1   **Isotope dilution liquid chromatography-tandem mass spectrometry for simultaneous**

2   **identification and quantification of beta-casomorphin 5 and beta-casomorphin 7 in**

3   **yoghurt**

4   **D. D. Nguyen[a], V. A. Solah[a], S. K. Johnson[a], J. W. A. Charrois[b] and F. Busetti[b*]**

5   [a]Food Science and Technology Program, School of Public Health Faculty of Health Sciences

6   Curtin University, GPO Box U1987, Perth, Western Australia, 6845, Australia

7   [b]Curtin Water Quality Research Centre, Department of Chemistry, Curtin University, GPO

8   Box U1987, Perth, Western Australia, 6845, Australia

9   [*]**Corresponding Author**: (Dr F. Busetti) Curtin Water Quality Research Centre, Department

10   of Chemistry, Curtin University, GPO Box U1987, Perth, Western Australia 6845, Australia.

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

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13

14   **Abstract:** A highly selective and sensitive liquid chromatography-tandem mass spectrometry

15   method was developed and validated for the simultaneous identification and quantification of

16   beta-casomorphin 5 (BCM5) and beta-casomorphin 7 (BCM7) in yoghurt. The method used

17   deuterium labelled BCM5-d_{10} and BCM7-d_{10} as surrogate standards for confident

18   identification and accurate and quantitation of these analytes in yoghurt. Linear responses for

19   BCM5 and BCM7 (R^2 = 0.9985 and 0.9986, respectively) was observed in the range 0.01–10

20   ng/µL. The method limits of detection (MLDs) in yoghurt extracts were found to be 0.5 ng/g

21   and 0.25 ng/g for BCM5 and BCM7, respectively. Analyses of spiked samples were used to

22   provide confirmation of accuracy and precision of the analytical method. Recoveries relative

23   to the surrogate standards of these spikes were in the range of 95 - 106 % for BCM5 and 103

24   - 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.

123 The aim of this study was to develop a sensitive and selective method for the accurate

124 determination of BCM5 and BCM7 in yoghurt. For separation and detection, the method

125 employed LC coupled to MS/MS operated in MRM mode. Stable isotope-labelled BCM5 and

126 BCM7 were used as surrogate standards for confident identification and accurate and

127 quantitation purposes. The LC-MS/MS method was also tested for the identification and

128 quantitation of target BCMs in commercial and laboratory prepared yoghurts.

129

130 **2.   Materials and methods**

131 *2.1 Chemicals and materials*

132 Yoghurt culture (YO-MIX^{TM}) containing *Streptococcus thermophilus* and *Lactobacillus*

133 *delbrueckii* ssp. *bulgaricus* was purchased from Danisco Australia Pty Ltd (Botany, NSW,

134 Australia). UHT milk and A1 skim milk powder were supplied by A2 Dairy Products

135 Australia Pty Ltd (Botany, NSW, Australia). BCM5 (purity 97.8%), BCM7 (purity >98.7%)

136 and the deuterated standards [^{2}H10] BCM5 (BCM5-d$_{10}$) and [^{2}H10] BCM7 (BCM7-d$_{10}$) -

137 deuterium enrichment > 99%, ^{2}H enrichment at phenylalanine position - were obtained from

138 Auspep Pty (Tullamarine, Victoria, Australia). Ultrapure water used for laboratory purposes

139 as well as LC mobile phase was purified using an IBIS Technology (Perth, Australia) Ion

140 Exchange System followed by Elga Purelab Ultra System (Sydney, Australia). Methanol

141 (ChromAR HPLC grade) was purchased from Mallinckrodt (New Jersey, USA); formic acid

142 (purity 99%) was purchased from Ajax FineChem (Sydney, Australia).

143 *2.2 Solutions and calibration standards*

144 Stock solutions of analytes of BCM5, BCM7 and deuterated standards BCM5-d$_{10}$, BCM7-d$_{10}$

145 were prepared by dissolving 5 mg of each compound in a 5 ml flask of ultrapure water

146 (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 ℃ 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

172   tube, then centrifuged at 15,000×g at 4 ^{0}C for 30 min. The supernatant was decanted into a

173   clean centrifuge tube and the extract was adjusted to pH 4.6 with 1M HCl (or 1M NaOH).

174   After re-centrifugation at 14,000×g for 20 min at 4 ^{0}C, the supernatant was collected by

175   decantation and filtered through a 0.45 μm membrane filter. The filtered extract was

176   evaporated to a final volume of 1 mL using a gentle nitrogen flow over the extract surface.

177   The concentrated extract was filtered through a 0.45 μm membrane filter into a vial.

178   *2.5 Liquid chromatography-tandem mass spectrometry conditions*

179   The LC-MS/MS system used in this study consisted of an 1100 Agilent (Palo Alto, CA,

180   USA) LC system and a Micromass (Manchester, UK) Quattro Ultima Triple Quadrupole

181   Mass spectrometer fitted with an electrospray ion source (ESI) operated in positive ionisation

182   mode. Chromatographic separation was achieved using a Kinetex C18 LC-MS column (100

183   mm × 2.1 mm, 2.6 μm, 100 Å) from Phenomenex (Sydney, Australia) at a flow rate of 100

184   μL/min. A KrudKatcher Ultra HPLC in-line filter (0.5 μm depth filter x 0.004 inch ID) was

185   used to protect the analytical column. The mobile phase was methanol (MeOH) (A) and

186   ultrapure water (B) both containing 0.1% of formic acid. Chromatographic runs began with

187   30% (A) for 3 min, followed by a 15 min linear gradient to 100% (A). The mobile phase

188   remained at 100% (A) for 15 min to elute analytes from the column. Afterwards, the initial

189   conditions were re-established within 1 min and the column re-equilibrated for 15 min before

190   injecting the next sample. To minimise potential carryover, before and after each injection,

191   the needle of the injector was rinsed for 30 seconds in the injection port with MeOH. The

192   injected volume was 5 μL.

193   Optimum MS signals were achieved by tuning the ESI capillary and cone voltages to 3.25

194   KV and 25 V, respectively. Hexapole1, aperture and hexapole2 were set to 0.0 V, 0.1 V and

195   0.2 V, respectively. Desolvation temperature and source temperature were 325 ºC and 135 ºC,

196   respectively. Cryogenic liquid nitrogen gas (BOC Gases, Perth, Australia) was used as

197 desolvation and nebulizer gas; cone gas flow was set to 40 L/h, while the desolvation gas

198 flow was set to 765 L/h. High purity Argon (99.997% purity) (BOC Gases, Perth, Australia)

199 was used as collision gas ($P = 2.7 \times 10^{-4}$ kPa). Both quadrupoles (Q1 and Q3) were set at unit

200 mass resolution; ion energy on Q1 and Q3 was set to 1.0, while the multiplier was set at 750

201 V. Surrogate standards, BCM5-d_{10} and BCM7-d_{10} were included in the analytical method to

202 correct results for any losses encountered during sample extraction and to correct for matrix

203 effects (Jessome & Volmer, 2006). Quantification was performed by rationing the peak area

204 of the analytes to the peak area of the corresponding deuterated standards. Concentrations

205 were calculated by QuanLynx 4.0 software comparing the peak area ratios from the extracts

206 to peak area ratios from the calibration standards.

207 *2.6 Validation of the analytical method*

208 Evaluation of instrumental linearity, instrumental detection limits (IDLs) instrumental

209 quantification limits (IQLs), peak identification criteria (retention time and MRM ratio),

210 accuracy, precision, inter-day reproducibility, method limits of detection (MLDs), method

211 limits of quantitation (MLQs), and matrix effects were undertaken to validate the analytical

212 procedure.

213 2.6.1 LC-MS/MS performance

214 Repetitive injections of standard solutions were used to assess LC-MS/MS instrument

215 performance. The linear range was tested using calibration standards spanning from 0.01

216 ng/μL up to 25 ng/μL. Injections of low concentrations standard solutions were used to

217 assess IDLs and IQLs. IDLs were estimated at signal-to-noise (S/N) ratio equal to 3 while

218 IQLs were estimated at S/N=10. Repeat injections (n=15) of a solution at 1 ng/μL were used

219 to determine the variability of the MRM ratio and of the retention time.

220 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)

245 Where: As is the area of the analyte spiked in ultrapure water (standard solution) and Am is

246 the area of the analyte spiked in a yoghurt blank extract (matrix).

247 *2.7 Analysis of commercial and laboratory prepared samples of yoghurt and reconstituted*

248 *milk*

249 Ten commercial yoghurts were purchased from a local supermarket located in Perth, Western

250 Australia. Three yoghurts contained *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, other

251 yoghurts contain *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus, L. acidophilus* and

252 Bifidobacterium or contain *L. acidophilus*, Bifidobacterium and *L. casei*. The expiry dates of

253 all yoghurts ranged from 13 and 29 days. The samples were extracted immediately after

254 purchasing.

255 Commercial and laboratory prepared yoghurts and reconstituted milk samples were prepared

256 as described in Section 2.4. The laboratory prepared yoghurts and reconstituted milk were

257 thawed at room temperature and thoroughly mixed before sampling. Ten grams of

258 reconstituted milk was gradually acidified with 1M HCl to pH 4.6 and allowed to rest for 10

259 minutes. Before extraction, yoghurt and reconstituted milk samples were spiked with

260 surrogate standards BCM5-d_{10} and BCM7-d_{10} at a concentration level of 100 ng/g. In

261 addition, 10 g of yoghurt and 10 g of reconstituted milk spiked with both 1000 ng of BCM5-

262 d_{10} and 1000 ng of BCM7-d_{10} as well as 100 ng of BCM5 and 100 ng of BCM7 were used as

263 quality control samples (QCs). The QCs were extracted as described in Section 2.4. BCM5

264 and BCM7 were analysed using the validated LC-MS/MS method as described in Section

265 2.5. All samples were extracted and analysed in duplicate.

266

267 **3. Results and discussions**

268 *3.1 Optimisation of MS/MS conditions*

269   Solutions consisting of BCM5 and BCM7 at 5 ng/µL in MeOH:H$_2$O (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

317   amount of BCM5 and BCM7 (Table 3). Relative standard deviation values were 6.4% and

318   6.1% for BCM5 and BCM7 respectively; indicating good reproducibility of the method.

319   *3.5 Method limits of detection (MLDs) and method limits of quantitation (MLQs)*

320   Average sample based MLDs and MLQs in yoghurt extracts were calculated from analysis of

321   samples spiked with 1 ng/g and 10 ng/g of BCM5 and BCM7. MLDs were found to be 0.5

322   ng/g for BCM5 and 0.25 ng/g for BCM7. Meanwhile, MLQs for BCM5 and BCM7 were

323   found to be 1.7 ng/g and 0.85 ng/g, respectively. These results are difficult to compare with

324   published methods because, to the best of our knowledge, MLDs and MLQs for BCM5 and

325   BCM7 in yoghurt have not been reported previously (Nguyen, Johnson, Busetti, & Solah,

326   2013).

327   *3.6 Evaluation of matrix effects affecting detection of BCM5 and BCM7 in yoghurt*

328   The efficiency of ESI sources to ionise polarisable analytes in real samples, is often affected

329   by the matrix, which can be responsible for suppressing the absolute response of analytes

330   when analysed in LC-MS/MS. If no corrections are made, matrix effects often lead to an

331   underestimation in the concentration of the analytes of interest in real samples. In order to

332   address matrix effects, different approaches have been proposed (Jessome & Volmer, 2006).

333   For example, De Noni and Cattaneo, (2010) reported the use of matrix matched calibration

334   standards to take into account the presence of the matrix when quantifying BCM7 in UHT

335   milk. This is a valid and effective method to account for matrix effects especially when

336   deuterated standards are unavailable or prohibitively expensive.

337   In the present work, matrix effects were corrected for with the inclusion of deuterated

338   homologues. This approach is by far the most effective method to account for matrix effects

339   as the analytes and the corresponding co-eluting deuterated homologues are theoretically

340   subject to identical matrix effects. In order to study the degree of signal suppression caused

341   by the presence of the matrix, blank yoghurt extracts were spiked at 1000 ng/mL with BCM5

342   and BCM7 and analysed by LC-MS/MS. Results were compared to injected analytical

343   standards at the same concentration levels. Matrix effects were responsible for causing about

344   26% signal suppression for BCM5 and about 40% signal suppression for BCM7. It is

345   possible that additional clean up (e.g. on-line or off-line solid-phase extraction) for sample

346   matrix would decrease observed signal suppression, which could improve detection limits.

347   *3.7 Application of the validated method*

348   The validated method was applied to the identification and quantification of BCM5 and

349   BCM7 in ten commercial yoghurts, laboratory prepared yoghurt and reconstituted milk.

350   Neither BCM5 nor BCM7 was detected in commercial products, however both peptides were

351   identified and quantified in reconstituted milk and laboratory prepared yoghurts. The content

352   of BCM5 decreased from 1.3 ng/g in milk to 1.1 ng/g in yoghurt at 0 day storage and to less

353   than the MLQ at 1 and 7 day storage. BCM7 content decreased from 1.9 ng/g in milk to less

354   than the MLQ in yoghurts at all storage days (Table 5). It appears, BCM5 and BCM7 present

355   in the milk have degraded into small peptides during fermentation and storage of yoghurt.

356   Recently, there have been a few studies measuring BCM5 and BCM7 in commercial

357   yoghurts. De Noni and Cattaneo (2010) attempted to measure both peptides in these products,

358   but neither BCM5 nor BCM7 was found. Nevertheless, BCM7 has been quantified between

359   0.86 to 2.45 μg/g in natural and probiotic yoghurt, levels decrease considerably during

360   storage (Jarmolowska, 2012).

361   Kunda et al. (2012) have identified several di-peptides such as β-CN fragment 60-61 and β-

362   CN fragment 62-63 in commercial yoghurt. As can been seen in Fig.1, amino acids at

363   position 61 and 63 on the chain of parent β-CNs are proline residues. This finding shows that

364   yoghurt bacterial enzymes are likely to digest the peptide bonds between proline and other

365 amino acid residues, leading to degradation of BCM5 and BCM7 during storage. The

366 degradation of these peptides during yoghurt processing may involve the activity of X-prolyl

367 dipeptidyl aminopeptidase (PepX), which is an enzyme derived from LAB that specifically

368 hydrolyses peptide bonds between proline and other residues (Gobbetti, 2002). Furthermore,

369 PepX activity in dairy products fermented with LAB has demonstrated to increase during

370 storage (Otte, Lenhard, Flambard & Sørensen, 2011).

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372 **4. Conclusions**

373 A highly selective and sensitive analytical method employing LC-MS/MS was successfully

374 developed and validated for the simultaneous determination of BCM5 and BCM7 in a

375 complex matrix, yoghurt. For the highest confidence in the identification and quantification

376 of BCM5 and BCM7, two isotopically labelled homologues were included in the method as

377 surrogate standards. The method showed very low MLQs, (1.7 ng/g for BCM5 and 0.85ng/g

378 for BCM7)making it suitable for the analysis of the low level of BCMs in yoghurt .

379 This validated method was applied to analyse BCMs in commercial and laboratory prepared

380 yoghurts and in milk. The absence of BCM5 and BCM7 in commercial yoghurts tested may

381 have been due to degradation of these peptides during processing or storage. In the laboratory

382 prepared yoghurt and milk tested, fermentation and storage lowered the content of BCM5 and

383 BCM7.

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487 **Table 1.** Mass spectrometry: precursor ions, product ions and collision energy values

488 optimised for the analysis of BCM5 and BCM7 under ESI(+) MS/MS in Multiple Reaction

489 Monitoring (MRM) mode. MRM transitions were grouped in one window. Dwell time was

490 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

491 [a]The product ions from the MRM transitions used for quantification.

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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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536 **Table 5.** BCM5 and BCM7 concentrations in reconstituted milk and laboratory prepared

537 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]

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

539 recoveries (%) are the average ± RSD% of n=2 yoghurt samples made from reconstituted milk spiked at 100

540 ng/g.

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556   **Fig. 1**. Structure of BCM5 (A) and BCM7 (B) (adapted from Juan-García, Font, Juan, &

557   Picó, 2009).

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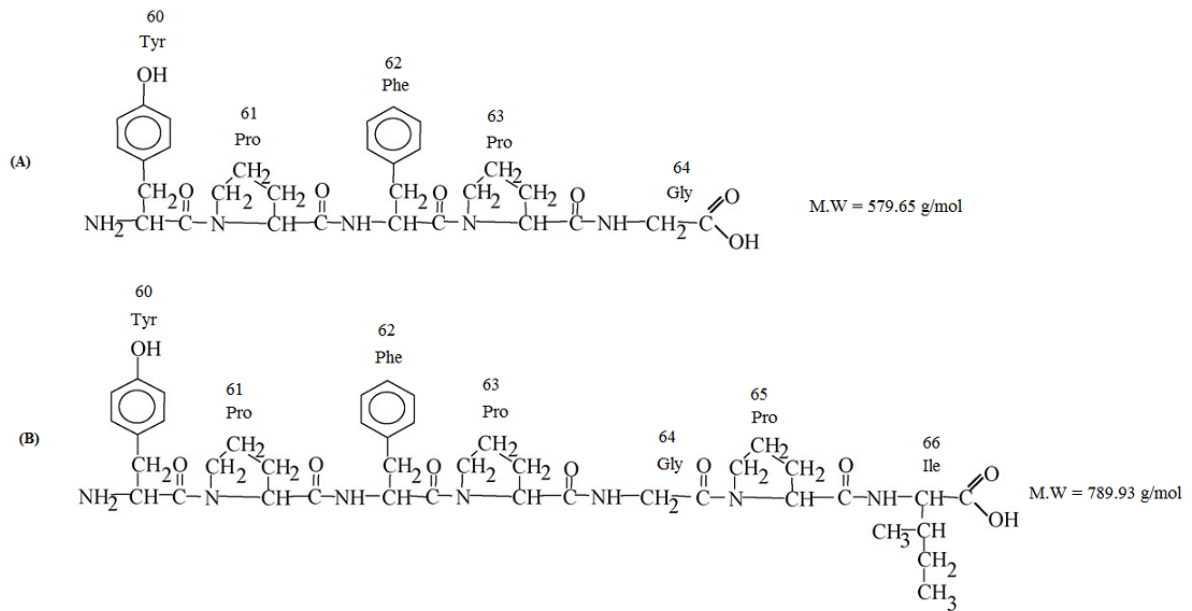
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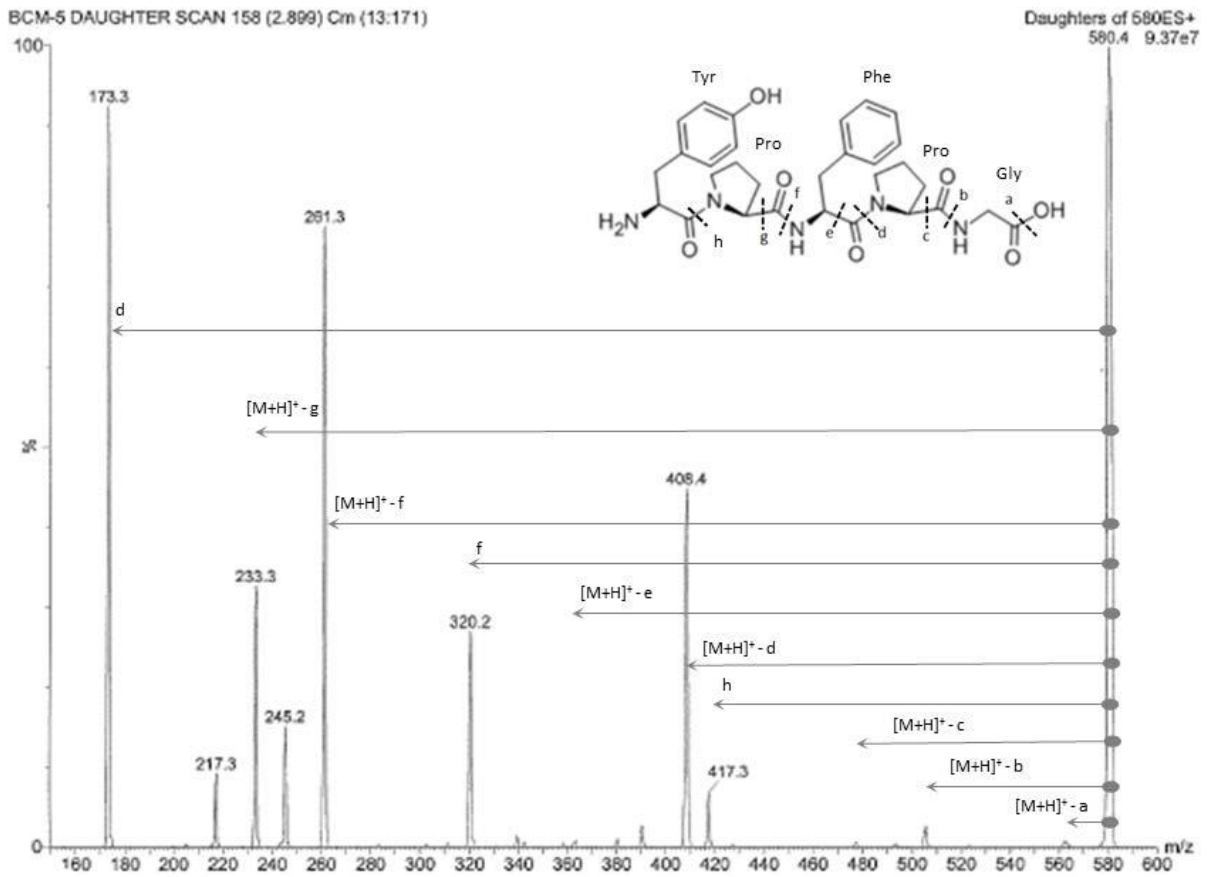
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571 **Fig. 2.** Product ions spectrum showing the fragmentation of BCM5 obtained at variable

572 collision energy values. The amino acids tyrosine (Tyr), proline (Pro), phenylalanine (Phe),

573 and glycine (Gly) are also indicated in the chemical structure of BCM5.
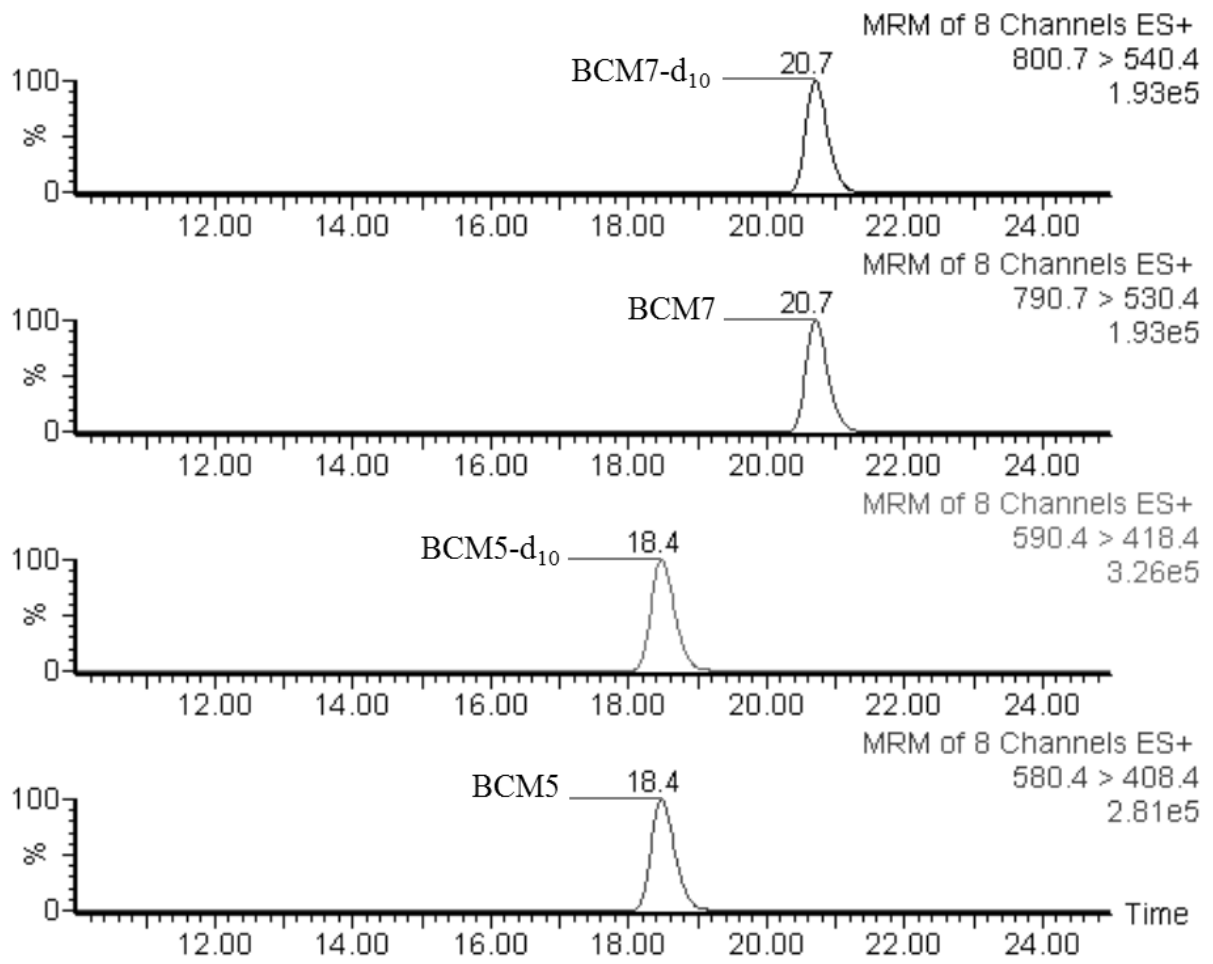

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581   **Fig. 3.** Typical LC-MS/MS chromatograms of BCM5 and BCM7 and their corresponding

582   deuterated homologues BCM5-d_{10} and BCM7-d_{10}. The ion source was an ESI operated in

583   positive ion mode, while the mass spectrometer was a triple quadrupole operated in MRM

584   mode.

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