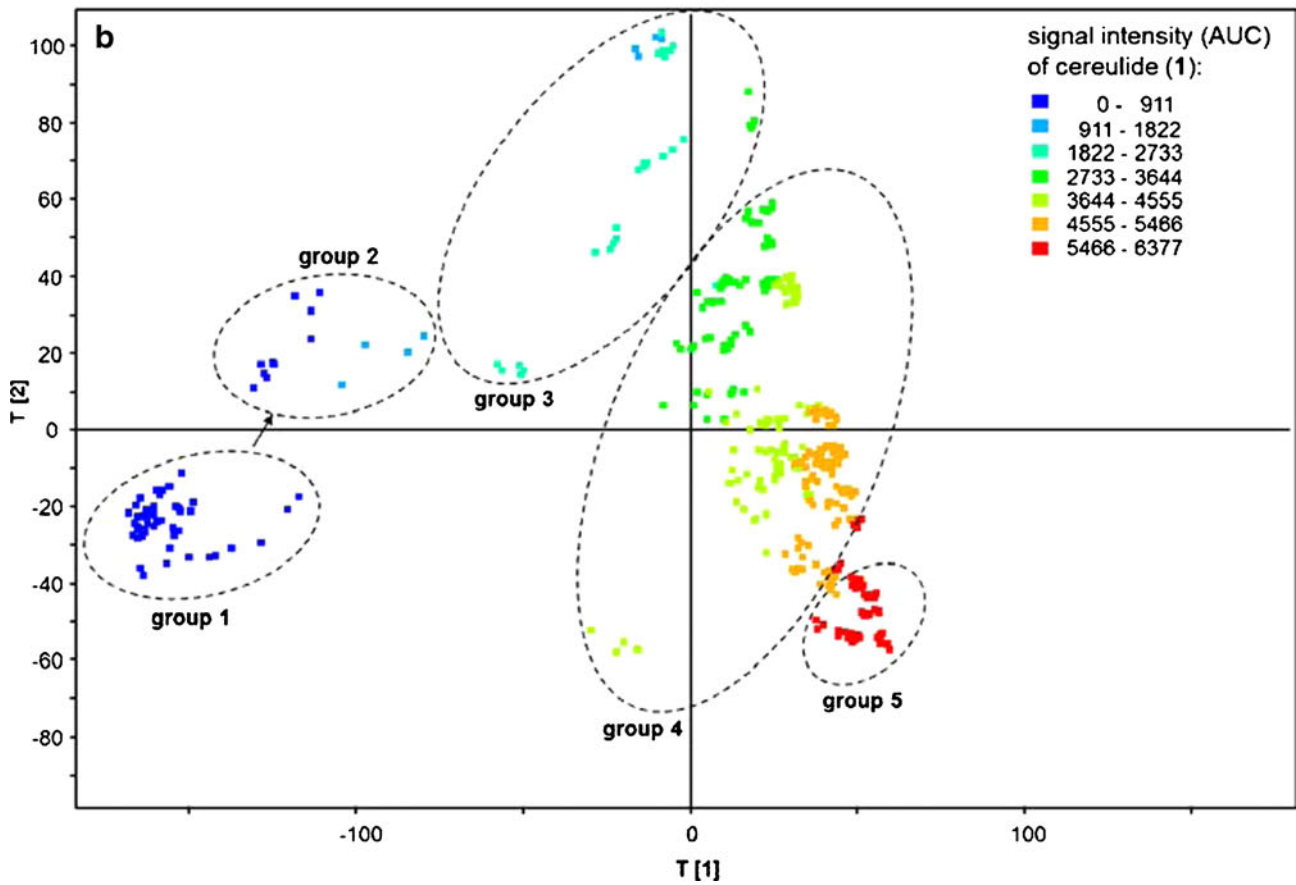
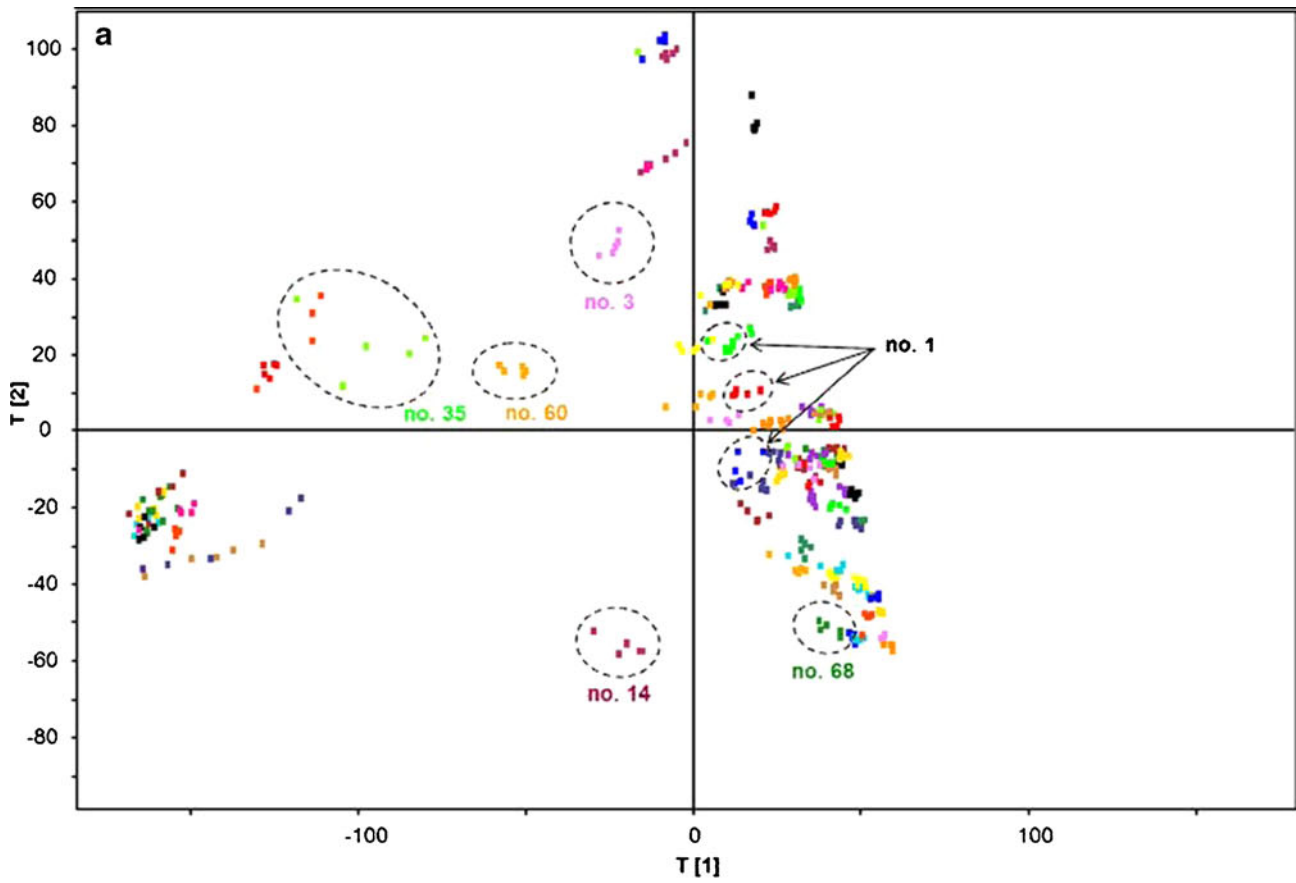


Fig. 1 Score plot (comp[1] vs. comp[2]) of UPLC–TOF MS full scan analysis (50–1,300 Da, ESI⁺, resolution mode) of **a** 78 *B. cereus* strains (five replicates per sample) and **b** 78 *B. cereus* strains with color-coded signal intensity of the accurate mass of pseudomolecular ion of cereulide (m/z 1,175.6608, [M+Na]⁺), r.t. 5.49 min, from group 1 showing the lowest up to group 5 exhibiting the highest signal intensity

production of the emetic toxin cereulide [19, 20], show toxin production under laboratory conditions. However, previous studies, including only a small number of emetic *B. cereus* strains, point towards an extreme intraspecies variation in toxin production capacities [12, 21]. Therefore, this work aims to gain a profound knowledge and a more systematic overview on variations of cereulide production capability within the group of emetic *B. cereus* by employing a UPLC–TOF MS-based approach. Such data are of utmost importance and form the starting point for deciphering the molecular basis of the highly diverging toxigenic potential of strains.

A previously described Microsoft Access database [12] was used to generate a panel of 78 strains representative for different sources (clinic, food intoxications, food, environment) and derived from diverse geographical origins (Table 1). Ethanolic extracts prepared from the *B. cereus* strains grown under defined conditions were analysed by means of UPLC–TOF MS (ESI⁺), and the MS data were processed to build a matrix comprising data on accurate mass, retention time and frequency of

each metabolite, followed by data normalisation and PCA. As shown in Fig. 1a, the score plot demonstrated large differences in the metabolite profile of the ethanolic extracts prepared from the various *B. cereus* strains. Analysis of each sample in five replicates showed a rather good clustering of each replicate in close proximity as highlighted for strains no. 3, 14, 35, 60 and 68 (Fig. 1a), thus confirming the reproducibility of the UPLC–TOF MS profiling method. Moreover, the biological deviation between three independent biological replicates of the reference strain F4810/72 (strain no. 1) was found to be rather low, e.g. the three biological replicates, with five analytical repetitions each, clustered in close proximity (Fig. 1a).

Chromatography of an authentic cereulide sample as well as co-chromatography with the pooled sample of all *B. cereus* strains confirmed the retention time of 5.49 min and the accurate mass of m/z 1,175.6678 for the pseudomolecular ion ([M+Na]⁺) of cereulide. Color coding of the signal intensity recorded for this pseudomolecular ion indicated a clear clustering of the *B. cereus* strains with regard to their activity in cereulide production (Fig. 1b), e.g. from group 1 with the lowest up to group 5 with by far the highest signal intensity of the cereulide pseudomolecular ion.

To visualize similarities and differences between the samples, S-plots of data pairs of accurate mass and retention time of each metabolite were calculated based on orthogonal partial least squares discriminant analysis from all the

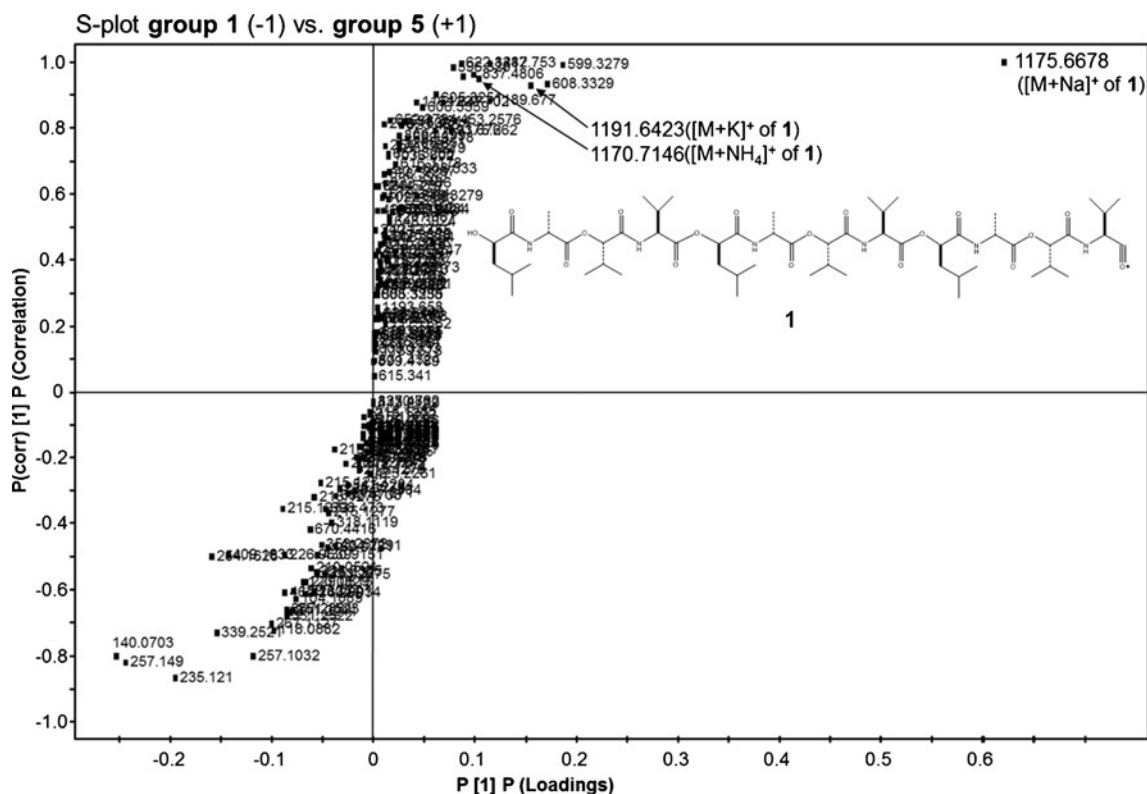


Fig. 2 S-Plot of *B. cereus* strain group 1 vs. group 5 and [M+Na]⁺ ion of cereulide

samples of group 1 and 5. As the y -axis of the S-plot denotes confidence of a metabolite's contribution to the group difference and the x -axis denotes the contribution of a particular metabolite to the group difference, both S-plots indicate the ion m/z 1,175.6678 showing by far the highest difference in abundance in group 1 and 5 (Fig. 2). In addition, the ions m/z 1,191.6423 and 1,170.7146 were found to clearly contribute to the differences between group 1 and 5. Almost identical S-plots were obtained when comparing single strains from group 1 with single strains from group 5 (data not shown).

On the basis of the accurate mass data, the sum formula of the ions showing m/z 1,191.6423, 1,170.7146 and 1175.6678 were calculated to be $C_{57}H_{96}N_6O_{18}K$ (+0.5 ppm), $C_{57}H_{100}N_7O_{18}$ (+2.1 ppm) and $C_{57}H_{96}N_6O_{18}Na$ (-0.1 ppm), fitting well to cereulide's pseudomolecular ions $[M+K]^+$, $[M+NH_4]^+$ and $[M+Na]^+$, respectively. Comparison of the base peak ion chromatograms recorded for strains no. 10, 37,

55, 69 and 76 revealed that cereulide was not detectable in the chromatograms recorded for strains 37 and 69. Confirming the data of the color-coded PCA (Fig. 1b), low levels of cereulide were found in the incubation of strain no. 10, and comparatively high levels were produced from strains no. 55 and 76, respectively (Fig. 3).

In order to consider possible variations of the cereulide content due to different growth behaviour of *B. cereus* strains, the optical density of the cultures was determined after 24 h of incubation. The optical density (OD_{600}) of the various *B. cereus* cultures, each incubated under identical conditions, was in a rather narrow range between 14 and 19 (16.5 ± 1.8 RSD). The frequency of the $[M+Na]^+$ cereulide ion was then normalized for the optical density measured for each single strain (Fig. 4). Again, the reproducibility of this quantitative profiling was checked by three independent incubations of the reference strain no. 1 each injected in five replicates. The ratio of the area under the curve (AUC)

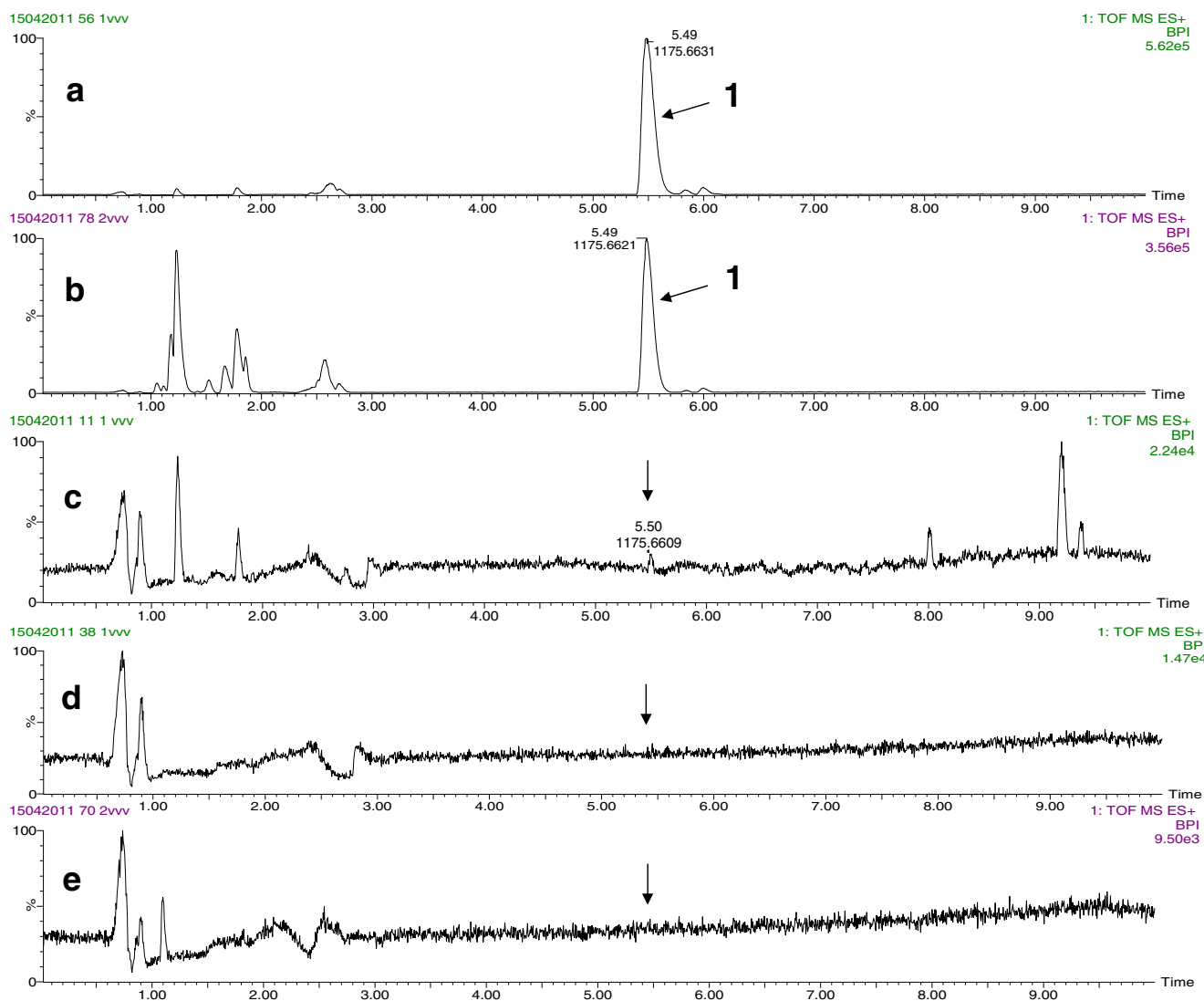


Fig. 3 Base peak ion mass chromatograms of strains 55 (a), 77 (b), 10 (c), 37 (d) and 69 (e), respectively

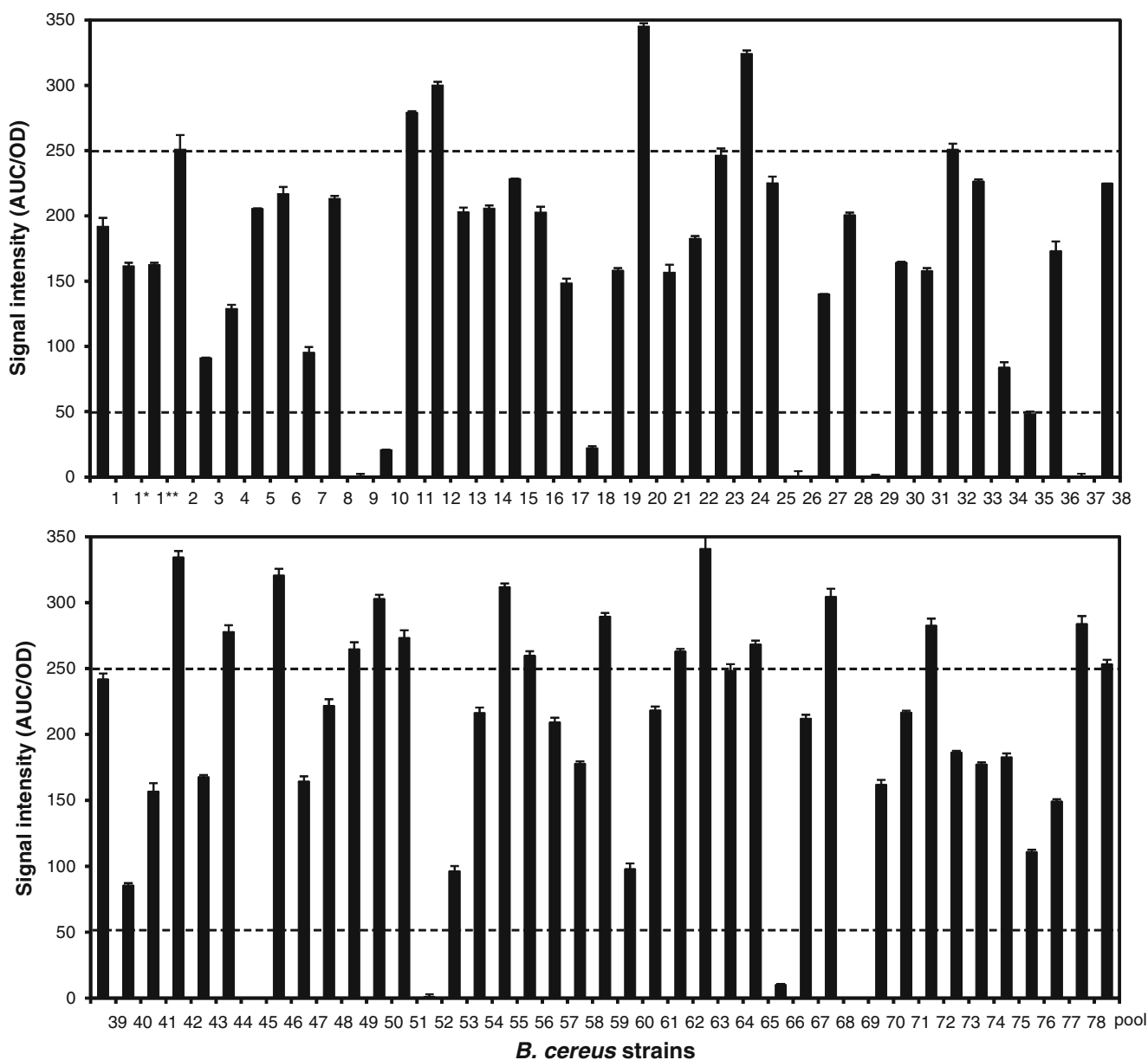


Fig. 4 Ratio of MS signal intensity (AUC) and optical density of the $[M+Na]^+$ ion of cereulide measured from 78 *B. cereus* strains (five replicates each). The sample 1, 1* and 1** are three biological replicates of strain no. 1, each analysed five times

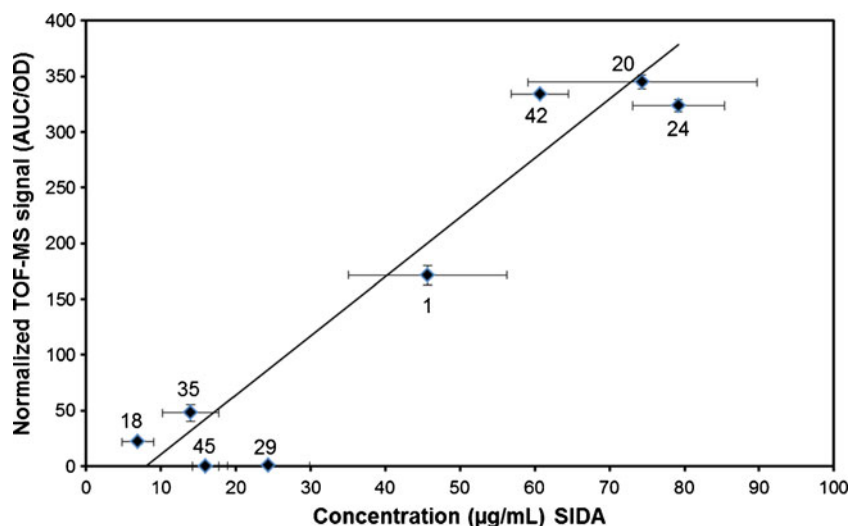
and the optical density (OD_{600}) calculated for cereulide in the cultures of the strain no. 1 was 171.7 ± 15.1 showing good reproducibility with a low relative standard deviation of 8.8 %.

Cereulide was either not detectable or present in trace levels in extracts prepared from strains no. 9, 26, 29, 37, 45, 52 and 69, respectively (Fig. 4), which is in accordance to molecular data. For instance, the strains 69 and 37 were negatively tested by *ces*-specific PCR for the nonribosomal peptide synthetase encoding the biosynthetic machinery for cereulide production [19, 20, 22] and strain no. 9, which shows a point mutation in the structural *ces* genes, is known for its very low cereulide production [12, 23].

Most of the strains investigated showed medium activity in cereulide production with AUC/OD ratios between 50 and 250, following by the group of high producers (AUC/OD >250), amongst which strains no. 20, 24, 42, 46, 50, 55, 63 and 68 contained the highest levels of cereulide (Fig. 4). It is interesting to notice that all the *B. cereus* strains isolated from vomit (no. 1, 4, 11 and 25) or faeces (no. 6, 12, 17, 44 and 76) from patients showing symptoms of intoxication were among the group of medium or high producers.

In order to validate the suitability of the UPLC–TOF MS method for microbial strain profiling, the absolute concentration of cereulide was quantified from three independent biological replicates of the no/low producers no. 18, 29, 35

Fig. 5 Correlation of the TOF MS signal intensity normalized for optical density (AUC/OD) of the $[M+Na]^+$ ion of cereulide with the absolute cereulide concentration determined by means of HPLC–MS/MS–SIDA



and 45, the medium producer reference strain F4810/72 (strain no. 1) and the high producers no. 20, 24 and 42 by means of stable isotope dilution analysis (SIDA) and HPLC–MS/MS. To achieve this, cultures were autoclaved, centrifuged, the pellets were suspended in ethanol, spiked with defined amounts of the internal standard $^{13}C_6$ cereulide, and after equilibration and centrifugation, supernatants were membrane filtered and analysed by means of HPLC–MS/MS without further sample clean-up. At least two mass transitions of each compound were selected for the final acquisition method, typically the most intensive mass transition was used for quantitation, and a second transition was selected for unequivocal identification of cereulide [10]. Following the precise concentration analysis of standard calibration solutions by means of quantitative NMR spectroscopy [18], a calibration curve was recorded, and peak area ratios of analyte and the corresponding internal standard were plotted against concentration ratios from 0.02 to 50 followed by linear regression ($R > 0.9999$).

Comparison of the data found by means of UPLC–TOF MS and HPLC–MS/MS–SIDA demonstrated a good correlation for the detected levels of cereulide with some small deviations (Fig. 5). HPLC–MS/MS–SIDA revealed only low cereulide concentrations ($< 30 \mu\text{g/mL}$) in ethanolic extracts of strains no. 18, 29, 35 and 45, which had been classified as no/low producers by means of UPLC–TOF MS. As expected, the emetic reference strain F4801/72 (strain no. 1) contained cereulide in a medium concentration of about $45 \mu\text{g/mL}$, and the high producer strains no. 20, 24 and 42 generated cereulide in concentrations of above $45 \mu\text{g/mL}$.

In order to gain a more detailed insight into the accuracy and reliability of cytotoxicity assays for quantitative analysis of cereulide, the concentration of the toxin was determined by means of the HEp-2 bioassay [7] in ethanolic extracts or autoclaved cell culture samples obtained from liquid cultures of the no/low producers no. 18, 29, 35 and 45, the medium

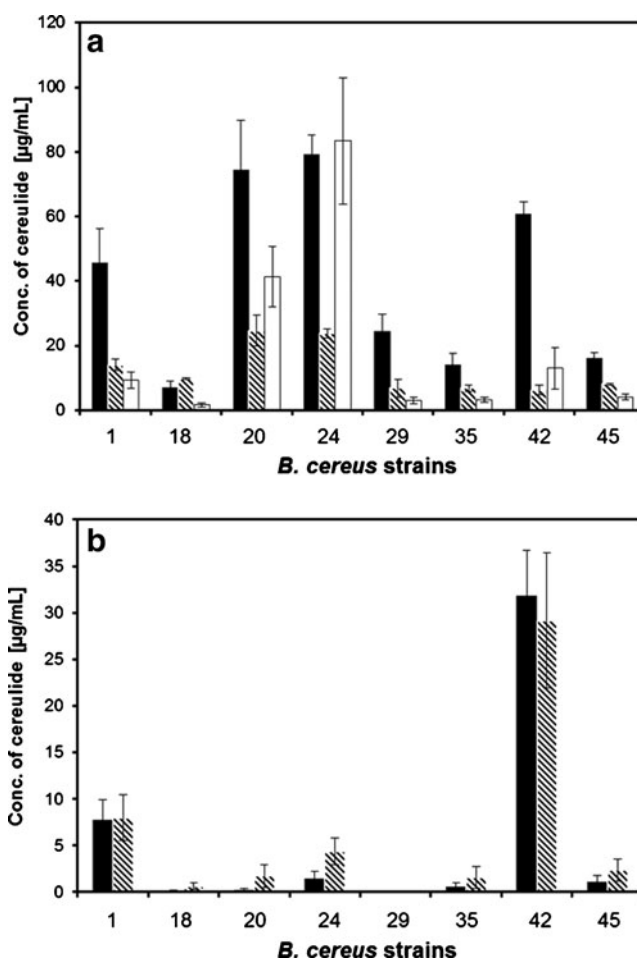


Fig. 6 Comparison of cereulide quantification using HPLC–LC–MS/MS–SIDA and a cell culture bioassay. **a** *B. cereus* strains cultivated 24 h at 24°C in LB broth with the addition of 0.2 % glucose and 0.5 % NaCl. Black bars represent cereulide concentrations in the ethanolic extracts quantified by LC–MS/MS. Striped bars show cereulide concentrations in the complete culture samples quantified by HEp-2 assays. White bars show cereulide concentrations in ethanolic extracts quantified by HEp-2 assays. **b** *B. cereus* strains cultivated for 24 h at 24°C in LB broth. Values represent means and error (SD) of three independent experiments

producer reference strain no. 1 and the high producers no. 20, 24 and 42 grown in LB broth containing 0.2 % D-glucose and 0.5 % NaCl (Fig. 6a). Comparison of the bioassay data to those found by means of HPLC–MS/MS–SIDA showed that, for all the samples analysed, cereulide concentrations obtained by SIDA were clearly above those found by means of the HEp-2 bioassay. This is most likely because the HEp-2 bioassay is based on the toxic action of cereulide to mitochondria of eukaryotic cells rather than on a direct measurement of the toxin. In conclusion, the bioassay seems to underestimate the cereulide concentrations in a sample when compared to the HPLC–MS/MS–SIDA method. With the exception of strain no. 24, the bioassay data found for the ethanolic extracts were rather similar to those obtained from the autoclaved cell samples.

In another set of experiments, the *B. cereus* strains were cultivated for 24 h at 24 °C in LB broth with 1 % NaCl but lacking glucose, and then, cereulide concentrations were determined in ethanolic extracts by means of HPLC–MS/MS–SIDA and in the culture samples by means of the HEp-2 assay (Fig. 6b). For all samples, the concentrations of cereulide generated under glucose-free conditions in the presence of 1 % NaCl were lower when compared to the medium containing glucose and 0.5 % NaCl, thus demonstrating the influence of the medium composition on cereulide productivity. Interestingly, the differences between the data obtained by LC–MS/MS and the HEp-2 bioassay were rather small under these glucose-free conditions. Future studies need to clarify as to whether the HEp-2 bioassay is affected by the medium composition and/or the metabolic state of the cell line.

Conclusions

Compared to a previous attempt using ion trap mass spectrometry with a rather limited linear calibration range [24], a fast and robust high-throughput UPLC–TOF MS profiling method was developed and successfully applied to discriminate *B. cereus* strains into no/low, medium and high cereulide producers. It is interesting to notice that all the *B. cereus* strains isolated from vomit (no. 1, 4, 11 and 25) or faeces (no. 6, 12, 17, 44 and 76) from patients showing symptoms for intoxication were among the group of medium or high producers. The data obtained by UPLC–TOF MS profiling were in alignment with absolute quantitative data obtained by means of a stable isotope dilution assay using HPLC–MS/MS. Comparison of HEp-2 bioassay data with those determined by means of mass spectrometry showed significant differences, most likely because the HEp-2 bioassay is based on the toxic action of cereulide to mitochondria of eukaryotic cells rather than on a direct measurement of the toxin. In conclusion, the UPLC–TOF MS and the HPLC–MS/MS–SIDA analyses seem to be promising tools for the robust

high-throughput analysis of cereulide in *B. cereus* from diverse origin, such as clinical setting, foods and environment.

Acknowledgments This research project (project AiF 16845 N) was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn). We are grateful to Sofie Lösch for technical assistance.

References

- Anonymous (2009). The community summary report on food-borne outbreaks in the European Union in 2007, EFSA J 271.
- Stenfors Arnesen LP, Fagerlund A, Granum PE (2008) FEMS Microbiol Rev 32:579–606
- Ehling-Schulz M, Fricker M, Scherer S (2004) Mol Nutr Food Res 48:479–487
- Agata N, Mori M, Ohta M, Suwan S, Ohtani I, Isobe M (1994) FEMS Microbiol Lett 121:31–34
- Isobe M, Suwan S, Agata N, Ohta M (1995) Bioorg Med Chem Lett 5:2855–2858
- Suwan Sathorn MI, Ohtani I, Agata N, Mori M, Ohta M (1995) J Chem Soc Perkin Trans 1:765–775
- Finlay WJJ, Logan NA, Sutherland AD (1999) Appl Environ Microbiol 65:1811–1812
- Kawamura-Sato K, Hiramata Y, Agata N, Ito H, Torii K, Takeno A, Hasegawa T, Shimomura Y, Ohta M (2005) Microbiol Immunol 49:25–30
- Hoonstra D, Andersson MA, Mikkola R, Salkinoja-Salonen MS (2003) Toxicol in Vitro 17:745–751
- Bauer T, Stark T, Hofmann T, Ehling-Schulz M (2010) J Agric Food Chem 58:1420–1428
- Apetroaie C, Andersson MA, Sproer C, Tsitko I, Shaheen R, Jaaskelainen EL, Wijnands LM, Heikkilä R, Salkinoja-Salonen MS (2005) Arch Microbiol 184:141–151
- Carlin F, Fricker M, Pielaat A, Heisterkamp S, Shaheen R, Salonen MS, Svensson B, Nguyen-the C, Ehling-Schulz M (2006) Int J Food Microbiol 109:132–138
- Rajkovic A, Uyttendaele M, Ombregt SA, Jaaskelainen E, Salkinoja-Salonen M, Debevere J (2006) J Food Prot 69:847–852
- Stark T, Wollmann N, Lösch S, Hofmann T (2011) Anal Chem 83:3398–3405
- Lang R, Wahl A, Stark T, Hofmann T (2011) Mol Nutr Food Res 55:1613–1623
- Ehling-Schulz M, Svensson B, Guinebretiere MH et al (2005) Microbiology 151:183–197
- Dietrich R, Fella C, Strich S, Martlbauer E (1999) Appl Environ Microbiol 65:4470–4474
- Korn M, Frank O, Hofmann T, Rychlik M (2011) Anal Biochem 419:88–94
- Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Martlbauer E, Scherer S (2005) Appl Environ Microbiol 71:105–113
- Ehling-Schulz M, Fricker M, Grallert H, Rieck P, Wagner M, Scherer S (2006) BMC Microbiol 6:20
- Andersson MA, Jääskeläinen EL, Shaheen R, Pirhonen T, Wijnands LM, Salkinoja-Salonen MS (2004) Int J Food Microbiol 94:175–183
- Magarvey NA, Ehling-Schulz M, Walsh CT (2006) J Am Chem Soc 128:10698–10699
- Dommel M, Lücking G, Scherer S, Ehling-Schulz M (2011) Food Microbiol 28:284–290
- Hägglöf MM, Apetroaie C, Andersson MA, Salkinoja-Salonen MS (2002) Appl Environ Microbiol 68:2479–2483ss
- Thorsen L, Budde BB, Henrichsen L, Martinussen T, Jakobsen M (2009) Int J Food Microbiol 134:133–139