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Analytical Methods

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 quantification 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 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 nor BCM7 was 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 days 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 63–70. 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; Kamiński et al., 2007; Kálmán, Cserháti, Valkó, & Neubert, 1992). 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

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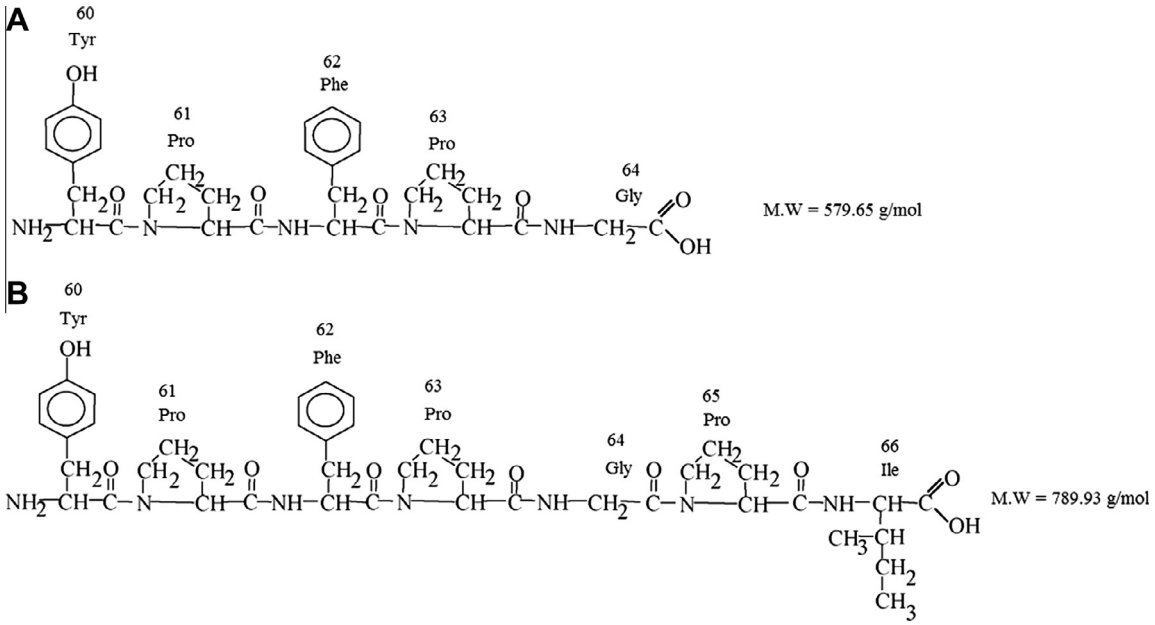

Fig. 1. Structure of BCM5 (A) and BCM7 (B). (Adapted from Juan-García, Font, Juan, & Picó, 2009.)

commercial yoghurt (Jarmolowska, 2012). In contrast, De Noni and 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, chap. 6). 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 physicochemical and spectrophotometric absorption properties can coelute 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 (Cieślińska et al., 2012; Jarmolowska, 2012; Sienkiewicz-Szłapka et al., 2009). 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 ionisation (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 quantification of BCM7 in plasma at low concentrations (ng/ml).