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

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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_{10} and BCM7-d_{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-CNs) (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).

99 Currently, there is no report in the literature on LC-MS/MS applications for the simultaneous
100 quantification of BCM5 and BCM7 in different processing stages of yoghurt manufacture.

101 The choice of calibration method plays an important role in LC-MS quantitative analysis.
102 External calibration using standards can be used. However, this approach could result in ion
103 suppression, leading to a decrease in the response of target analytes (Jessome & Volmer,
104 2006). In addition, using external standards requires that calibration samples are identical in
105 composition to test samples, to compensate fully for matrix effects (Jessome & Volmer,
106 2006). To date, the use of UHT milk extract for dissolving synthesised BCM5 and BCM7 as
107 calibration samples has been reported by De Noni and Cattaneo (2010) who used LC-MS/MS
108 coupled to electrospray ionization (ESI) for quantifying BCM5 and BCM7 in yoghurt. The
109 extracts of UHT milk, however, are not identical to those of yoghurt because after
110 fermentation, many compounds in milk are degraded into different ones in yoghurt, for
111 instance, lactose is degraded into lactic acid. Therefore, the difference in matrix may affect
112 ion suppression, leading to variable results.

113 Inclusion of deuterated homologues in LC-MS/MS quantitative analysis is an alternative
114 technique that allows easy identification and quantification of target analytes in complex
115 matrices. Stable isotope-labelled compounds allow compensation for matrix effects and loss
116 of target analytes during sample preparation, so their use can significantly reduce data
117 variability and improve accuracy and precision of the analytical determination (Jessome &
118 Volmer, 2006). Recently, Song et al. (2012) used LC-MS/MS and applied stable isotope-
119 labelled BCM7 as the surrogate standard for quantitation of BCM7 in plasma at low
120 concentrations (ng/ml). To date, however, there are no reported studies using stable isotope-
121 labelled BCM7 and BCM5 as the surrogate standards for simultaneous determination of these
122 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^{TM}) 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%, ^{2}H 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

147 prepared by diluting stock solutions in ultrapure water. Serial mixed standard solutions of
148 BCM5 and BCM7 ranging from 0.01 to 25 ng/µL (nominal concentrations 0.01, 0.025, 0.05,
149 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
150 solutions. A working solution (nominal concentration of 10 ng/µL) of deuterated standards
151 was also prepared by dilution of BCM5-d_{10} and BCM7-d_{10} stock solutions. All the solutions,
152 as well as analytical and surrogate standards were stored at –20 ºC to minimise degradation.

153 *2.3 Laboratory-prepared yoghurts*

154 Yoghurt was prepared by inoculating YO-MIX^{TM} into the UHT milk preheated at 43 ^{0}C in an
155 incubator. The inoculated milk was then incubated at 43 ^{0}C until pH 4.6 was reached. After
156 fermentation, yoghurt was cooled down and stored at 4 ^{0}C. This product was used for
157 validation of analytical method and is referred to as "laboratory prepared yoghurt". In order
158 to prepare yoghurt for the application of analytical method, 65 grams of A1 skim milk
159 powder was dissolved in 0.5 litre of deionised water that was pre-boiled for 10 minutes and
160 then cooled down to 55 ^{0}C. After thorough mixing, 100 ml of reconstituted milk was placed
161 into two tightly capped centrifuge tubes and stored at -20 ^{0}C prior to analysis. The rest of the
162 reconstituted milk was cooled down to 43 ^{0}C and inoculated with 0.003% (w/v) of YO-
163 MIX^{TM}. The inoculated milk was placed into seven centrifuge tubes, which were then capped,
164 and fermented at 43^{0}C until the pH reached the value of 4.6. One tube was used to measure
165 pH during the course of fermentation. When pH reached the required value, two tubes were
166 stored -20 ^{0}C for further analysis. Two tubes were stored for 1 day and two others were stored
167 for 7 days at 4 ^{0}C. All these tubes were stored -20 ^{0}C until extraction of water soluble
168 peptides.

169 *2.4 Extraction of water soluble peptides*

170 The preparation of yoghurt extracts was carried out as described in Donkor et al. (2007) with
171 some minor modifications. Ten grams of yoghurt were weighted (± 0. 1g) into a centrifuge

tube, then centrifuged at 15,000×g at 4 ^{0}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 ^{0}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 ºC and 135 ºC, 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 ($P = 2.7 \times 10^{-4}$ 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-d_{10} and BCM7-d_{10} 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

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

229 2.6.3 Inter-day reproducibility

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

233 2.6.4 Method limit of detection and method limit of quantitation

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

239 2.6.5 Evaluation of matrix effects

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

244 Matrix effects (%) = (As-Am)/As X 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-d_{10} and BCM7-d_{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-d_{10} and 1000 ng of BCM7-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

269 Solutions consisting of BCM5 and BCM7 at 5 ng/µL in MeOH:H_2O (50:50) with 0.1% of
270 formic acid were infused into the mass spectrometer to optimise: 1) formation of protonated
271 parent ions minimising secondary adducts; 2) fragmentation of selected parent ions (MS/MS)
272 and 3) detection of product ions in MRM mode.

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

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

289 *3.2 Instrumental performance and peaks identification criteria*

290 Injections of standard solutions were used to assess LC-MS/MS instrument performance. An
291 example of a LC-MS/MS chromatogram of a 1 ng/µL standard solution of BCM5, BCM5-
292 d_{10}, BCM7 and BCM7-d_{10} is showed in Fig. 3.

293 The linear range was tested using calibration standards spanning from 0.01 ng/μL (0.05 ng
294 on-column) to 25 ng/μL (125 ng on-column). For both BCM5 and BCM7 tested, calibration
295 curves showed good linearity in the range 0.05-50 ng on-column, with R^2 values typically
296 higher than 0.9985 (Table 2). Injections of low concentrations standard solutions were used
297 to assess IDLs and IQLs. IDLs for BCM7 and BCM5, estimated at S/N ratio equal to 3,
298 ranged from 0.007 to 0.010 ng on-column, respectively. For BCM7 and BCM5, IQLs were
299 estimated at S/N=10, and ranged from 0.024 to 0.036 ng on-column, respectively. Repeat
300 injections (n=15) of a solution at 1 ng/μL were used to determine the variability of the MRM
301 ratio and of the retention time. MRM ratio variability, measured as %RSD, was less than
302 8.8% indicating reproducible fragmentation of the parent ions in the collision cell; t_R
303 variability, measured as standard deviation (SD) was less than 12 seconds, indicating a
304 reproducible chromatographic separation.

305 *3.3 Accuracy and precision*

306 Accuracy and precision of the analytical method were determined by experiments on yoghurt
307 samples spiked with increasing concentration of BCM5 and BCM7. Accuracy was expressed
308 as recovery percentage relative to the surrogate standards while precision was expressed as
309 %RSD. Accuracy was in the range 95 - 106 % for BCM5 and 103 - 109% for BCM7 (Table
310 3). Relative standard deviation of these recoveries was between 1-6% for BCM7, and 1-7%
311 for BCM5 exception for the value at spiked concentration of 1 ng/g (%RSD = 16) (Table 3).
312 No corrections to recovery were necessary since blank samples were found not to contain
313 BCM5 and BCM7 in detectable amounts.

314 *3.4 Inter-day reproducibility*

315 The inter-day reproducibility of the LC-MS/MS analytical method was determined by
316 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.

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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.

Compounds	Precursor ion (*m/z*)	Product ions (*m/z*)	Collision energy (arbitrary units)
BCM5	580.4	173.3[a]	30
		408.4	25
BCM5-d_{10}	590.4	173.3[a]	30
		418.4	25
BCM7	790.7	229.3[a]	50
		530.4	35
BCM7-d_{10}	800.7	229.3[a]	50
		540.4	35

[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.

Compounds	Linear range[a]	R^2	IDL[a,b] S/N = 3	IQL[a,c] S/N = 10	MRM ratio (± %RSD)	t_R (min±SD)
BCM-5	0.05–50	0.9985	0.010	0.036	3.0±7.8	18.2±0.2
BCM-7	0.05–50	0.9986	0.007	0.024	2.6±8.8	20.6±0.1

[a]ng on-column; [b] IDL: Instrumental Detection Limit; [c] IQL: Instrumental Quantitation Limit; MRM: Multiple Reaction Monitoring; t_R: Retention Time.

516 **Table 3.** Recovery and precision study for BCM5 and BCM7.

Compounds	Spiked concentration (ng/g)	Recovery (%) n=3	Precision (%RSD) n=3
BCM5	1	106	16
	10	104	7
	100	106	2
	1000	95	2
	2500	97	1
BCM7	1	103	5
	10	109	4
	100	107	6
	1000	106	1
	2500	104	1

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524 **Table 4.** Inter-day reproducibility data determined by repeat measurements on two different
525 days of yoghurt samples (n=3) spiked at three different concentrations of BCM5 and BCM7.

Compounds	Nominal spiked concentration (ng/g)	Analysis day 1 (ng/g) n=3	Analysis day 2 (ng/g) n=3	Average (ng/g)	Inter-day reproducibility (%RSD)
BCM5	10	10.4	11.2	10.8	6.4
	100	106	106	106	2.0
	1000	950	1000	974	3.6
BCM7	10	10.7	11	10.8	6.1
	100	110	111	110	3.4
	1000	1040	1080	1060	3.2

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Table 5. BCM5 and BCM7 concentrations in reconstituted milk and laboratory prepared yoghurts. Results for Quality Control (QC) samples are also reported.

Samples	BCM5 (ng/g)	BCM7 (ng/g)
Reconstituted milk	1.3[a]	1.9[a]
Yogurt, 0 day storage	1.1[a]	<MLQ
Yoghurt, 1 day storage	<MLQ	<MLQ
Yoghurt, 7 day storage	<MLQ	<MLQ
QC (reconstituted milk)	94±3[b]	93±1[b]
QC (yoghurt produced from reconstituted milk)	95 ±1[c]	98±1[c]

[a]mean values, n=2; [b]recoveries (%) are the average ± RSD% of n=2 milk samples spiked at 100 ng/mL; [c] 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).

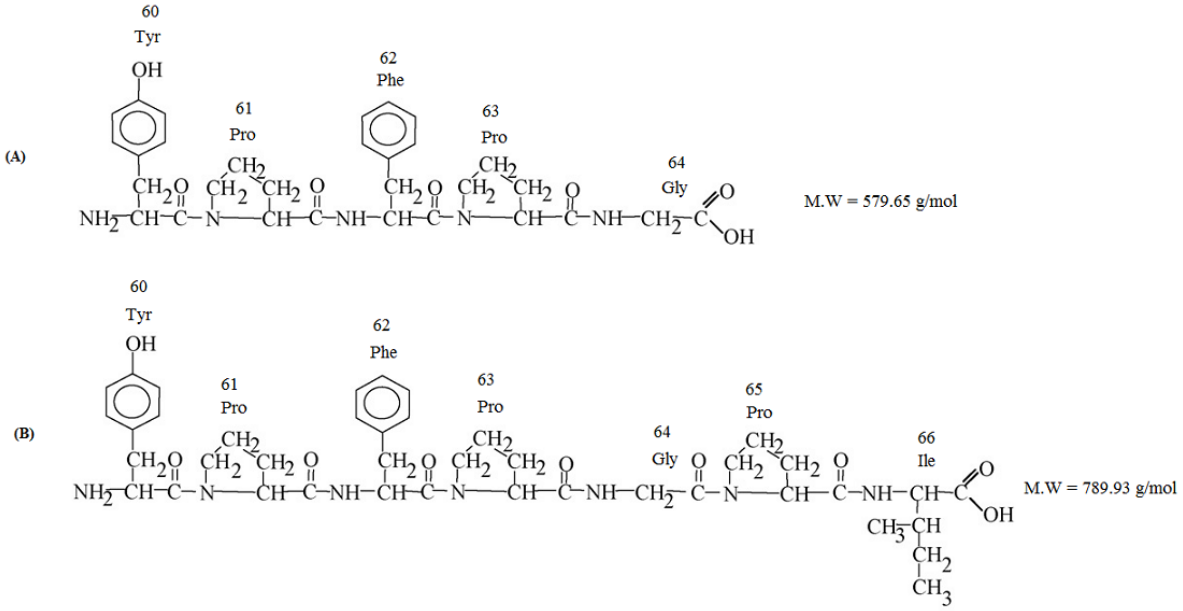

Fig. 2. Product ions spectrum showing the fragmentation of BCM5 obtained at variable collision energy values. The amino acids tyrosine (Tyr), proline (Pro), phenylalanine (Phe), and glycine (Gly) are also indicated in the chemical structure of BCM5.

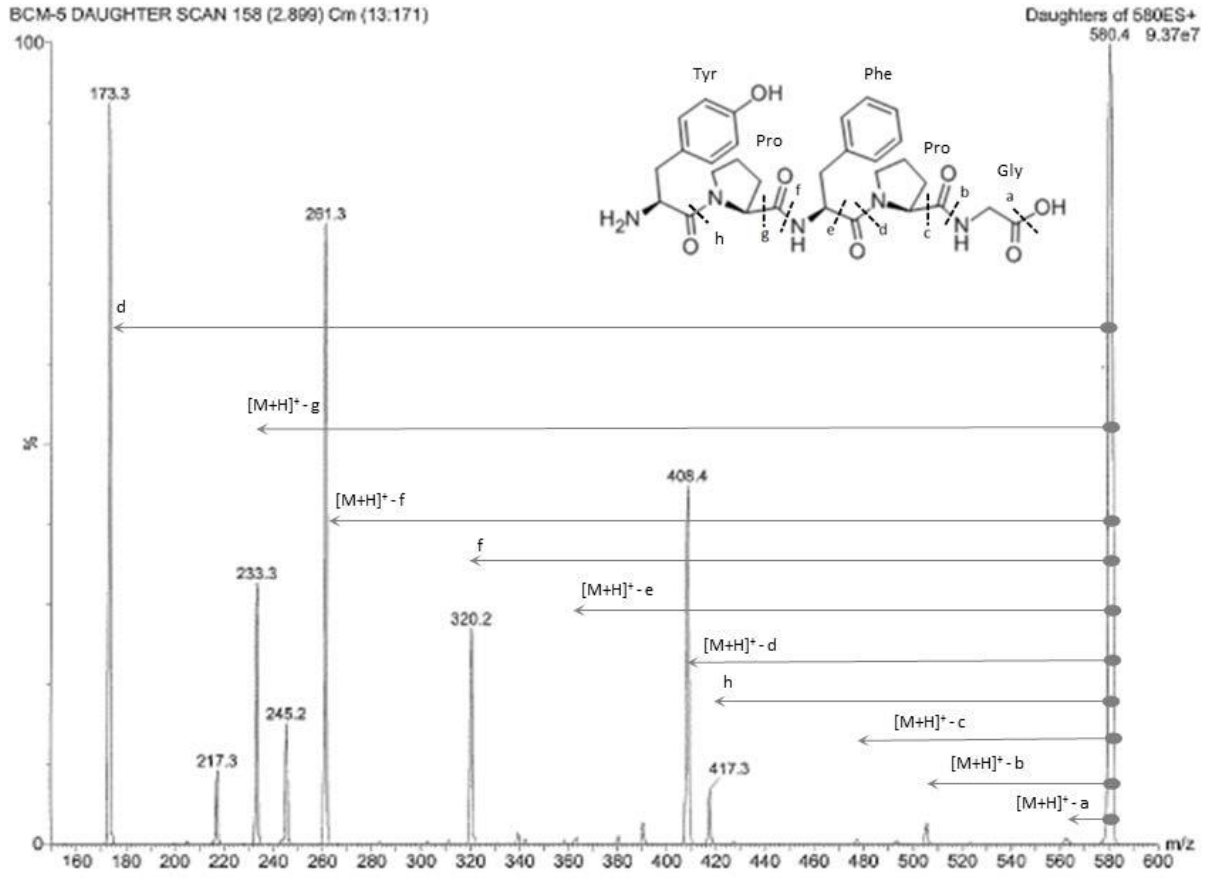

Fig. 3. Typical LC-MS/MS chromatograms of BCM5 and BCM7 and their corresponding deuterated homologues BCM5-d_{10} and BCM7-d_{10}. The ion source was an ESI operated in positive ion mode, while the mass spectrometer was a triple quadrupole operated in MRM mode.