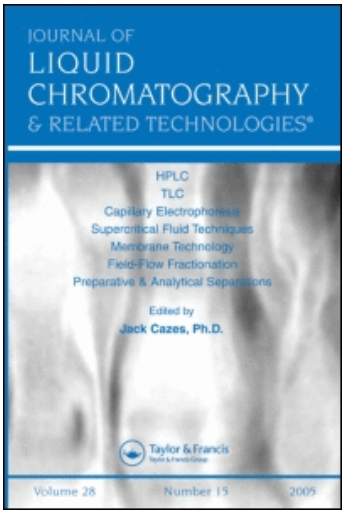

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Quantification of *Bacillus cereus* Emetic Toxin (Cereulide) in Figs Using LC/MS

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ABSTRACT

An LC/MS method is described for the quantification of cereulide, the emetic toxin of *Bacillus cereus* in figs. The method can also be used for the determination of cereulide in rice. The sample was extracted with a mixture of acetone–tetrahydrofurane, methanol, and water, after which the organic layer was separated from water with chloroform and evaporated to dryness. The dry residue was diluted in chloroform–hexane and purified using a silica solid phase extraction column. The detection limit was 1 ng/g.

Key Words: *Bacillus cereus*; Emetic toxin; Figs; Cereulide; LC/MS.

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INTRODUCTION

Bacillus cereus causes two different types of foodborne illness; the diarrheal type and the emetic type.[1–3] The diarrheal type is caused by enterotoxin(s), produced during vegetative growth of *B. cereus* in the small intestine, and the emetic toxin is produced by bacterial cells growing in the food before consumption. The documented biological activities of the emetic toxin, cereulide (CRL), are described,[4] and include emesis in primates,[5,6] swelling of mitochondria in HEp-2 cells,[7] and in hepatocytes of a fatally food-poisoned patient.[8] The structure of the emetic toxin, has been described.[4,9] CRL is a cyclic dodecadepsipeptide, (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$, and is synthesized non-ribosomally by a peptide synthetase.[9] CRL is resistent to heat, proteolysis and is pH stable. CRL is insoluble in water because it is highly hydrophobic. After the toxin is produced, no treatment will destroy this stable molecule, including the stomach acid and the proteolytic enzymes of the intestinal tract.[10] Heat-treating of food will kill vegetative bacteria, but cereulide will not be destroyed. In such cases, microbiological analyses will be insufficient. Consequently, there is a need for methods that directly detect the toxin in food samples.[11]

In December 2002, two cases of foodborne illness after consumption of dried figs were reported in Norway. The clinical symptoms and results from microbiological examinations linked these cases to the emetic toxin produced by *B. cereus.*

To determine the presence of CRL in food, a bioassay based on inhibition of boar spermatozoa was used.[11] An LC/MS method for CRL from *B. cereus* culture extracts has been published,[12] but no chemical method has been published for the determination of CRL in figs or other food matrices.

The purpose of the present study, was to develop a rapid, simple, and specific LC/MS method for the determination of CRL in figs, with a sensitivity, which would at least meet requirements set by the National Food Control Authority in Norway.

EXPERIMENTAL

Materials and Reagents

Samples of toxin-free figs were used in the spiked experiments. All chemicals and solvents were of analytical or HPLC grade (BDH Laboratory Supplies, Poole, England). CRL was obtained from *B. cereus* F-4810/72, John Kramer (Public Health Central, London, UK). Bacteria were grown on tryptic soy agar plates at 28°C for 10 days. Colonies were scraped from the

agar. To 500 mg bacteria, 1 mL water was added, mixed, and then stored by freezing (−20°C) for 2 hr. To the thawed sample, 8 mL acetone–tetrahydrofurane (6 + 4) was added. The sample was shaken vigorously for ∼30 sec, and then centrifuged for 5 min at 4500 rpm. The supernatant was transferred to another centrifuge tube. The bacteria precipitate was re-extracted with 2 × 2 mL acetone–tetrahydrofurane. The supernatants were combined and evaporated to ∼5 mL under a stream of N_2, using a reacti-therm heating module at 60°C and a reacti-vap evaporating unit. After addition of 5 mL $CHCl_3$, the sample was shaken for ∼10 sec and centrifuged for 3 min. The upper layer (water) was discarded and the organic phase was transferred to another glass-stoppered tube with a Pasteur pipette to avoid water residues. The organic layer was evaporated to dryness. The dry residue was vortex-mixed for 20 sec in 3 mL hexane and centrifuged for 2 min. The hexane was filtered through a Spin-X centrifuge filter unit (0.22 μm, nylon type from Costar, Cambridge, USA). The hexane sample was evaporated to dryness and the residue was weighed. The CRL weight was 0.6 mg.

The CRL stock solution (1 mg/mL) and working standard solutions (0.1 μg/mL) were prepared by dilution with methanol. The stock solution was stored in a freezer (−20°C) and the working standard solution was stored in a refrigerator (+4°C).

Extraction columns Bond Elut (1 cc/100 mg) silica cartridges (SI) for solid phase extraction (SPE) were purchased from Varian (Harbor City, USA).

Chromatographic Conditions

The analyses were performed on a Applied Biosystems LC/MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler (Perkin Elmer, Norwalk, CT). The acquired data were entered into a Model 8500 Apple Power Macintosh, and processed with either Multiview 1.4 or MacQuant 1.6 software packages (Applied Biosystems), for spectral information and quantification data processing. An API 100 LC/MS system (Applied Biosystems, Ontario, Canada) single quadrupole mass spectrometer with a turbo-ion spray inlet for the API LC/MS system was used for this study. The turbo probe of the instrument was maintained at 200°C, the probe air flow-rate was 6 L/min. The LC/MS was set to collect single-ion data in positive ion mode at m/z 1170.9 for CRL. The entrance electrode voltages and the position of the ion spray inlet were adjusted to provide optimum intensity for the molecular ion.

The analytical column, Zorbax SB-C18 Rapid Resolution HT 4.6 mm × 50 mm, was packed with 1.8 μm particles (Agilent Technologies, USA) and was operated with a constant temperature of 30°C. The column

was connected to an A-318 precolumn filter on line, with an A-102 frits (Upchurch Scientific, Oak Harbor, WA). The mobile phase for CRL was a mixture of 92% methanol and 8% water containing 100 μL/L trifluoroacetic acid (Rathburn, Walkerburn, Scotland). The pump was operated isocratically at a flow-rate of 0.8 mL/min. The room temperature was 22°C. Beforehand, the eluent was split post-column, ~1 : 20, so that ca. 50 μL flowed into the ion-spray ion source with a flow rate of 1 mL/min.

Sample Pretreatment

Volumes of 1 mL methanol or standard (the total volume should always be 1 mL), 2 mL water, and 6 mL acetone–tetrahydrofuran (6 + 4) were added to 3 g of sample. The mixture was homogenized for ~6–7 sec with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Germany). After centrifugation for 5 min at 5000 rpm, 3 mL of supernatant (corresponding to 0.75 g sample) was transferred to a glass-stoppered centrifuge tube, and 4 mL $CHCl_3$ was added. The mixture was shaken vigorously for ~15 sec, and centrifuged for 3 min at 3500 rpm. The upper layer (water) was discarded and the organic layer was transferred to another glass-stoppered tube with a Pasteur pipette (to avoid water residues). The organic layer was evaporated to ~50 μL followed by addition of 1 mL hexane. The sample was evaporated to dryness under a stream of air using a reacti-therm heating module at 60°C. After the sample had achieved room temperature, the dry residue was dissolved in 200 μL $CHCl_3$; thereafter, 3 mL hexane was added. The mixture was mixed and centrifugated for 4 min at 4500 rpm. The hexane sample extract was then loaded into a conditioned SI column.

Clean-Up on SPE-Column

The SI column was conditioned with 1 mL hexane at a vacuum of −5 inches Hg, using a Vac Master system (International Sorbent Technology) and the sample extract was loaded into the column. The flow rate of the sample through the column was with low vacuum (dropwise). The column was washed (vacuum of −5 inches Hg) with 1 mL hexane, 3 × 1 mL hexane–diethyl ether (9 + 1), 2 × 1 mL hexane–diethyl ether (8 + 2), 1 mL dichloromethane–ethanol (96%) (99.5 + 0.5 mL) and 1 mL chloroform. Afterwards, the SPE column was suctioned to dryness. The column was then eluted with 3 × 1 mL CH_2Cl_2–CH_3OH (90 + 10), with a vacuum of −5 inches Hg. The eluate was collected and evaporated to dryness. The dry residue was dissolved (vortex-mixed) in 50 μL CH_3OH, and then 250 μL CH_3OH–H_2O (6 + 4) was added and mixed with a whirlimixer for 3 sec. After centrifugation

for 4 min (4500 rpm), the methanol based supernatant was centrifuged for 10 min (10,000 rpm) through a Spin-X centrifuge filter.

Aliquots of 30 μL were injected into the LC/MS at intervals of 10 min for the determination of CRL.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for CRL from figs were determined from figs spiked with standard solution to yield 0, 1, 2, 3, 5, 7, 10, 15, and 20 ng/g. The recovery rates were determined by comparing analysis of spiked figs with those of standard solutions. The linearity of the standard curve for CRL in figs, was calculated using peak area measurements.

For the determination of recovery rates, the volumes of standard solutions were doubled. Consequently, the total volumes were increased from 300 to 600 μL (see Sample Pretreatment) with CH_3OH-H_2O (6 + 4).

RESULTS AND DISCUSSION

For CRL in figs, the standard curve was linear in the investigated areas from 2 to 20 ng/g. The corresponding correlation coefficient (for CRL in figs) was 0.999. The recovery and repeatabilities for CRL are shown in Table 1.

Chromatograms obtained from toxin-free fig samples, and from the corresponding samples spiked with CRL, are shown in Fig. 1.

Using an automate-pipette with tips, the addition of acetone–tetrahydrofurane in the sample pretreatment is not accurate. This problem is avoided by using a bottle top dispenser.

In many laboratories, a stream of nitrogen is used to evaporate samples to dryness. The use of air produced from a central air compressor for evaporating

Table 1. Recovery and repeatability for CRL from spiked samples of figs.

Sample (g)	*n*	Amount of toxin (ng/g)	CRL (%)	
			S.D	RC
3	5	5	1.8	93
	5	10	1.3	89
	5	20	2.7	93

Note: *n*, number of samples; S.D., standard deviation; RC, recovery.

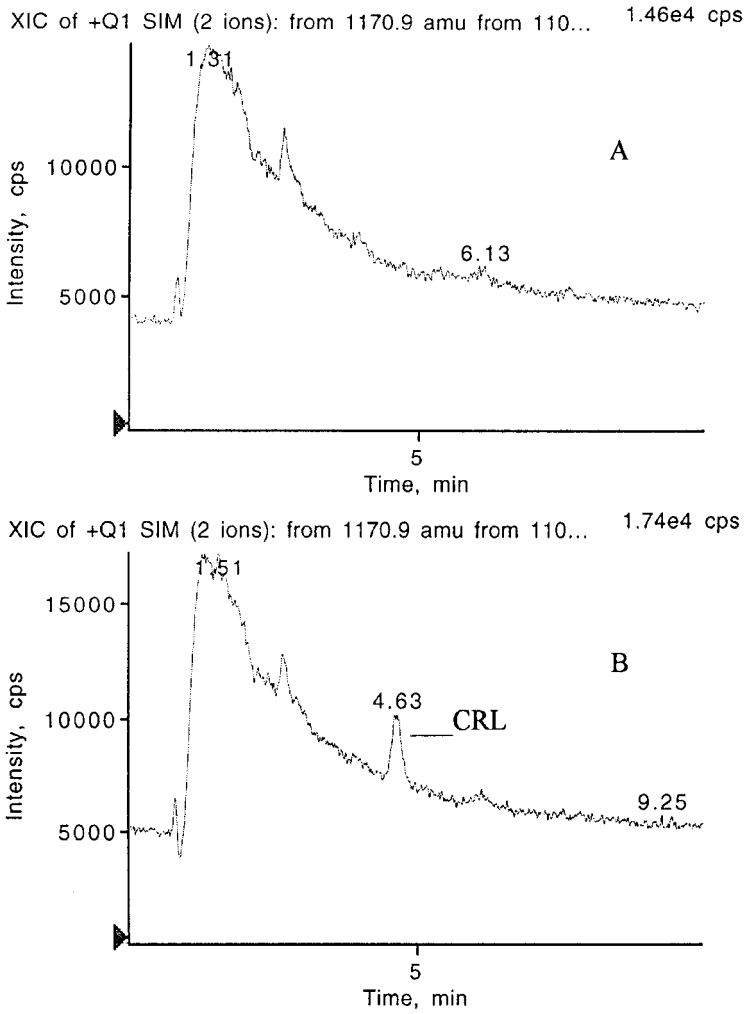

Figure 1. Chromatograms of extract from fig. (A) Toxin-free fig; (B) fig spiked with CRL (3 ng/g).

CRL from figs is an economically favourable alternative compared with nitrogen. In the present study, nitrogen was used for evaporation under the preparation of CRL standard from *B. cereus*.

Häggblom et al.[12] describe a quantitative LC/MS (electrospray ion trap) for analysis of cereulide from culture extract produced under various

conditions. The published method is not applicable for the determination of cereulide from fig fruits. The method described in this paper, includes extraction of CRL from a food matrix, and it requires a more complex clean-up process than described for bacterial cultures.

The method presented in this paper is selective, robust, sensitive, and accurate. It can also be used for the determination of CRL in rice with a good baseline resolution, but this method was not validated in the present study.

The limit of detection was calculated as three times the peak-to-peak baseline noise ($S/N = 3$) from toxin-free fig fruits. The limit of detection was 1 ng/g, while the limit of quantification was 2 ng/g. No interference was seen during analysis, neither when calibrating the curves, nor when performing the recovery studies.

The detection limit of the assay depends mainly on the sensitivity of the LC/MS. This, in turn, could be influenced by such factors as the position of the ion spray inlet, cleanliness of the LC/MS, the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

The advantage of the LC/MS technique lies in the combination of the separation capabilities of HPLC, with the power of MS as an identification and confirmation method with high sensitivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. Thus, LC/MS seems to provide a better alternative than HPLC for CRL analysing in fig fruits.

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