Isotope dilution liquid chromatography-tandem mass spectrometry for simultaneous identification and quantification of beta-casomorphin 5 and beta-casomorphin 7 in yoghurt

D. D. Nguyen\textsuperscript{a}, V. A. Solah\textsuperscript{a}, S. K. Johnson\textsuperscript{a}, J. W. A. Charrois\textsuperscript{b} and F. Busetti\textsuperscript{b}\textsuperscript{*}

\textsuperscript{a}Food Science and Technology Program, School of Public Health Faculty of Health Sciences Curtin University, GPO Box U1987, Perth, Western Australia, 6845, Australia

\textsuperscript{b}Curtin Water Quality Research Centre, Department of Chemistry, Curtin University, GPO Box U1987, Perth, Western Australia, 6845, Australia

\textsuperscript{*}Corresponding Author: (Dr F. Busetti) Curtin Water Quality Research Centre, Department of Chemistry, Curtin University, GPO Box U1987, Perth, Western Australia 6845, Australia.

Tel.: +61 8 9266 3273; fax: +61 8 9266 2300; e-mail address: f.busetti@curtin.edu.au

Keywords: beta-casomorphin 5, beta-casomorphin 7, LC-MS/MS, yoghurt.

Abstract: A highly selective and sensitive liquid chromatography-tandem mass spectrometry method was developed and validated for the simultaneous identification and quantification of beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in yoghurt. The method used deuterium labelled BCM5-d\textsubscript{10} and BCM7-d\textsubscript{10} as surrogate standards for confident identification and accurate and quantitation of these analytes in yoghurt. Linear responses for BCM5 and BCM7 (\(R^2 = 0.9985\) and 0.9986, respectively) was observed in the range 0.01–10 ng/µL. The method limits of detection (MLDs) in yoghurt extracts were found to be 0.5 ng/g and 0.25 ng/g for BCM5 and BCM7, respectively. Analyses of spiked samples were used to provide confirmation of accuracy and precision of the analytical method. Recoveries relative to the surrogate standards of these spikes were in the range of 95 - 106 % for BCM5 and 103 - 109% for BCM7. Precision from analysis of spiked samples was expressed as relative
standard deviation (%RSD) and values were in the range 1-16% for BCM5 and 1-6% for BCM7. Inter-day reproducibility was between 2.0-6.4% for BCM5 and between 3.2-6.1% for BCM7. The validated isotope dilution LC-MS/MS method was used to measure BCM5 and BCM7 in ten commercial and laboratory prepared samples of yoghurt and milk. Neither BCM5 and BCM7 were detected in commercial yoghurts. However, they were observed in milk and laboratory prepared yoghurts and interestingly their levels decreased during processing. BCM5 decreased from 1.3 ng/g in milk to 1.1 ng/g in yoghurt made from that milk at 0 day storage and < MLQ at 1 and 7 day storage. BCM7 decreased from 1.9 ng/g in milk to < MLQ in yoghurts immediately after processing. These preliminary results indicate that fermentation and storage reduced BCM5 and BCM7 concentration in yoghurt.

1. Introduction

Beta-casomorphins (BCMs) are a group of structurally similar peptides containing a sequence of 4-11 amino acids (Kamiński, Cieślińska, & Kostyra, 2007). The first three amino acids, tyrosine, proline and phenyalanine in the peptide, are conserved (Muehlenkamp & Warthesen, 1996) and arise from enzymatic hydrolysis of beta-caseins (β-CN) (De Noni & Cattaneo, 2010). These peptides are released from the parent protein by cleavage at position 60 (tyrosine), and a second cleavage of residues at positions 63 to 70. For example, a peptide with cleavages at position 60 (tyrosine) and position 66 (isoleucine) is beta-casomorphin 7 (Fig. 1), which was first isolated from bovine casein (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). BCMs have morphine-like activity, and therefore are classified as opioid peptides (Kamiński et al., 2007). BCM5 and BCM7 (Fig. 1) have strong opioid activity (Brantl, Teschemacher, Bläsig, Henschen, & Lottspeich, 1981; Kálmán, Cserháti, Valkó, & Neubert, 1992; Kamiński et al., 2007). Epidemiological evidence suggests that consumption of milk containing A1 beta-casein, which releases BCM7 on hydrolysis, is linked to an
increased risk of type-1 diabetes and heart disease (Elliott, Harris, Hill, Bibby, & Wasmuth, 1999; Laugesen & Elliott, 2003; McLachlan, 2001). However, the European Food Safety Authority (EFSA) concluded there were insufficient data to determine a causal relationship between exposure to BCM7 and other related BCMs and non-communicable diseases (EFSA, 2009). Therefore, the reported presence of BCM5 and BCM7 in dairy products needs further research due to their putative link to elevated chronic disease risk.

BCM7 is found in bovine milk (Cieślińska, Kaminski, Kostyyra, & Sienkiewicz-Szłapka, 2007; Cieślińska et al., 2012), in human milk (Jarmołowska et al., 2007), in cheeses (De Noni & Cattaneo, 2010; Jarmolowska, Kostyra, Krawczuk, & Kostyra, 1999; Norris, Coker, Boland, & Hill, 2003; Sienkiewicz-Szłapka et al., 2009) and in commercial yoghurt (Jarmolowska, 2012). In contrast, De Noni & Cattaneo (2010) showed that BCM7 was not present in yoghurt purchased from a market. Therefore, the presence of BCM7 in yoghurt may be affected by processing steps in yoghurt manufacturing or levels may be below the limit of detection of previous analytical methods used.

Yoghurt is a popular dairy product usually fermented by two lactic acid bacteria (LAB), *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Tamime & Robinson, 1999). In addition to containing angiotensin-converting enzyme inhibitory (ACE-I) peptides (Donkor, Henriksson, Singh, Vasiljevic, & Shah, 2007; Kunda et al., 2012), antihypertensive peptides (Kunda et al., 2012; Muguerza et al., 2006; Schieber & Brückner, 2000), and antioxidant peptides (Sabeena Farvin, Baron, Nielsen, Otte, & Jacobsen, 2010), yoghurt may also contain BCM peptides (Jarmolowska, 2012). However, yoghurt is a complex food matrix (EFSA, 2009), in which protein, sugar or lactic acid can interfere with the identification and quantification of target analytes such as peptides.

Reverse-phase high performance liquid chromatography (RP-HPLC) coupled to an ultraviolet-visible (UV-Vis) detector is used widely for separation and quantification of
BCM5 and BCM7 in milk and dairy products (Jarmołowska et al., 2007; Muehlenkamp & Warthesen, 1996). A limitation of RP-HPLC-UV for detection and quantification of BCMs is that peptides with similar physico-chemical and spectrophotometric absorption properties can co-elute with BCM5 and BCM7, increasing their apparent absorption values (Muehlenkamp & Warthesen, 1996; Sienkiewicz-Szłapka et al., 2009) resulting in an overestimation of BCM7 (Cass et al., 2008; Sienkiewicz-Szłapka et al., 2009) and BCM5 concentration (Sienkiewicz-Szłapka et al., 2009). Additionally, analytical methods employing UV-Vis detector may lack the sensitivity required to quantify the low levels (e.g. 2 µg/ml of cheese extract) of BCM7 and BCM5 found in dairy products (Muehlenkamp & Warthesen, 1996).

More recently, enzyme–linked immune sorbent assays (ELISA) have been applied to detect and quantify BCM5 and BCM7 in bovine milk and dairy products (Sienkiewicz-Szłapka et al., 2009; Cieślińska et al., 2012; Jarmolowska, 2012). However, during milk processing, heat treatment of milk may modify the conformation of BCM7 by interaction between lactose and amino acid residues, leading to a reduction in the binding affinity of the modified BCM7 to the ELISA antibody, resulting in an underestimation of the BCM7 concentration (Cieślińska et al., 2012). RP-HPLC coupled with mass spectrometry (MS) represents “the-state-of-the-art” method for identification and quantification of peptides in complex matrices. Cass et al. (2008) applied matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) to analyse opioid-derived exogenous or endogenous peptides in urine, and found that peaks previously analysed by RP-HPLC-UV were erroneously identified as BCM7. Alternatively, tandem mass spectrometry (MS/MS) operating in multiple reaction monitoring (MRM) mode allows accurate quantification of BCM7 in plasma at low levels (ng/ml) (Song, Zaw, Amirkhani, Clarke, & Molloy, 2012).
Currently, there is no report in the literature on LC-MS/MS applications for the simultaneous quantification of BCM5 and BCM7 in different processing stages of yoghurt manufacture. The choice of calibration method plays an important role in LC-MS quantitative analysis. External calibration using standards can be used. However, this approach could result in ion suppression, leading to a decrease in the response of target analytes (Jessome & Volmer, 2006). In addition, using external standards requires that calibration samples are identical in composition to test samples, to compensate fully for matrix effects (Jessome & Volmer, 2006). To date, the use of UHT milk extract for dissolving synthesised BCM5 and BCM7 as calibration samples has been reported by De Noni and Cattaneo (2010) who used LC-MS/MS coupled to electrospray ionization (ESI) for quantifying BCM5 and BCM7 in yoghurt. The extracts of UHT milk, however, are not identical to those of yoghurt because after fermentation, many compounds in milk are degraded into different ones in yoghurt, for instance, lactose is degraded into lactic acid. Therefore, the difference in matrix may affect ion suppression, leading to variable results.

Inclusion of deuterated homologues in LC-MS/MS quantitative analysis is an alternative technique that allows easy identification and quantification of target analytes in complex matrices. Stable isotope-labelled compounds allow compensation for matrix effects and loss of target analytes during sample preparation, so their use can significantly reduce data variability and improve accuracy and precision of the analytical determination (Jessome & Volmer, 2006). Recently, Song et al. (2012) used LC-MS/MS and applied stable isotope-labelled BCM7 as the surrogate standard for quantitation of BCM7 in plasma at low concentrations (ng/ml). To date, however, there are no reported studies using stable isotope-labelled BCM7 and BCM5 as the surrogate standards for simultaneous determination of these peptides in yoghurt.
The aim of this study was to develop a sensitive and selective method for the accurate determination of BCM5 and BCM7 in yoghurt. For separation and detection, the method employed LC coupled to MS/MS operated in MRM mode. Stable isotope-labelled BCM5 and BCM7 were used as surrogate standards for confident identification and accurate and quantitation purposes. The LC-MS/MS method was also tested for the identification and quantitation of target BCMs in commercial and laboratory prepared yoghurts.

2. Materials and methods

2.1 Chemicals and materials

Yoghurt culture (YO-MIX™) containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* was purchased from Danisco Australia Pty Ltd (Botany, NSW, Australia). UHT milk and A1 skim milk powder were supplied by A2 Dairy Products Australia Pty Ltd (Botany, NSW, Australia). BCM5 (purity 97.8%), BCM7 (purity >98.7%) and the deuterated standards [^2]H10 BCM5 (BCM5-d_{10}) and [^2]H10 BCM7 (BCM7-d_{10}) - deuterium enrichment > 99%, ^2H enrichment at phenylalanine position - were obtained from Auspep Pty (Tullamarine, Victoria, Australia). Ultrapure water used for laboratory purposes as well as LC mobile phase was purified using an IBIS Technology (Perth, Australia) Ion Exchange System followed by Elga Purelab Ultra System (Sydney, Australia). Methanol (ChromAR HPLC grade) was purchased from Mallinckrodt (New Jersey, USA); formic acid (purity 99%) was purchased from Ajax FineChem (Sydney, Australia).

2.2 Solutions and calibration standards

Stock solutions of analytes of BCM5, BCM7 and deuterated standards BCM5-d_{10}, BCM7-d_{10} were prepared by dissolving 5 mg of each compound in a 5 ml flask of ultrapure water (nominal concentration of 1 mg/ml). Working stock solutions (10 ng/µL and 25 ng/µL) were
prepared by diluting stock solutions in ultrapure water. Serial mixed standard solutions of BCM5 and BCM7 ranging from 0.01 to 25 ng/μL (nominal concentrations 0.01, 0.025, 0.05, 0.1, 0.25, 0.75, 1, 2.5, 5, 7.5, 10, 12.5 and 25 ng/μL) were prepared from the working stock solutions. A working solution (nominal concentration of 10 ng/μL) of deuterated standards was also prepared by dilution of BCM5-d10 and BCM7-d10 stock solutions. All the solutions, as well as analytical and surrogate standards were stored at –20 ºC to minimise degradation.

2.3 Laboratory-prepared yoghurts

Yoghurt was prepared by inoculating YO-MIX™ into the UHT milk preheated at 43 °C in an incubator. The inoculated milk was then incubated at 43 °C until pH 4.6 was reached. After fermentation, yoghurt was cooled down and stored at 4 °C. This product was used for validation of analytical method and is referred to as “laboratory prepared yoghurt”. In order to prepare yoghurt for the application of analytical method, 65 grams of A1 skim milk powder was dissolved in 0.5 litre of deionised water that was pre-boiled for 10 minutes and then cooled down to 55 °C. After thorough mixing, 100 ml of reconstituted milk was placed into two tightly capped centrifuge tubes and stored at -20 °C prior to analysis. The rest of the reconstituted milk was cooled down to 43 °C and inoculated with 0.003% (w/v) of YO-MIX™. The inoculated milk was placed into seven centrifuge tubes, which were then capped, and fermented at 43°C until the pH reached the value of 4.6. One tube was used to measure pH during the course of fermentation. When pH reached the required value, two tubes were stored -20°C for further analysis. Two tubes were stored for 1 day and two others were stored for 7 days at 4 °C. All these tubes were stored -20 °C until extraction of water soluble peptides.

2.4 Extraction of water soluble peptides

The preparation of yoghurt extracts was carried out as described in Donkor et al. (2007) with some minor modifications. Ten grams of yoghurt were weighted (± 0.1g) into a centrifuge
tube, then centrifuged at 15,000×g at 4 °C for 30 min. The supernatant was decanted into a clean centrifuge tube and the extract was adjusted to pH 4.6 with 1M HCl (or 1M NaOH). After re-centrifugation at 14,000×g for 20 min at 4 °C, the supernatant was collected by decantation and filtered through a 0.45 μm membrane filter. The filtered extract was evaporated to a final volume of 1 mL using a gentle nitrogen flow over the extract surface. The concentrated extract was filtered through a 0.45 μm membrane filter into a vial.

2.5 Liquid chromatography-tandem mass spectrometry conditions

The LC-MS/MS system used in this study consisted of an 1100 Agilent (Palo Alto, CA, USA) LC system and a Micromass (Manchester, UK) Quattro Ultima Triple Quadrupole Mass spectrometer fitted with an electrospray ion source (ESI) operated in positive ionisation mode. Chromatographic separation was achieved using a Kinetex C18 LC-MS column (100 mm × 2.1 mm, 2.6 μm, 100 Å) from Phenomenex (Sydney, Australia) at a flow rate of 100 μL/min. A KrudKatcher Ultra HPLC in-line filter (0.5 μm depth filter x 0.004 inch ID) was used to protect the analytical column. The mobile phase was methanol (MeOH) (A) and ultrapure water (B) both containing 0.1% of formic acid. Chromatographic runs began with 30% (A) for 3 min, followed by a 15 min linear gradient to 100% (A). The mobile phase remained at 100% (A) for 15 min to elute analytes from the column. Afterwards, the initial conditions were re-established within 1 min and the column re-equilibrated for 15 min before injecting the next sample. To minimise potential carryover, before and after each injection, the needle of the injector was rinsed for 30 seconds in the injection port with MeOH. The injected volume was 5 μL.

Optimum MS signals were achieved by tuning the ESI capillary and cone voltages to 3.25 KV and 25 V, respectively. Hexapole1, aperture and hexapole2 were set to 0.0 V, 0.1 V and 0.2 V, respectively. Desolvation temperature and source temperature were 325 °C and 135 °C, respectively. Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as
desolvation and nebulizer gas; cone gas flow was set to 40 L/h, while the desolvation gas flow was set to 765 L/h. High purity Argon (99.997% purity) (BOC Gases, Perth, Australia) was used as collision gas (P = 2.7 × 10⁻⁴ kPa). Both quadrupoles (Q1 and Q3) were set at unit mass resolution; ion energy on Q1 and Q3 was set to 1.0, while the multiplier was set at 750 V. Surrogate standards, BCM5-d₁₀ and BCM7-d₁₀ were included in the analytical method to correct results for any losses encountered during sample extraction and to correct for matrix effects (Jessome & Volmer, 2006). Quantification was performed by rationing the peak area of the analytes to the peak area of the corresponding deuterated standards. Concentrations were calculated by QuanLynx 4.0 software comparing the peak area ratios from the extracts to peak area ratios from the calibration standards.

### 2.6 Validation of the analytical method

Evaluation of instrumental linearity, instrumental detection limits (IDLs) instrumental quantification limits (IQLs), peak identification criteria (retention time and MRM ratio), accuracy, precision, inter-day reproducibility, method limits of detection (MLDs), method limits of quantitation (MLQs), and matrix effects were undertaken to validate the analytical procedure.

#### 2.6.1 LC-MS/MS performance

Repetitive injections of standard solutions were used to assess LC-MS/MS instrument performance. The linear range was tested using calibration standards spanning from 0.01 ng/μL up to 25 ng/μL. Injections of low concentrations standard solutions were used to assess IDLs and IQLs. IDLs were estimated at signal-to-noise (S/N) ratio equal to 3 while IQLs were estimated at S/N=10. Repeat injections (n=15) of a solution at 1 ng/μL were used to determine the variability of the MRM ratio and of the retention time.

#### 2.6.2 Accuracy and precision
Accuracy and precision of the analytical method were assessed by recovery experiments of BCMs in yoghurt. Yoghurt samples (10 g) were spiked with standard solutions of BCM5 and BCM7 at five different levels of concentration (1, 10, 100, 1000 and 2500 ng/g) in triplicate. Surrogate standards were also spiked at a concentration level of 100 ng/g. Accuracy was expressed as recovery relative to the surrogate standards, while precision was expressed as relative standard deviation (%RSD). Blank samples (10 g of yoghurt spiked with 100 ng/g of deuterated standards) were also processed in triplicate along with each batch of samples for QA/QC purposes.

2.6.3 Inter-day reproducibility

Repeated measurements on two different days of three yoghurt samples spiked with 10, 100 and 1000 ng/g of BCM5 and BCM7 were conducted. The %RSD of these measurements was used to assess the inter-day reproducibility of the analytical method.

2.6.4 Method limit of detection and method limit of quantitation

Yogurt samples spiked with low levels of BCM5 and BCM7 (1 ng/g and 10 ng/g) were extracted and analysed; the results were used to estimate MLDs and MLQs. MLDs were calculated from the concentration equivalent to S/N of three, while MLQs were calculated from the concentration equivalent to S/N of ten (Busetti, Linge, Blythe, & Heitz, 2008; Foley & Dorsey, 1984) on smoothed chromatographic traces.

2.6.5 Evaluation of matrix effects

In order to study the degree of signal suppression caused by the presence of the matrix, blank yoghurt extracts were spiked at 1000 ng/mL with BCM5 and BCM7 and analysed by LC-MS/MS. Results were compared to injections of analytical standards at the same concentration levels. Matrix effects were calculated as followed (Eq. 1):

\[
\text{Matrix effects (\%)} = \frac{(\text{As}-\text{Am})}{\text{As}} \times 100 (1)
\]
Where: As is the area of the analyte spiked in ultrapure water (standard solution) and Am is the area of the analyte spiked in a yoghurt blank extract (matrix).

2.7 Analysis of commercial and laboratory prepared samples of yoghurt and reconstituted milk

Ten commercial yoghurts were purchased from a local supermarket located in Perth, Western Australia. Three yoghurts contained *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, other yoghurts contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. acidophilus* and Bifidobacterium or contain *L. acidophilus*, Bifidobacterium and *L. casei*. The expiry dates of all yoghurts ranged from 13 and 29 days. The samples were extracted immediately after purchasing.

Commercial and laboratory prepared yoghurts and reconstituted milk samples were prepared as described in Section 2.4. The laboratory prepared yoghurts and reconstituted milk were thawed at room temperature and thoroughly mixed before sampling. Ten grams of reconstituted milk was gradually acidified with 1M HCl to pH 4.6 and allowed to rest for 10 minutes. Before extraction, yoghurt and reconstituted milk samples were spiked with surrogate standards BCM5-\textsubscript{d\textsubscript{10}} and BCM7-\textsubscript{d\textsubscript{10}} at a concentration level of 100 ng/g. In addition, 10 g of yoghurt and 10 g of reconstituted milk spiked with both 1000 ng of BCM5-\textsubscript{d_{10}} and 1000 ng of BCM7-\textsubscript{d_{10}} as well as 100 ng of BCM5 and 100 ng of BCM7 were used as quality control samples (QCs). The QCs were extracted as described in Section 2.4. BCM5 and BCM7 were analysed using the validated LC-MS/MS method as described in Section 2.5. All samples were extracted and analysed in duplicate.

3. Results and discussions

3.1 Optimisation of MS/MS conditions
Solutions consisting of BCM5 and BCM7 at 5 ng/µL in MeOH:H₂O (50:50) with 0.1% of formic acid were infused into the mass spectrometer to optimise: 1) formation of protonated parent ions minimising secondary adducts; 2) fragmentation of selected parent ions (MS/MS) and 3) detection of product ions in MRM mode.

Initial experiments were conducted in MS scan mode (50-1000 m/z). In the presence of 0.1% of formic acid, the most intense precursor ion observed for BCM5 and BCM7 was the proton adduct [M+H]^+; other characteristic ESI precursor ions such as the sodium or MeOH adducts usually present in non-acidic/methanolic solutions were substantially weaker. The [M+H]^+ precursor ions were therefore selected for further MS/MS experiments. Fragmentation experiments were conducted in product ion scan mode. While the MS/MS spectra of BCM7 have been reported previously (De Noni, 2008), the fragmentation spectrum of BCM5 is reported in Fig. 2 along with assignment of the main product ions observed.

After optimisation of the collision energy, which controls the degree of fragmentation of the parent compound in the collision cell (Q2), the most intense characteristic transitions were identified from the product ion spectra (data not shown). Two MRM transitions (one parent ion fragmenting into two product ions) were used for the identification of each compound and surrogate standard (Table 1). This approach provides four identification points, which are sufficient to fulfil the criteria for identifying and confirming the presence of a target analyte in food matrices (EC, 2002). In addition, the MRM ratio and retention time (tᵣ) were also monitored against standard solutions for additional confidence.

3.2 Instrumental performance and peaks identification criteria

Injections of standard solutions were used to assess LC-MS/MS instrument performance. An example of a LC-MS/MS chromatogram of a 1 ng/µL standard solution of BCM5, BCM5-d₁₀, BCM7 and BCM7-d₁₀ is showed in Fig. 3.
The linear range was tested using calibration standards spanning from 0.01 ng/μL (0.05 ng on-column) to 25 ng/μL (125 ng on-column). For both BCM5 and BCM7 tested, calibration curves showed good linearity in the range 0.05-50 ng on-column, with \( R^2 \) values typically higher than 0.9985 (Table 2). Injections of low concentrations standard solutions were used to assess IDLs and IQLs. IDLs for BCM7 and BCM5, estimated at S/N ratio equal to 3, ranged from 0.007 to 0.010 ng on-column, respectively. For BCM7 and BCM5, IQLs were estimated at S/N=10, and ranged from 0.024 to 0.036 ng on-column, respectively. Repeat injections (n=15) of a solution at 1 ng/μL were used to determine the variability of the MRM ratio and of the retention time. MRM ratio variability, measured as %RSD, was less than 8.8\% indicating reproducible fragmentation of the parent ions in the collision cell; \( t_R \) variability, measured as standard deviation (SD) was less than 12 seconds, indicating a reproducible chromatographic separation.

3.3 Accuracy and precision

Accuracy and precision of the analytical method were determined by experiments on yoghurt samples spiked with increasing concentration of BCM5 and BCM7. Accuracy was expressed as recovery percentage relative to the surrogate standards while precision was expressed as %RSD. Accuracy was in the range 95 - 106 % for BCM5 and 103 - 109% for BCM7 (Table 3). Relative standard deviation of these recoveries was between 1-6% for BCM7, and 1-7% for BCM5 exception for the value at spiked concentration of 1 ng/g (%RSD = 16) (Table 3). No corrections to recovery were necessary since blank samples were found not to contain BCM5 and BCM7 in detectable amounts.

3.4 Inter-day reproducibility

The inter-day reproducibility of the LC-MS/MS analytical method was determined by repeated measurements on two different days of three yoghurt samples spiked with different...
amount of BCM5 and BCM7 (Table 3). Relative standard deviation values were 6.4% and 6.1% for BCM5 and BCM7 respectively; indicating good reproducibility of the method.

3.5 Method limits of detection (MLDs) and method limits of quantitation (MLQs)

Average sample based MLDs and MLQs in yoghurt extracts were calculated from analysis of samples spiked with 1 ng/g and 10 ng/g of BCM5 and BCM7. MLDs were found to be 0.5 ng/g for BCM5 and 0.25 ng/g for BCM7. Meanwhile, MLQs for BCM5 and BCM7 were found to be 1.7 ng/g and 0.85 ng/g, respectively. These results are difficult to compare with published methods because, to the best of our knowledge, MLDs and MLQs for BCM5 and BCM7 in yoghurt have not been reported previously (Nguyen, Johnson, Busetti, & Solah, 2013).

3.6 Evaluation of matrix effects affecting detection of BCM5 and BCM7 in yoghurt

The efficiency of ESI sources to ionise polarisable analytes in real samples, is often affected by the matrix, which can be responsible for suppressing the absolute response of analytes when analysed in LC-MS/MS. If no corrections are made, matrix effects often lead to an underestimation in the concentration of the analytes of interest in real samples. In order to address matrix effects, different approaches have been proposed (Jessome & Volmer, 2006). For example, De Noni and Cattaneo, (2010) reported the use of matrix matched calibration standards to take into account the presence of the matrix when quantifying BCM7 in UHT milk. This is a valid and effective method to account for matrix effects especially when deuterated standards are unavailable or prohibitively expensive.

In the present work, matrix effects were corrected for with the inclusion of deuterated homologues. This approach is by far the most effective method to account for matrix effects as the analytes and the corresponding co-eluting deuterated homologues are theoretically subject to identical matrix effects. In order to study the degree of signal suppression caused
by the presence of the matrix, blank yoghurt extracts were spiked at 1000 ng/mL with BCM5 and BCM7 and analysed by LC-MS/MS. Results were compared to injected analytical standards at the same concentration levels. Matrix effects were responsible for causing about 26% signal suppression for BCM5 and about 40% signal suppression for BCM7. It is possible that additional clean up (e.g. on-line or off-line solid-phase extraction) for sample matrix would decrease observed signal suppression, which could improve detection limits.

3.7 Application of the validated method

The validated method was applied to the identification and quantification of BCM5 and BCM7 in ten commercial yoghurts, laboratory prepared yoghurt and reconstituted milk. Neither BCM5 nor BCM7 was detected in commercial products, however both peptides were identified and quantified in reconstituted milk and laboratory prepared yoghurts. The content of BCM5 decreased from 1.3 ng/g in milk to 1.1 ng/g in yoghurt at 0 day storage and to less than the MLQ at 1 and 7 day storage. BCM7 content decreased from 1.9 ng/g in milk to less than the MLQ in yoghurts at all storage days (Table 5). It appears, BCM5 and BCM7 present in the milk have degraded into small peptides during fermentation and storage of yoghurt.

Recently, there have been a few studies measuring BCM5 and BCM7 in commercial yoghurts. De Noni and Cattaneo (2010) attempted to measure both peptides in these products, but neither BCM5 nor BCM7 was found. Nevertheless, BCM7 has been quantified between 0.86 to 2.45 µg/g in natural and probiotic yoghurt, levels decrease considerably during storage (Jarmolowska, 2012).

Kunda et al. (2012) have identified several di-peptides such as β-CN fragment 60-61 and β-CN fragment 62-63 in commercial yoghurt. As can been seen in Fig.1, amino acids at position 61 and 63 on the chain of parent β-CNs are proline residues. This finding shows that yoghurt bacterial enzymes are likely to digest the peptide bonds between proline and other
amino acid residues, leading to degradation of BCM5 and BCM7 during storage. The degradation of these peptides during yoghurt processing may involve the activity of X-prolyl dipeptidyl aminopeptidase (PepX), which is an enzyme derived from LAB that specifically hydrolyses peptide bonds between proline and other residues (Gobbetti, 2002). Furthermore, PepX activity in dairy products fermented with LAB has demonstrated to increase during storage (Otte, Lenhard, Flambard & Sørensen, 2011).

4. Conclusions

A highly selective and sensitive analytical method employing LC-MS/MS was successfully developed and validated for the simultaneous determination of BCM5 and BCM7 in a complex matrix, yoghurt. For the highest confidence in the identification and quantification of BCM5 and BCM7, two isotopically labelled homologues were included in the method as surrogate standards. The method showed very low MLQs, (1.7 ng/g for BCM5 and 0.85ng/g for BCM7) making it suitable for the analysis of the low level of BCMs in yoghurt. This validated method was applied to analyse BCMs in commercial and laboratory prepared yoghurts and in milk. The absence of BCM5 and BCM7 in commercial yoghurts tested may have been due to degradation of these peptides during processing or storage. In the laboratory prepared yoghurt and milk tested, fermentation and storage lowered the content of BCM5 and BCM7.

Acknowledgements

This study was a part of PhD project sponsored by the Vietnamese Government and Curtin University. The authors also thank A2 Dairy products Australia Pty Ltd for providing UHT milk and skim milk powder.
References


Table 1. Mass spectrometry: precursor ions, product ions and collision energy values optimised for the analysis of BCM5 and BCM7 under ESI(+) MS/MS in Multiple Reaction Monitoring (MRM) mode. MRM transitions were grouped in one window. Dwell time was 80 ms for all the transitions monitored.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Precursor ion</th>
<th>Product ions</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(m/z)</td>
<td>(m/z)</td>
<td>(arbitrary units)</td>
</tr>
<tr>
<td>BCM5</td>
<td>580.4</td>
<td>173.3(^a)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>408.4</td>
<td>25</td>
</tr>
<tr>
<td>BCM5-d(_{10})</td>
<td>590.4</td>
<td>173.3(^a)</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>418.4</td>
<td>25</td>
</tr>
<tr>
<td>BCM7</td>
<td>790.7</td>
<td>229.3(^a)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>530.4</td>
<td>35</td>
</tr>
<tr>
<td>BCM7-d(_{10})</td>
<td>800.7</td>
<td>229.3(^a)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>540.4</td>
<td>35</td>
</tr>
</tbody>
</table>

\(^a\)The product ions from the MRM transitions used for quantification.
Table 2. Instrument performance parameters obtained by repeat injections of standard solutions into the LC-MS/MS system.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Linear range&lt;sup&gt;a&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>IDL&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>IQL&lt;sup&gt;a,c&lt;/sup&gt;</th>
<th>MRM ratio (± %RSD)</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCM-5</td>
<td>0.05–50</td>
<td>0.9985</td>
<td>0.010</td>
<td>0.036</td>
<td>3.0±7.8</td>
<td>18.2±0.2</td>
</tr>
<tr>
<td>BCM-7</td>
<td>0.05–50</td>
<td>0.9986</td>
<td>0.007</td>
<td>0.024</td>
<td>2.6±8.8</td>
<td>20.6±0.1</td>
</tr>
</tbody>
</table>

<sup>a</sup>ng on-column; <sup>b</sup>IDL: Instrumental Detection Limit; <sup>c</sup>IQL: Instrumental Quantitation Limit; MRM: Multiple Reaction Monitoring; t<sub>R</sub>: Retention Time.
Table 3. Recovery and precision study for BCM5 and BCM7.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Spiked concentration (ng/g)</th>
<th>Recovery (%)</th>
<th>Precision (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCM5</td>
<td>1</td>
<td>106</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>104</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>106</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>95</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>97</td>
<td>1</td>
</tr>
<tr>
<td>BCM7</td>
<td>1</td>
<td>103</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>109</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>107</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>106</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>104</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 4. Inter-day reproducibility data determined by repeat measurements on two different days of yoghurt samples (n=3) spiked at three different concentrations of BCM5 and BCM7.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Nominal spiked concentration (ng/g)</th>
<th>Analysis day 1 (ng/g)</th>
<th>Analysis day 2 (ng/g)</th>
<th>Average (ng/g)</th>
<th>Inter-day reproducibility (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCM5</td>
<td></td>
<td>n=3</td>
<td>n=3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.4</td>
<td>11.2</td>
<td>10.8</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>106</td>
<td>106</td>
<td>106</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>950</td>
<td>1000</td>
<td>974</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>BCM7</td>
<td></td>
<td>n=3</td>
<td>n=3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10.7</td>
<td>11</td>
<td>10.8</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>110</td>
<td>111</td>
<td>110</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1040</td>
<td>1080</td>
<td>1060</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>
Table 5. BCM5 and BCM7 concentrations in reconstituted milk and laboratory prepared yoghurts. Results for Quality Control (QC) samples are also reported.

<table>
<thead>
<tr>
<th>Samples</th>
<th>BCM5 (ng/g)</th>
<th>BCM7 (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted milk</td>
<td>1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yogurt, 0 day storage</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;MLQ</td>
</tr>
<tr>
<td>Yogurt, 1 day storage</td>
<td>&lt;MLQ</td>
<td>&lt;MLQ</td>
</tr>
<tr>
<td>Yogurt, 7 day storage</td>
<td>&lt;MLQ</td>
<td>&lt;MLQ</td>
</tr>
<tr>
<td>QC (reconstituted milk)</td>
<td>94±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>93±1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>QC (yoghurt produced from reconstituted milk)</td>
<td>95 ±1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98±1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean values, n=2; <sup>b</sup> recoveries (%) are the average ± RSD% of n=2 milk samples spiked at 100 ng/mL; <sup>c</sup> recoveries (%) are the average ± RSD% of n=2 yoghurt samples made from reconstituted milk spiked at 100 ng/g.
Fig. 1. Structure of BCM5 (A) and BCM7 (B) (adapted from Juan-García, Font, Juan, & Picó, 2009).
Fig. 2. Product ions spectrum showing the fragmentation of BCM5 obtained at variable collision energy values. The amino acids tyrosine (Tyr), proline (Pro), phenylalanine (Phe), and glycine (Gly) are also indicated in the chemical structure of BCM5.
Fig. 3. Typical LC-MS/MS chromatograms of BCM5 and BCM7 and their corresponding deuterated homologues BCM5-d$_{10}$ and BCM7-d$_{10}$. The ion source was an ESI operated in positive ion mode, while the mass spectrometer was a triple quadrupole operated in MRM mode.