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Analytical Methods

Analysis of lysozyme in cheese samples by on-line combination of capillary zone electrophoresis and mass spectrometry

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ABSTRACT

Some methodological aspects of an on-line combination of capillary zone electrophoresis with mass spectrometric detection (CZE–QqQ–MS) were studied in this work as well as the possibilities of using this combination for analysis of the high-molecular mass compounds present in multi-component matrices. All experiments using an on-line combination of capillary electrophoresis with mass spectrometric detection were carried out in cationic mode in covalently-coated capillary. The optimised electrolyte system consisted of 100 mmol/L formic acid. Prior to the CZE–QqQ–MS analysis, an extraction of lysozyme from cheese samples using 1 mol/L of acetic acid was performed. The LOD was 3.6 mg lysozyme per kg and the LOQ was 10.9 mg lysozyme per kg. The concentration range of the lysozyme determined in four cheese samples analysed in this work was from 0.5 to 3.3 g of lysozyme per kg. The values of the relative standard deviations thus obtained were from 4.6% to 9.3% depending on the cheese sample.

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1. Introduction

Hen egg-white lysozyme, also known as muramidase, is a hydrolase enzyme with an extensive bacteriostatic activity. The molecular mass of egg-white lysozyme, calculated from the sequence of 129 amino acid residues, is 14,307 Da. These residues are cross-linked by four disulphide bridges (Mine, Ma, & Lauriau, 2004). The mechanism of the bacteriostatic activity of lysozyme is in the combination of the enzymatic hydrolysis of *N*-glycosidic linkages in the microbial cells and non-enzymatic damage of the cytoplasmic membrane by direct binding of the protein (Yeh, Dodds, Zuo, & Johnson, 1997). This hydrolase enzyme exhibits lytic activity on the cell-wall of Gram-positive bacteria. Hence, lysozyme is used as a preservative agent in the cheesemaking process to prevent the late gas-blowing defect caused by *Clostridium tyrobutyricum* (Kvasnička, 2003; Schneider, Becker, & Pischetsrieder, 2010; Schneider, Werkmeister, Becker, Pischetsrieder, 2011). Lysozyme is also added as the preservative agent in the manufacturing process of soya milk, sushi and Chinese noodles. Fresh vegetables, fish, meat and seafood have been preserved by coating the surface of the food with lysozyme (Mine et al., 2004). In winemaking, the lysozyme is used to control spontaneous lactic acid-producing bacteria (LAB), many of which cause wine spoilage (Guzzo, Cappello, Azzolini, Tosi, & Zapparoli, 2011; Tirelli & De Noni, 2007; Tolin et al., 2012). However, lysozyme can cause allergic reactions in susceptible individuals. For this reason, it is desirable to monitor (detect) its amount in food (NDA, 2005).

Lysozyme present in food can be determined by high-performance liquid chromatography (Guarino, Fuselli, La Mantia, & Longo, 2011; Pellegrino & Tirelli, 2000; Schneider et al., 2011; Tirelli & De Noni, 2007). Schneider (Schneider et al., 2011) detailed the use of high-performance liquid chromatography combined with fluorescence detection (HPLC–FLD) for the analysis of lysozyme in cheese samples, using a reversed-phase polymeric column. An extraction of lysozyme from cheese samples using 1 mol/L solution of sodium chloride was performed prior to the direct HPLC–FLD analysis. The limit of detection was calculated as 7.3 mg of lysozyme per kg of cheese. Lysozyme can also be determined by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) (Thammasirirak et al., 2010; Tolin et al., 2012), or mass spectrometry alone (Alomiraha, Allia, & Konishib, 2000; Léonil, Gagnaire, Molleé, Pezennec, & Bouhallab, 2000; Schneider et al., 2010). Schneider et al. (2010) described a method combining immuno-capture purification and direct mass-spectrometric analysis (MALDI–TOF–MS) for the detection of lysozyme in cheese samples. Cheese extracts were treated with magnetic particles coated with a monoclonal antibody directed against lysozyme. Lysozyme was finally detected by MALDI–TOF–MS. The limit of detection of lysozyme in cheese was approximately 5 mg/kg.

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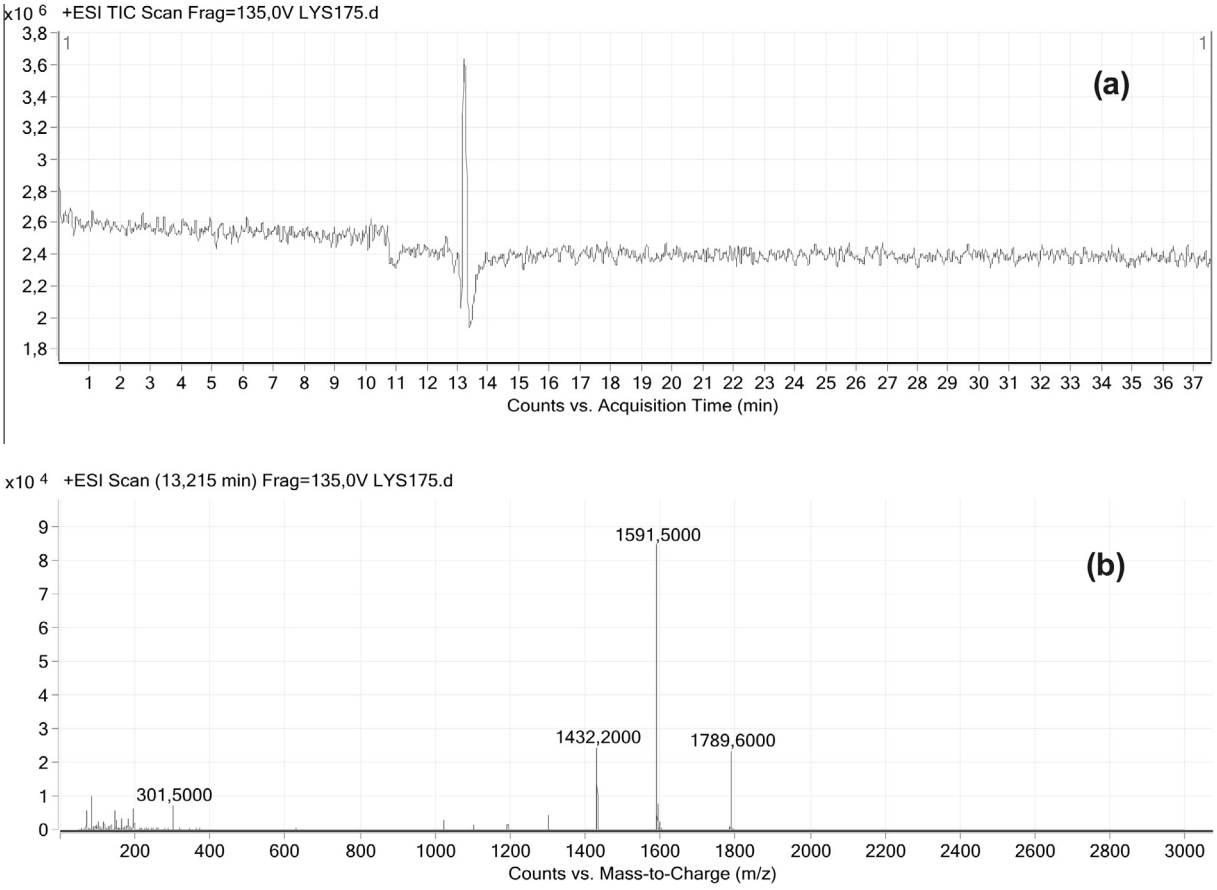

Fig. 1. TIC record (a) and MS spectrum (b) obtained from CZE–QqQ–MS analysis of lysozyme standard.

Another approach to the determination of the lysozyme was described by Kvasnička (2003). He detailed the use of an on-line coupling of capillary isotachophoresis with capillary zone electrophoresis (CITP–CZE) for the determination of lysozyme in selected food products, using ammonium hydroxide with acetic acid as the leading electrolyte and ε-aminocaproic acid with acetic acid as the terminating electrolyte. The limit of detection was 0.25 mg/L. An extraction of lysozyme from selected food samples using 1 mol/L acetic acid was performed prior to the direct CITP–CZE separation.

Capillary zone electrophoresis (CZE) represents a high-resolution separation technique which requires very small volumes of the analysed samples (nL–μL) and a short separation time (10 s–30 min) and can be combined with mass spectrometry (MS) (Ahmed, 2009). MS in comparison with other commonly used detection techniques in CZE provides information on the structure of the analyte, especially when MS/MS and MS^n instrumentation is used, and also gives information on the molecular mass of the studied analyte (Nielen et al., 2006). A suitable combination of CZE and MS affords the benefits of each of the techniques, i.e., high-efficiency separations of the analytes from the sample constituents and, at the same time, obtaining information on the molecular masses and/or structures of the analytes during the analysis (Cai & Henion, 1995; Desiderio, Rossetti, Iavarone, Messana, & Castagnola, 2010). The combination of CZE–MS has several advantages over the HPLC–MS combination, i.e., (1) a much smaller sample is needed (nL versus μL); (2) higher separation efficiencies can be obtained; (3) CZE works in a single-phase separation system unlike HPLC where at least two phases are used and, accordingly, the optimal separation conditions can be more readily and speedily found; (4) CZE separates the charged compounds and the neutral compounds present in the sample do not interfere in the detection whereas HPLC separates practically all the compounds present in the sample and there is a high potential of interferences from the matrix; (5) the sample pretreatment procedures used in CZE are much simpler than the complicated protocols used prior to HPLC; (6) minimum matrix effects have been described in the literature to date while the negative impact of the matrix effects is well-known in the HPLC–MS combination. A detailed summary of the use of CZE–MS in food analysis and its advantages can be found in the works of Ravelo-Pérez, Asensio-Ramos, Hernández-Borges, and Rodríguez-Delgado (2009) and Klepárnik (2013).

This work sought the development of an analytical procedure suitable for the analysis of lysozyme in various cheese samples using the on-line combination of capillary zone electrophoresis with triple-stage mass-spectrometric detection (CZE–QqQ–MS) and a fast and simple method for the determination of lysozyme as an allergen.

2. Materials and methods

2.1. Instruments

The experiments were performed using an Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany). Fused-silica capillaries were obtained from MicroSolv Technologies (Eatontown, NJ, USA). All CZE–QqQ–MS experiments were performed in a polyacrylamide-coated fused silica capillary

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