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Identification and quantification of lichenysin – a possible source of food poisoning

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Abstract

Lichenysin which is produced by 53 different *Bacillus licheniformis* strains has been structurally examined with a qualitative liquid chromatography – tandem mass spectrometry (LC-MS/MS) method using quadrupole – time of flight mass spectrometry. The same lichenysin isoforms are produced from all strains, indicating that the growth conditions have a stronger influence on the lipopeptide production than the genotype. A rapid method for the quantification of lichenysin from bacterial cell cultures with LC-MS/MS after a simple methanol extraction has been refined. For the first time, commercially available lichenysin has been used as calibrant, making quantification more accurate. The trueness for C15-lichenysin has been improved to 94% using matrix-matched calibration with lichenysin compared to 30% using solvent calibration with surfactin. The quantitative method was fully validated based on Commission Decision 2002/657/EC. The LOD of the method was below 1 µg g^{-1} and the repeatability ranged from 10% to 16%.

Keywords: lichenysin; LC-MS/MS; quantification; validation; *Bacillus licheniformis*

Introduction

Bacillus licheniformis is a saprophytic soil bacterium that is widespread in nature due to its endospore forming properties. It is widely used in the fermentation industry in the production of enzymes (proteases and amylases), antibiotics (Schallmey et al. 2004) and probiotics (Cutting 2011). *B. licheniformis* is not considered a human pathogen although it has been isolated from several human infections, in all of which the inflicted humans where immunocompromised (Idelevich et al. 2013, Lepine et al. 2009, Park et al. 2006). The first reports of the involvement of B. licheniformis in food poisoning came in England in the 1970s, but the virulence factor(s) were neither detected nor described (Kramer and Gilbert 1989). Large amounts of *B. licheniformis* have been associated with a few cases of food poisoning (intoxications), one of which had a fatal outcome (Salkinoja-Salonen et al. 1999). It has also been involved in animal abortions and bovine mastitis where lichenysin-producing strains were detected in mastitic milk (Agerholm et al. 1995, Johnson et al. 1994, Nieminen et al. 2007, Syrjälä et al. 2007). The exact mechanism and role of *B. licheniformis* as causative agent of food poisoning is unknown, although lichenysin has been proposed as the virulence factor (From et al. 2005, Mikkola et al. 2000, Salkinoja-Salonen et al., 1999). Toxic lichenysin was detected in baby milk formula associated with the death of an infant, indicating that lichenysin is indeed the source of food poisoning (Mikkola et al., 2000). Further investigations are needed to clarify the role of lichenysin in food poisoning.

Lichenysin is a lipopeptide produced by most, if not all *B. licheniformis* strains (Madslien et al. 2013). It is an excellent surfactant and a good chelating agent for Ca^{2+}

and Mg^{2+} (Grangemard et al. 2001, Javaheri et al. 1985, McInerney et al. 1990). Lichenysin is also shown to have anti-inflammatory, antimicrobial, antitumor and immunosuppressive properties but is also haemolytic (Grangemard, Wallach, Maget-Dana and Peypoux 2001). These characteristics are to a wide extent caused by the amphiphilic nature of the lipopeptide; it consists of a peptide moiety comprised by seven amino acids and a β-hydroxy fatty acid with 12 – 17 carbon atoms with possible normal, iso and anteiso branching (Grangemard et al. 1999, Hasumi et al. 1995, Horowitz and Griffin 1991, Jenny et al. 1991, Konz et al. 1999, Mikkola, Kolari, Andersson et al., 2000, Trischman et al. 1994, Yakimov et al. 1999, Yakimov et al. 1995). Several isoforms and homologues of lichenysin are found in nature, both amino acid substitutions and alterations in the length and branching of the fatty acid chain occurs. The most abundant isoform is known as lichenysin A (Figure 1) (Yakimov et al., 1999) where the amino acid sequence is Gln – Leu – D-Leu – Val – Asp – D-Leu – Ile (Konz et al., 1999, Mikkola, Kolari et al., 2000, Yakimov et al., 1999). Surfactin, another lipopeptide produced by *Bacillus subtilis*, is very similar in structure to lichenysin A (Figure 1) and differ only with the substitution of glutamine with glutamic acid in the first amino acid position (AA1) (Konz et al., 1999, Peypoux et al. 1999). This small difference however, increases the surfactant properties of lichenysin significantly; the critical micelle concentration (CMC) is 22 μM for lichenysin and 220 μM for surfactin, 100 % haemolysis is obtained with 15 μM lichenysin and 200 μM surfactin and the association constant with Ca^{2+} is four times higher for lichenysin than surfactin and 16 times higher for Mg^{2+} (Grangemard et al., 2001).

Several different detection methods for lichenysin have been developed including cytotoxicity methods like the sperm motility assay (Andersson et al. 1998, Hoornstra et al. 2003) and the Vero cell assay (Sandvig and Olsnes 1982), and PCR

(Madslien et al., 2013, Nieminen et al., 2007, Tapi et al. 2010, Turgay and Marahiel 1994, Wu et al. 2015). None of these methods proves the presence of lichenysin, only a possible effect of the lipopeptide or the genes encoding lichenysin synthetase. Analytical methods utilising LC-MS detect lichenysin directly by separating the analyte of interest from interfering matrix components both based on differences in hydrophobicity and molecular mass. Several qualitative mass spectrometry based methods have been published for the detection of lichenysin (Andersson et al., 1998, From et al. 2007, Grangemard et al., 1999, Guo et al. 2014, Horowitz and Griffin 1991, Jenny et al., 1991, Li et al. 2008, Mikkola et al. 1999, Yakimov et al., 1999, Yang et al. 2006) but only a few quantitative methods, none of which uses commercially available lichenysin for calibration (Madslien et al., 2013, Zhang et al. 2014).

The use of *Bacillus* species as additives in the production of animal feed is regulated by the EFSA panel on additives and products or substances used in animal feed (FEEDAP). Until 2013 the FEEDAP scientific opinion stated that a test for haemolysis together with PCR screening for non-ribosomal peptide synthase genes were sufficient to reveal the potential of lipopeptide production in the strains of interest (EFSA FEEDAP Panel: Technical guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition 2011). After the revelation that most, if not all, *Bacillus licheniformis* strains produces lichenysin and several strains are non-haemolytic (Madslien et al., 2013), together with indications of the same behaviour in *Bacillus subtilis* strains (Dybwad et al. 2012), FEEDAP endorsed the need for a revision of the current scientific opinion resulting in a revised scientific opinion published in 2014 (EFSA FEEDAP Panel: Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition 2014, EFSA FEEDAP Panel: The need to revise the Technical Guidance on the assessment of the toxigenic potential of *Bacillus*

species used in animal nutrition 2013). It recommends that the cytotoxicity of all *Bacillus* strains (non-*B. cereus*) considered in feed production should be evaluated by an *in vitro* cell-based method like the Vero cell assay. In case of proven cytotoxicity is the strain not suitable for use as a feed additive.

We have earlier shown that the cytotoxicity alone is insufficient as a marker for the lichenysin content in cell extracts (Madslien et al., 2013). The risk of illness resulting from the presence of lichenysin in food products is also yet to be evaluated. To ensure an accurate measurement of the lichenysin production from different bacterial strains it is therefore important not only to verify the cytotoxicity but also quantify the amount of lichenysin present in the cell extracts. Quantitative methods suitable for routinely use are necessary to enable this. To ensure an accurate quantification the choice of calibration and a proper validation of the method is of importance.

In this study 53 *Bacillus licheniformis* strains have been qualitatively analysed to compare the occurrence of the different lichenysin isoforms. We have also sought to further improve a quantitative method to determine the lichenysin content in bacterial cell extracts. For the first time commercially available lichenysin has been used for calibration to ensure more accurate quantification. The method has been fully validated based on Commission Decision 657/2002.

Materials and methods

This method, both the sample preparation, chromatography and quantitative mass spectrometry, is a modified version of the one described by Madslien et al., (2013).

Chemicals and reagents

All chemicals were of at least HPLC-grade and supplied by VWR (West Chester, PA, USA) except lichenysin A (98.2 %, Lipofabrik, Villeneuve-d'Ascq, France), surfactin (≥ 98 %, Sigma, Steinheim, Germany) and heptafluorobutyric acid (Fluka, Buchs, Switzerland). The water used was grade 1 purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Sample preparation

Bacterial strains were grown for 10 days at 37°C on trypticase soy agar (TSA) plates (Merck KGaA, Darmstadt, Germany). The biomass was collected and weighed before addition of 2 equivalents of methanol and homogenisation by vortex mixing for 5 min (VXR basic Vibrax, IKA Werk, Staufen, Germany). Equivalents of 50 mg biomass were weighed in centrifuge tubes. The cells were lysed by boiling in 1 mL methanol for 30 min, resulting in partially evaporation of the methanol. The residue was added 500 µL methanol and vortex mixed for 3 min before centrifugation at 14 000 x *g* for 3 min. The supernatant was transferred to a 12 mL centrifuge tube and evaporated to dryness at 80°C under a stream of air using a Pierce Reacti-Therm heating module (Pierce, Rockford, IL, USA). The dry residue was reconstituted in 200 µL methanol and filtered through a 0.22 µm nylon spin filter (Costar Spin-x, Costar, Corning Incorporated, Corning, NY, USA). Aliquots of 10 µL were injected on column for qualitative analysis and 1 µL for quantitative analysis.

Liquid chromatography

The instrumentation used for the qualitative analysis was an Agilent 1260 SL system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, thermostatted autosampler kept at 4°C and column compartment kept at 35°C. The

separation was performed on an RRHD Zorbax Eclipse Plus C18 column, 100 x 2.1 mm id, with 1.8 µm particles (Agilent Technologies, Palo Alto, CA, USA). Mobile phase A consisted of 2 mM ammonium acetate and 0.2 % heptafluorobutyric acid in water and mobile phase B was acetonitrile and methanol (1+1). The flow rate was 0.3 mL/min with a linear gradient from 90 – 93 % B in 6 min. Total time of analysis was 12 min. The autosampler temperature was 4°C.

The quantitative analysis was performed with an Agilent 1290 Infinity system (Agilent Technologies, Waldbronn, Germany) with the same conditions as the quantitative analysis only with flow rate 0.4 mL/min with a linear gradient from 90 – 93 % B in 4 min. Total time of analysis was 8 min.

Qualitative mass spectrometry

The structure elucidation of lichenysin was done on a G6520 quadrupole-time of flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) operated in 4 GHz, high resolution mode. The ionisation was done with dual electrospray in positive mode with reference mass correction. The fragmentor voltage was 175 V, gas temperature 325°C, drying gas flow 5 L/min, nebulizer pressure 30 psi, capillary voltage 4000 V and collision energy 25 V and 35 V. The acquisition mode was targeted MS/MS in scan area 50-1200 Da with recorded 3 spectra/s in both MS and MS/MS. The targeted list is shown in Table 1. The delta retention time was 2 min for all ions.

Quantitative mass spectrometry

Lichenysin was quantified using a G6490 triple quadrupole mass spectrometer (Agilent Technologies, Singapore) equipped with a Jet Stream electrospray ion source. Data was acquired in positive multiple reaction monitoring mode, MRM. The ion transitions monitored and their corresponding collision energies are listed in Table 2. Common

instrument settings for all ion transitions were fragmentor voltage 380 V, dwell time 20 ms, gas flow 14 L/min, gas temperature 250°C, nebulizer pressure 20 psi, sheath gas flow 11 L/min, sheath gas temperature 400°C, capillary voltage 4000 V and nozzle voltage 0 V.

Calibration

Matrix matched calibration was performed by adding lichenysin at five concentration levels (1, 10, 50, 100 and 500 µg/mL) to biomass from *Bacillus cereus* strain ATCC 14579 before the extraction. Calibration curves of both lichenysin and surfactin in pure solvent (methanol/water, 3+1) were prepared for comparison with matrix matched calibration. Five concentration levels (1, 10, 50, 100 and 500 µg/mL) were diluted with methanol from a stock solution of 1 mg/mL in acetonitrile (lichenysin) or ethanol (surfactin).

Validation

Bacillus cereus strain ATCC 14579 was used as matrix for the validation samples. The biomass from seven plates was pooled, added a known amount of methanol and homogenised to a slurry by vortexing before weighing in aliquots equivalent to 50 mg biomass. *Bacillus licheniformis* strain NVH1115 was used as positive control.

The validation of the quantitative method was done using the calibration curve method based on 2002/657/EC (Commission decision of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002) and an interpretation thereof (Antignac et al. 2003). Matrix matched calibration curves were prepared by adding lichenysin at five concentration levels (1, 10, 50, 100, 500 µg/g) to the samples before extraction. The regression coefficient (R^2) and the slope (a) of the calibration curve were used to assess

the linearity of the method together with the response factor test. The repeatability was evaluated by the RSD for 12 samples from three different sample sets spiked with 10 µg/g lichenysin. The limit of detection (LoD = 3 x SD_b, where SD_b is the standard deviation of the blank signal) and limit of quantification (LoQ = 10 x SD_b) assesses the sensitivity. The selectivity was evaluated from six negative samples.

Determination of the recovery was done by analysing pure lichenysin standard (A_S), matrix samples without fortification (A_B), prepared matrix samples fortified before injection on the LC-MS/MS (A_{MS}) and matrix samples fortified before the sample preparation (A_{SP}). Both total recovery ($R^T = (A_{SP}- A_B)/A_S$), recovery in the sample preparation ($R^{SP} = (A_{SP}-A_B)/(A_{MS}- A_B)$) and recovery of the LC-MS/MS method ($R^{MS} = (A_{MS}- A_B)/ A_S$) were established.

The lichenysin content in 21 positive samples from *Bacillus licheniformis* strain NVH1079 was used to evaluate the homogeneity. The biomass from 18 plates were pooled and treated in the same way as the validation samples.

Results and discussion

This study was done to fully validate and elaborate the quantitative method for lichenysin that we first published in 2013 (Madslien et al., 2013), as well as to confirm the structure of the lichenysin isoforms found by developing a qualitative LC-MS/MS method. The introduction of commercially available lichenysin as calibrant instead of surfactin has raised new methodological issues and a study of different quantification techniques has been applied. To ensure the validity of the quantification a validation study based on Commission decision 657/2002 has been applied (Commission decision of 12 August 2002 implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results 2002).

During the validation study it became clear that the lichenysin isoform with precursor ion m/z 993 was not expressed as well by the bacterial strain used for the calibration standard (ATCC 14580) as by the strains used in the preliminary experiments (Madslien et al., 2013). As a consequence, only one ion transition was sufficiently abundant to be evaluated for m/z 993 and it was not possible to establish any sensible validation data due to too few calibration levels above the LOD. The ion transition m/z 993 – 685 has been included in the study but is only to be regarded as qualitative.

Structural determination of lichenysin in cell extracts from different bacterial strains

The molecular structure of lichenysin has been described in several publications during the last two decades and, as pointed out in the introduction, several isoforms and homologues of lichenysin occur in nature (Grangemard et al., 1999, Hasumi et al., 1995, Horowitz and Griffin 1991, Jenny et al., 1991, Konz et al., 1999, Mikkola et al., 2000, Trischman et al., 1994, Yakimov et al., 1999, Yakimov et al., 1995). Different bacterial strains might produce different isoforms in different ratios. Whether this is enzymatically controlled, due to growth conditions or genetics or a combination, is not certain (Konz et al., 1999). Four major isoforms of lichenysin have been separated and identified in this study (Figure 2). They are all detected as their protonated ions $(M+H)^+$, their sodium adducts $(M+Na)^+$ and the three most abundant are also detected as their 2Na adducts $(M-H+2Na)^+$. The protonated ions are the most sensitive in the LC-MS/MS under the optimized conditions. The molecular structure of the three major peaks of lichenysin (m/z 1007.7, 1021.7 and 1035.7) in extracts from 53 different *B. licheniformis* strains was investigated. More information about the strains can be found in Madslien et al 2013 (Madslien et al., 2013). The product ion mass spectra from m/z

1007.67, m/z 1021.68 A and 1035.70 all show the same amino acid sequence with a good coverage of both y- and b-ions (Figure 3) after ring opening, only the b1 and y1 ions are missing from the expected ion series (Table 3). Most of the fragments are present in pairs with a mass difference of 18 Da, resulting from a possible dehydration at either end of the lipopeptide during the ring opening. From the y- and b-series of ions resulting from ring opening and dehydration at the N-terminal end, only b2, b3 and y3-6 are found. This is in accordance with the findings of Yakimov et al 1999 (Yakimov et al., 1999). y6 after dehydration at the N-terminal end, m/z 685.45, is by far the most abundant fragment ion from all precursors and represents the peptide moiety after ring opening and loss of the fatty acid chain and AA1 (Hue et al. 2001, Yakimov et al., 1999). The elucidated amino acid sequence for all three major peaks was the same: Gln – Leu/Ile - Leu/Ile – Val – Asp – Leu/Ile – Leu/Ile. This is indeed the established structure for lichenysin A; henceforth we will name the peaks C13-lichenysin (m/z 1007), C14-lichenysin (m/z 1021) and C15-lichenysin (m/z 1035).

As shown in Figure 2, there are two major peaks of about the same intensity from C14-lichenysin as opposed to the other three lichenysin isoforms that only show one major peak each. The product ion mass spectra from the two C14-lichenysin peaks labelled A and B (Figure 4) reveal the occurrence of an amino acid substitution where leucine in position AA7 has been substituted with valine in the second peak, 1021 B. The overall m/z of the isoform remains the same as for 1021 A, meanwhile the rest of the amino acid sequence remains the same; hence the fatty acid chain has gained one methyl group. This substitution has been reported earlier (Zhang et al., 2014), also for surfactin (Peypoux et al. 1991). However, here the product ion scan from precursor 1021 B shows that the fragment m/z 685 is also present alongside m/z 671.This suggests that peak 1021 B is comprised of two different lichenysin isoforms that have the same

retention properties on the C18 column, despite structural differences. This might be because the alterations only involves the addition and removal of two methyl groups, as opposed to the substitution of glutamine with glutamic acid in surfactin that leads to as much as two minutes reduction of the retention time. The product ion spectra of m/z 1021 A and B from all 53 *B. licheniformis* strains reveals a similar pattern: Peak A only contains the common lichenysin A amino acid sequence, while peak B contains a mixture of the AA7 leucine and AA7 valine isoforms, but in different ratios ranging from 50 % to 220 %. Overall, the production of the same lichenysin isoforms from all 53 bacterial strains investigated indicates that the growth conditions have a greater influence on which isoforms are produced than the genotype. This is in accordance with the findings of Li et al (Li et al., 2008) who found that *B. licheniformis* strain HSN221 produced different lichenysin homologues when grown in different media. The AA7 valine isoforms of lichenysin might also be present for the m/z 993, 1007 and 1035 isoforms, but due to low-intensity second peaks they have not been considered for structural determination.

Quantification with surfactin and lichenysin as calibration standards

Each of the four major isoforms of lichenysin was quantified separately. Since it is not known whether all isoforms are equally potent it is in general the total lichenysin concentration that is of interest. The certified amount of lipopeptide in commercially available lichenysin and surfactin is also given as the total amount, it is important to note that the distribution between the different isoforms present might differ.

Lichenysin has recently become commercially available and was used for calibration for the first time. Until now, surfactin (Madslien et al., 2013) and presumably in-house purified lichenysin (Zhang and Wu, 2014) have been used as calibration standard for lichenysin quantification. Surfactin and lichenysin are very

similar in structure (Figure 1) differing only with 1 Da in their molecular masses; hence they appear to be good calibrants for each other. However, the substitution of glutamic acid with glutamine at AA1 alters the physicochemical properties of the molecules as illustrated by the difference in their surface tension characteristics (Grangemard et al., 1999). Consequently they might be affected differently during the sample preparation and not be prone to the same matrix effects during the analysis on the LC-MS, the latter also due to the differences in retention times. These differences in retention times are what make surfactin a possible calibration standard for lichenysin: If they co-eluted it would be impossible to differentiate between the monoisotopic mass of surfactin and the first isotopic mass of lichenysin. To evaluate the suitability of surfactin as calibration standard for lichenysin we calculated the lichenysin content in twelve samples spiked with 10 µg/g lichenysin with pure solvent standards of both surfactin and lichenysin as calibrants (Table 4). The measured concentrations were 50 – 70 % lower with surfactin as calibrant compared to lichenysin as calibrant (Table 4). This reveals a substantial underestimation of the lichenysin concentration when surfactin is used as calibration standard.

Both lichenysin m/z 1021 Da and surfactin m/z 1022 Da are double peaks. As shown through the structural determination, these peaks are representing different isoforms of lichenysin but only one isoform of surfactin (data not shown). Consequently, the quantification of lichenysin with surfactin is a challenge for lichenysin m/z 1021. Not only because of the different isoforms, but also due to the difference in peak intensity: for surfactin the first peak is the less intense, while the first peak is the most intense for lichenysin (Figure 5). This pattern is seen in lichenysin produced from all 53 strains included in the study: the ratio between C14-lichenysin A and B varies but C14-lichenysin A is always the most intense. To overcome this

challenge we have earlier chosen to integrate and quantify these peaks as one (Madslien et al., 2013). However, the structure elucidation performed in this study revealed that the two peaks represent different isoforms; hence they should be quantified separately. The negative trueness found for m/z 1021 B with surfactin as calibration standard (Table 4) emphasises the shortcoming of surfactin as calibration standard for this lichenysin isoform.

Quantification with pure solvent standards and matrix matched calibration.

Quantification against a matrix matched calibration curve yields about 20 % higher lichenysin concentrations than calculated against pure solvent standards (Table 4). This demonstrates the significance of matrix matched calibration. In some cases, for instance at high concentration levels, it is necessary to dilute the samples to get within the concentration range of the calibration curve. A desirable side effect is the removal of possible matrix effects through dilution, and thus eliminating the difference between matrix matched calibration and calibration against pure solvent standards. The ratio was 93 % to 101 % (n = 7) between the two calibration techniques when both samples and calibration samples were diluted 100 times before injection on the LC-MS/MS (with surfactin as standard). This implies that in cases where the samples have to be diluted hundred times due to high concentration levels of lichenysin, the most correct result will be achieved from calibration against pure solvent standard instead of undiluted matrix matched standards.

Matrix matched calibration with surfactin as calibrant was carried out with *B. licheniformis* NVH 1079 grown according to protocol as sample matrix; hence the calibration samples all contained lichenysin as well. As mentioned earlier, a small peak with m/z 1035 eluted at the same time as C15-surfactin with m/z 1036. This is another

disadvantage for surfactin as calibrant for lichenysin but poses no difficulties for the selectivity of lichenysin as the monoisotopic mass is 1 Da lower than for surfactin.

Surfactin as internal standard

Zhang et al (Zhang et al., 2014) used C15-surfactin (m/z 1036) as an internal standard for the quantification of lichenysin. We find that C15-surfactin coelutes with a low-intensity peak representing an isoform of C15-lichenysin in extracts from *B. licheniformis*. These two peaks are isobaric and not separable in the mass spectrometer; hence C15-surfactin is not suitable for use as internal standard for lichenysin when several strains are being investigated. It would be possible to use one of the other surfactin isoforms as internal standard for all lichenysin isoforms but that would neither compensate possible matrix effects occurring at different retention times, nor variations throughout the sample preparation due to their different physicochemical properties. The validation data from this study shows that variation in extraction efficiency and matrix effects that are influencing the lichenysin isoforms differently are a higher contribution to the RSD than variation between samples due to common influences on all isoforms, for instance sample loss during the sample preparation. The best solution to overcome this challenge and improve the precision and trueness of the method would be to implement isotopically labelled internal standards for each lichenysin isoform.

Validation of the quantitative LC-MS/MS method

For all lichenysin isoforms, the most abundant fragment ion was chosen for the quantitative ion transition. The qualitative ion transition was chosen not only according to abundance, but also considering precision. For m/z 1021 B, neither the quantitative nor the qualitative fragment ion is present in the second isoform comprising the peak; subsequently only the AA7 leucine isoform is quantified.

We have earlier shown that all of the 53 *B. licheniformis* strains tested contained the lichenysin syntethase gene (*lchAA*) and produced lichenysin, including strains thought to be non-producers such as ATCC 14580 (Madslien et al., 2013). This is probably due to our prolonged growth period; ten days as opposed to the 24 hours used by Wu et al., (2015). As a result, none of the *B. licheniformis* strains could be used as negative control. To ensure the calibration samples did not contain any lichenysin another *Bacillus* sp., *Bacillus cereus* ATCC 14579, was grown according to protocol and used as negative control and blank matrix for the calibration samples.

The linearity was evaluated through the regression coefficient, R^2, and the response factor test (Table 5). The coefficient of determination, R^2, was ≥ 0.99 both with and without 1/x- weighting for all ion transitions except m/z 1035 - 685 and m/z 1035 - 240. This is the most sensitive precursor ion; hence the highest calibration point at 500 µg/g yields counts around the saturation limit of the detector. Without this level, with 1/x-weighting, R^2 is 0.995 and 0.994 for the ion transitions m/z 1035 – 685 and m/z 1035 – 240, respectively. The response factor test was < 15 % for all ion transitions. Six matrix samples without lichenysin were used to evaluate the selectivity; no interferences were discovered at the retention times of the analytes. For all three precursors m/z 1007, 1021 A and 1021 B, the LOQ is higher for the qualitative ion transitions than the one used for quantification (Table 5). This is as expected as the qualitative ion transitions are the least sensitive. With this method the LODs are all below 1 µg/g. If necessary, the injection volume can be increased to achieve a higher sensitivity. We have tried this without difficulties during the method development (data not shown).

Without a suitable internal standard, it was not possible to determine the reproducibility of the method. The samples deteriorate rapidly both when stored at room

temperature, + 4°C and - 21°C; hence without an internal standard to compensate for the variation in storage time, the RSDs are above acceptable levels when validation samples from different days are compared. As a consequence, the precision was evaluated through the repeatability in three sample sets prepared separately but within the same day by the same analyst. The RSD at 10 μg/g was between 10 % and 16 % (n = 12). The ion ratio of m/z 1021 B is 0.50 with an RSD of 15 %, whereas the remaining ion ratios are well within the limits stated in EC/657/2002 (Table 5). The retention times of all ion transitions in the samples are well within 2.5 % of the retention time of the standard; hence the requirement from EC/657/2002 is achieved.

According to EC/657/2002, the trueness should be within 80 – 110 %. Due to the lack of a certified reference material, the trueness was evaluated by spiking blank matrix samples with a known amount of lichenysin. The results are given for each ion transition in Table 5, ranging from 67 % to 121 % overall (n = 12). Both ion transitions from precursor ion m/z 1035 show the best trueness; 77 – 109 % and 79 – 113 %, respectively, but also these are slightly outside the limits. The poor trueness of the method is most likely a result of varying matrix effects and sample loss throughout the analysis. Careful studying of each measurement reveals that the different lichenysin isoforms are not influenced in the same way in each sample: The ratio in trueness ranges from 80 % to 111 % between two single measurements for the different lichenysin isoforms. Implementation of isotopically labelled internal standards representing each lichenysin isoform would probably improve the trueness of the method.

The total recovery of the method, R^T, was 74 % to 83 %. This is a result of the rapid and simple sample preparation; about one third of the lichenysin was lost during the extraction as shown through the R^{SP} ranging from 57 % to 68 %. The R^{MS} from

111% to 142 % indicates a signal enhancement that reduces the effect of the analyte loss during the sample preparation. A total recovery less than 100 % emphasises the importance of matrix matched calibration where the calibration samples undergoes the same extraction procedure as the unknown samples.

Biomass from 18 plates of *Bacillus licheniformis* strain NVH1079, a known lichenysin producer (Madslien et al., 2013) was pooled to achieve a homogenous sample material to be used as a positive control. The RSD (n = 21) was 30 % for C12-lichenysin, 18 % for C13-lichenysin, 16 % for C14-lichenysin A and B and 12 % for C15-lichenysin when samples prepared on three different days within one month were calculated together. Within each day (n = 7) the RSDs ranged from 4 - 8 %, 6 - 10 % and 16 - 23 %, without C12-lichenysin. This indicates that the highest contribution to the deviation is day-to-day variations and that the homogeneity of the sample material is satisfactory.

Conclusion

Lichenysin produced by 53 different *B. licheniformis* strains has been qualitatively examined. All strains produced the same lichenysin isoforms but in varying ratios. This indicates that lichenysin production is indeed more dependent on growth conditions than genotype. By introducing commercially available lichenysin as calibration standard and perform a complete validation study based on Commission Decision 657, we have refined a quantitative analytical method that ensures more accurate quantification of lichenysin in bacterial cell extracts.

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Table 1: Targeted precursors for product ion scan for qualitative LC-MS/MS analysis on LC-Q-TOF

	Precursor *m/z*	Retention time (min)
Lichenysin	993.65	4.8
	1007.67	5.7
	1021.68	7.0 and 7.5
	1035.70	8.6
Surfactin	994.64	3.7
	1008.65	4.3
	1022.67	5.4 and 5.7
	1036.68	6.7

Table 2: Ion transitions monitored and their corresponding collision energies for quantitative LC-MS/MS analysis on LC-QqQ

	Precursor *m/z*	Product *m/z*	CE (eV)	Retention time (min)
Lichenysin	993.4	685.2	40	3.3
	993.4	535.4	40	3.3
	1007.7	685.4	40	3.8
	1007.7	441.1	40	3.8
	1021.7	685.4	40	4.6 and 4.8
	1021.7	581.3	40	4.6 and 4.8
	1035.7	685.3	20	5.5
	1035.7	240.1	80	5.5
Surfactin	994.6	685.2	30	2.6
	994.6	441.2	40	2.6
	1008.6	685.2	30	3.0
	1008.6	441.2	40	3.0
	1022.6	685.2	30	3.6 and 3.8
	1022.6	201.0	80	3.6 and 3.8
	1036.6	685.3	30	4.4
	1036.6	227.2	50	4.4

Table 3: y- and b- ions found after ring opening and fragmentation of the peptide moiety of lichenysin. The first fragments are resulting from dehydration at the C-terminal end and the second fragments are products of dehydration at the N-terminal end. The fragments in brackets are from m/z 1021.68 B with valine in the AA7 position.

Ions found	m/z 1007.67	m/z 1021.68 A	m/z 1021.68 B	m/z 1035.70
b1	-	227	227	-
b2	341 / 323	355 / 337	355 / 337 (369)	369 / 351
b3	454 / 436	468 / 450	468 / 450 (482)	482 / 464
b4	567	581	581 (595)	595 / 578
b5	666	680	680 (694)	694
b6	781	795	795 (809)	809
b7	894	908	908 (922)	922
y2	227	227	227	227
y3	342 / 360	342 / 360	342 / 360	342 / 360
y4	441 / 459	441 / 459	441 / 459 (445)	441 / 459
y5	554 / 572	554 / 572	554 / 572	554 / 572
y6	667 / 685	667 / 685	667 / 685 (671)	667 / 685
y7	795	795	795	-

Table 4: Average trueness (%) of lichenysin (concentration 10 µg/g, n = 12) quantified against pure solvent standards of surfactin and lichenysin, and matrix matched calibration with lichenysin. The RSDs are given in brackets.

Ion transition	Surfactin, pure solvent	Lichenysin, pure solvent	Lichenysin, matrix matched
1007 – 685	20 (9)	68 (10)	89 (10)
A 1021 – 685	38 (5)	70 (12)	89 (12)
B 1021 – 685	-0.2 (-4)	67 (14)	89 (16)
1035 – 685	30 (12)	74 (11)	94 (11)

Table 5: Results of the validation calculated from three different sample sets and a total of 30 samples. LOD, LOQ and trueness are calculated with 1/x-weighting in linear regression. (The numbers in square brackets are calculated without + 500 µg/g.)

Ion transition	R^2	R^2 With 1/x-weighting	LOD (µg/g)	LOQ (µg/g)	Response factor test (%)	Repeatability RSD (%) n = 12	ΔRT (%) n = 30	Ion ratio (RSD %) n = 21	Trueness (%) n = 12
993 – 685*	0.973	0.854	- 4.0	17	60	67	2.1	4.2 (127)	-116 – 70
1007 – 685	0.994	0.993	0.4	1.0	12	10	0.2	0.37 (14)	72 – 102
1007 - 441	0.990	0.991	-0.1	2.5	14	16	0.3		67 – 117
A 1021 – 685	0.993	0.992	0.5	0.9	14	12	0.2	0.35 (9)	70 – 103
A 1021 – 581	0.991	0.992	0.5	1.0	13	13	0.2		83 – 121
B 1021 – 685	0.991	0.992	0.2	1.7	14	15	0.2	0.50 (15)	68 – 113
B 1021 – 581	0.995	0.994	0.8	2.1	13	13	0.3		79 – 121
1035 – 685	0.992	0.987 [0.995]	- 0.1	1.2	12	10	0.2	0.79 (5)	77 – 109 [70 – 98]
1035 - 240	0.991	0.986 [0.996]	-0.3	0.5	13	12	0.2		79 – 113 [71 – 100]

* The ion transition m/z 993 – 685 is only qualitative due to low content in the calibration standard. It is included in the table for information only.

Word count: 7608

Figure 1: Molecular structure of lichenysin A (monoisotopic mass 1020, R = NH_2) and surfactin (monoisotopic mass 1021, R = OH), differing only with a glutamine (lichenysin) / glutamic acid (surfactin) substitution at amino acid position AA1.

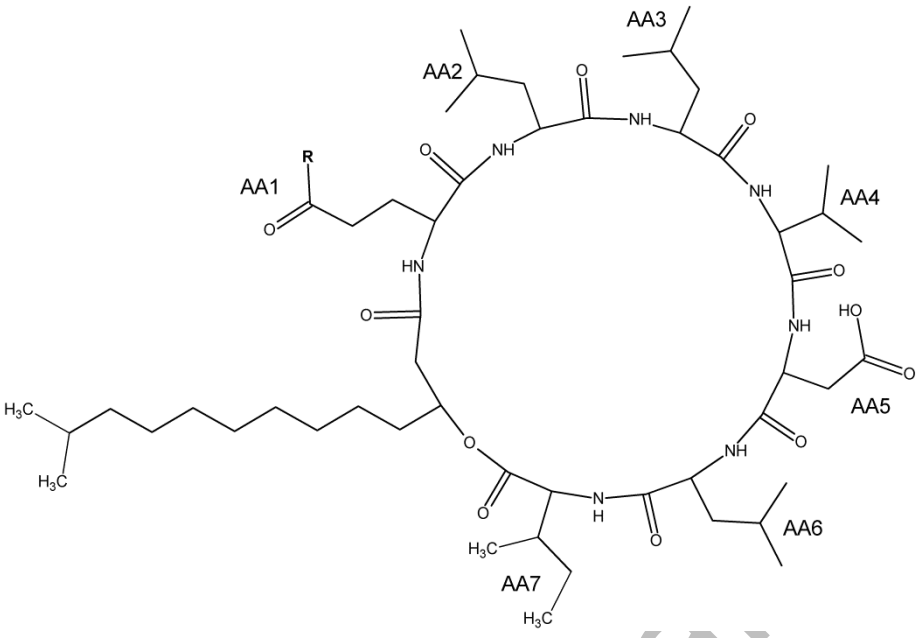

Figure 2: Extracted ion chromatograms, EIC, of the four major lichenysin isoforms found together with their mass spectra. (*B. licheniformis* ATCC 14580.)

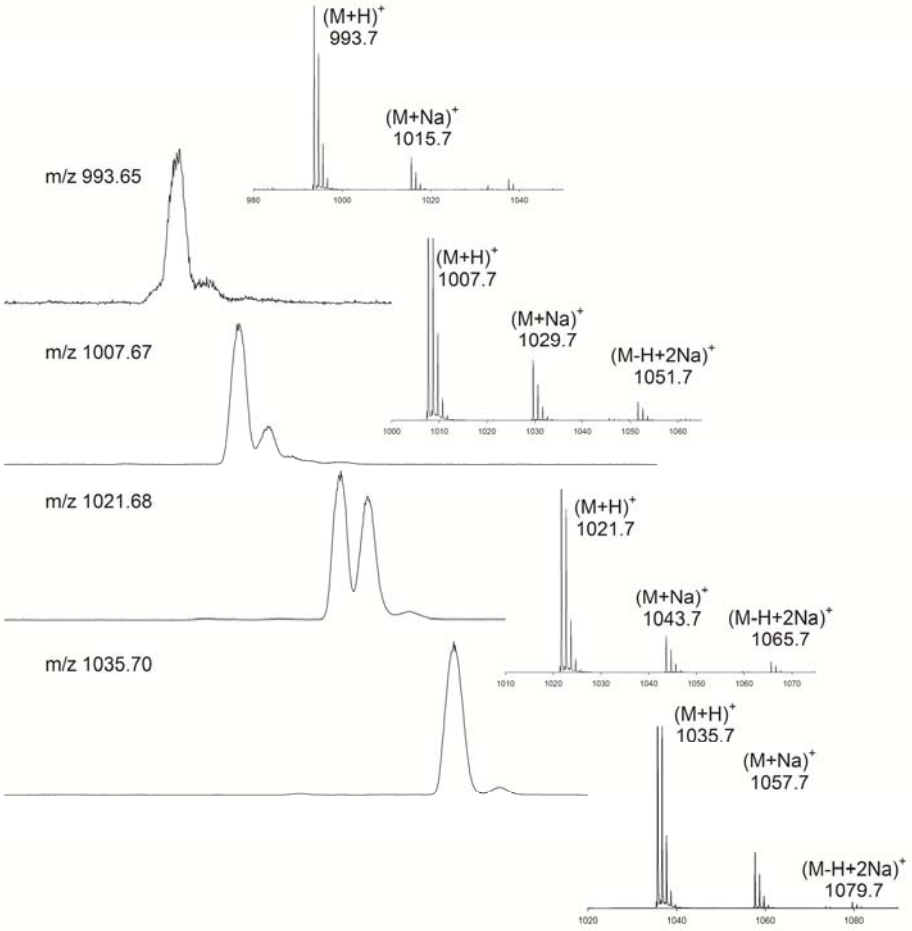

Figure 3: Q-TOF mass spectrum from lichenysin precursor m/z 1007.67 (*B. licheniformis* NVH 1115). Only the b1 and y1 ions are missing from the expected fragment ion series resulting from a ring opening and dehydration at the C-terminal end. From the fragment ion-series of ions resulting from ring opening and dehydration at the N-terminal end, only b2-H_2O and b3-H_2O and y3+$H_2$0, y4+H_2O, y5+H_2O and y6+H_2O are found. The latter is the most abundant fragment ion in the mass spectrum.

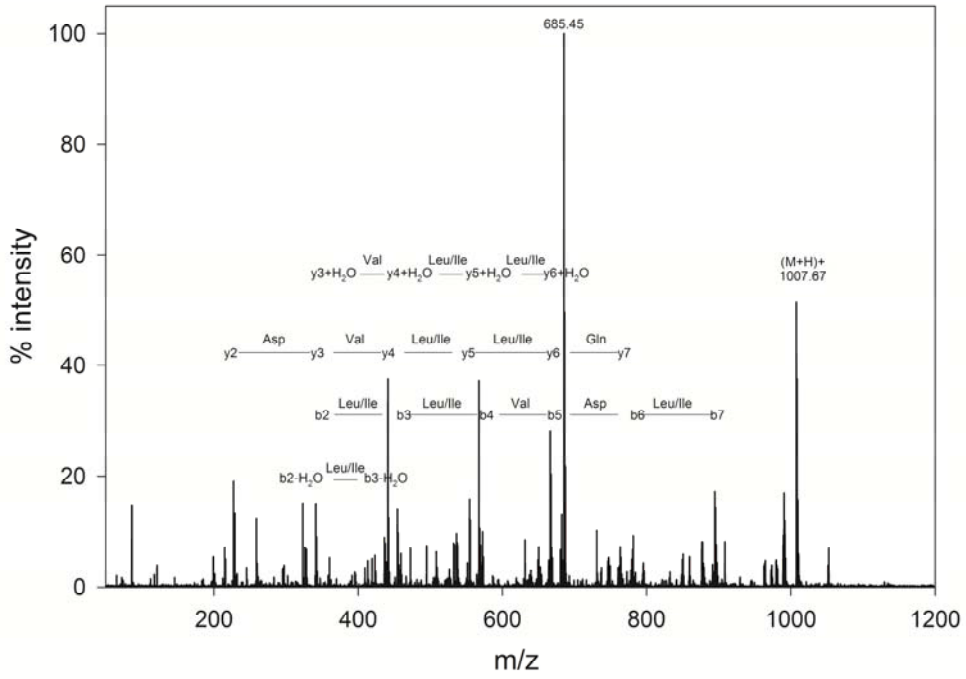

Figure 4: Product ion spectra from m/z 1021.68 A and B showing the complete b-ion series from the two different occurring isoforms: In m/z 1021 A is AA7 leucine, while in m/z 1021 B is both AA7 leucine and AA7 valine present. The most abundant fragment ion, y6 + H2O, represented by both m/z 671 and m/z 685 from precursor m/z 1021 B indicates the presence of both AA7 Leu and AA7 Val isoforms, whereas it is only present as the AA7 Leu isoform in m/z 1021 A.

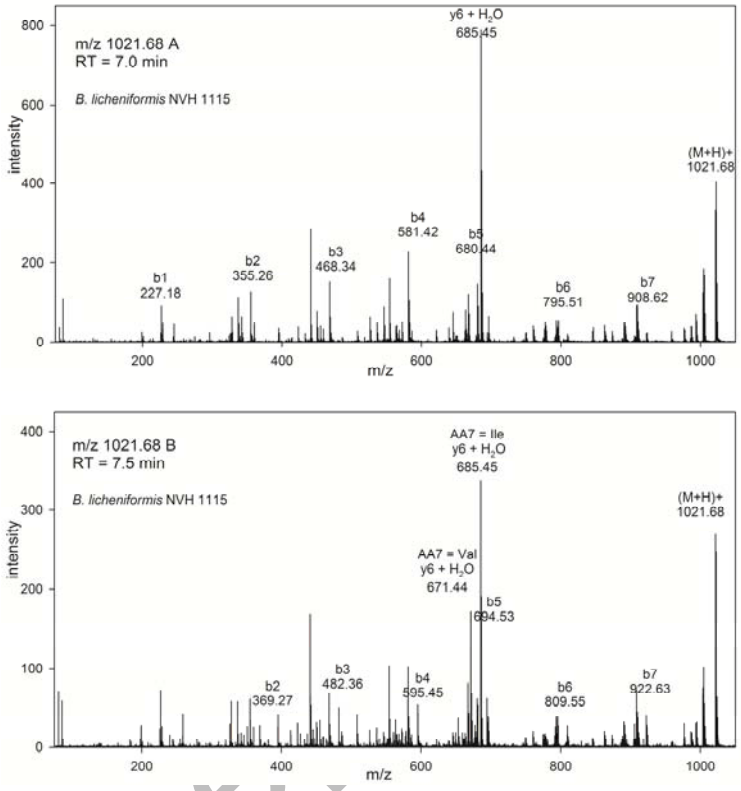

Figure 5: Peak intensity difference between C14-surfactin A and B and C14-lichenysin A and B. (Surfactin from *B. subtilis* and lichenysin from *B. licheniformis* ATCC 14580.)