Journal of Chromatography A, xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Evaluation of two sub-2 μm stationary phases, core–shell and totally porous monodisperse, in the second dimension of on-line comprehensive two dimensional liquid chromatography, a case study: Separation of milk peptides after expiration date

Eduardo Sommella[a], Giacomo Pepe[a], Giovanni Ventre[a], Francesco Pagano[a], Michele Manfra[c], Giuseppe Pierri[b], Omar Ismail[b], Alessia Ciogli[b], Pietro Campiglia[a,*]

[a] *Dipartimento DIFARMA, Università di Salerno, I-84084 Fisciano, Salerno, Italy*
[b] *Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, P.le Aldo Moro 5, 00185 Roma, Italy*
[c] *Department of Science, University of Basilicata, Viale dell'Ateneo Lucano 10, I-85100 Potenza, Italy*

ARTICLE INFO

Article history:
Received 26 July 2014
Received in revised form
24 November 2014
Accepted 27 November 2014
Available online xxx

Keywords:
Sub-2 μm particles
Peptides
LC × LC
UHPLC

ABSTRACT

Milk is a rich source of bioactive peptides of great interest for their healthy properties. These peptides are usually encrypted in the sequences of proteins and are released after time dependent proteolysis as very complex hydrolysates. In order to separate and identify the bioactive sequences, we developed an on-line comprehensive two dimensional liquid chromatography approach using the high performance combined with the ultra high performance conditions. A microbore reversed phase (C18 silica, 5 μm) column was employed in first dimension, while, in second dimension, two different UHPLC columns, packed with C18 silica, were tested: a new column based on monodisperse sub-2 μm fully porous particles with high surface area (50 mm × 3.0 mm, 1.9 μm d.p., from Supelco), and a column based on sub-2 μm core–shell particles (50 mm × 3.0 mm, 1.7 μm d.p, from Phenomenex®). Both set-ups were compared, showing high peak capacity values with respect to a high efficiency monodimensional method, maintaining the same analysis time. Satisfactory selectivity was obtained through the use of different pH between the two dimension, while a very fast continuous shifted gradient in second dimension ensured a good employment of the 2D separation space.

1. Introduction

Bovine milk proteins represent a very complex biological matrix, that possesses not only an important nutritional value but has a deep connection with many physiological processes. Milk proteins, which are constituted by two major families of proteins: caseins (insoluble) and whey proteins (soluble), are involved in a wide range of biochemical mechanisms, and exert many activities including: immunomodulatory, anti-hypertensive, anti-bacterial, opioid and also anti-carcinogenic [1]. The beneficial health effects are related to the bioactive peptides, encrypted in the amino-acid sequence of milk proteins. These peptides are latent until released by enzymatic proteolysis that occurs from bacterial activity or in the gastrointestinal digestion [2]. Fermented milk is a rich source of bioactive peptides; in fact the proteolytic system of bacteria, mainly endopeptidases and exopeptidases, enhances the degradation and leads to the release of peptides having various regulatory functions [3]. The identification of bioactive peptide sequences is an important step to understanding the biological function of these compounds and their possible employment in nutraceutical formulations as well as in enriched and personalized milks. Milk protein hydrolysates represent a complex sample containing up to 10^3 different peptides and, their identification is a challenging task. A variety of methods are employed to analyze milk peptides and proteins: electrophoretic techniques such as two dimensional gel electrophoresis (2D-GE), capillary zone electrophoresis (CZE) or gel free techniques like High-Performance Liquid Chromatography (HPLC) [4,5]. Among these, HPLC allows rapid and automated analysis, high resolution, repeatability and easy hyphenation with mass spectrometry (MS), which is the workhorse in the field of protein analysis. Despite the huge improvements in MS analyzers technology, which are more and more sensitive and accurate,

---

* Corresponding author. Tel.: +39 089969242.
E-mail address: pcampiglia@unisa.it (P. Campiglia).

http://dx.doi.org/10.1016/j.chroma.2014.11.072
0021-9673/

Please cite this article in press as: E. Sommella, et al., Evaluation of two sub-2 μm stationary phases, core–shell and totally porous monodisperse, in the second dimension of on-line comprehensive two dimensional liquid chromatography, a case study: Separation of milk peptides after expiration date, J. Chromatogr. A (2014), http://dx.doi.org/10.1016/j.chroma.2014.11.072

front-end separation techniques are still essential in order to not overwhelm the mass spectrometer source and prevent ion suppression phenomena which lead to impaired detection of low abundant peptides [6]. In this regard monodimensional (1D) approaches usually cannot provide adequate values of resolution and more often two dimensional liquid chromatography (2D-LC) methods are employed. 2D-LC, operated in the "comprehensive" mode (LC × LC) is the best way to obtain high values of peak capacity. In this approach the entire eluate from first dimension (^{1}D) is continuously collected and injected on-line into the second dimension (^{2}D) through an interface, usually represented by a multiport switching valve. Many on-line LC × LC systems have been developed for peptide analysis using a combination of separation techniques, such ion exchange-reversed phase (IEC × RP) [7,8], size exclusion-reversed phase (SEC-RP) [9], reversed phase-reversed phase (RP × RP), which represents the combination that provides the highest practical peak capacity values [10,11]. Recently, comprehensive LC × LC using reversed phase × hydrophilic liquid chromatography (RP × HILIC and HILIC × RP) was very promising for its high degree of orthogonality [12]. Furthermore in HILIC mode, highly organic mobile phases provide high desolvation efficiency, and thus better coupling with ESI-MS detection [13]. The development of sub-2 μm stationary phases and instruments capable of higher pressure limits led to the rapid expansion of ultra high performance liquid chromatography (UHPLC) methods, characterized, with respect to conventional HPLC methods, by superior resolution and very short analysis time. Nowadays, the employment of UHPLC conditions in LC × LC has proved to be a valuable tool in terms of speed and performance for the separation of pharmaceutical samples, complex natural matrices, polyphenols, and polymers [14–17]. In this contribution, we analyzed the low-medium molecular weight peptides derived from soluble fraction of semi-skimmed milk after four weeks from the expiration date. For this purpose, after an ultrafiltration through centrifugal filters with different cutoff and subsequent solid phase extraction (SPE) the peptide fraction with molecular weight <3000 Da was collected. A comprehensive RP × RP platform was developed, consisting of a microbore (5 μm, 1.0 mm I.D.) C18 column in ^{1}D, and in ^{2}D, two different (3.0 mm I.D) sub-2 μm stationary phases were investigated, based on 1.9 μm totally porous monodisperse particles, and 1.7 μm core–shell column, respectively. The two dimensions were coupled through an ultra high pressure 10 port-two position switching valve and two different modulation times (60 s and 45 s) were used together with a ^{2}D shifted gradient in order to maximize the peak capacity. Different pH conditions of mobile phase operated in the two dimensions (basic in ^{1}D and acidic in ^{2}D) allowed significant selectivity, despite the employment of the same separation mode (RP). Results were compared with those obtained with a high efficient monodimensional separation with the same analysis time, and corrected taking into account both the under-sampling effect, and the orthogonality of the system developed, showing how the LC × UHPLC system is a valuable tool, and is probably the first application of this technique for the study of time dependent degradation of proteins belonging to the soluble fraction milk.

2. Materials and methods

2.1. Chemicals

Ultra pure water (H_2O) was obtained by a Direct-8 Milli-Q system (Millipore, Milan, Italy), LC–MS grade acetonitrile (ACN) and additives formic acid (HCOOH), ammonium acetate (CH_3COONH_4) trifluoroacetic acid (TFA), reagent grade glacial acetic acid (CH_3COOH) and 1 M sodium-acetate (CH_3COONa) solution, the standard peptide mixture and the small molecule test mixture were all purchased from Sigma–Aldrich (St. Louis, Mo, USA). Centrifugal Filter Devices Amicon® Ultra-4 3 K and 10 K and Strata C18-SPE cartridge were purchased by Millipore© and Phenomenex® (Castel Maggiore, Bologna, Italy), respectively, while filter paper Whatman® 540 from Sigma–Aldrich.

2.2. Sampling and sample preparation

Commercial samples of semi-skim milk from Centrale del latte di Salerno (CdLdS) (Salerno, Campania, Italy) were analyzed; samples were stored at room temperature and the corresponding peptide fractions, generated by enzymatic hydrolysis in the products during fermentation, were extracted at four weeks after expiration date. Samples were prepared by separating the residual fat fraction from expired semi-skim milks: 50 mL of milk were subjected to centrifugation at 4000 × *g* for 30 min at 4 °C (Mikro 220R, Hettich, Germany). To harden the cream, the tubes were kept at −20 °C for 10 min and finally the cream was removed. Skim milk sample was diluted with distilled water (1:10, *v/v*) and the solution thus obtained was shake vigorously. The caseins were removed from skim milks by precipitation at pH 4.3 (20 °C) with addition of glacial acetic acid (10% *v/v*) and 1 M sodium-acetate solution. The acidified milk was centrifuged for 15 min at 3000 × *g* (20 °C) and subsequently the precipitated caseins were eliminated by double filtration using, firstly by filter paper (particle retention 22 μm) for retention of coarse and gelatinous precipitates and subsequently again by filter paper (8 μm), to a final volume of 40 mL. The obtained soluble fraction was processed to collect the low-medium molecular weight peptides (see afterwards).

2.3. Peptides isolation

The supernatant of skimmed milk samples were ultra-filtered by Millipore's Amicon® Ultra-4 centrifugal filter devices at different cutoffs. A preliminary filtration was carried out for all the samples using filters with 10,000 NMWL (Nominal Molecular Weight Limit) and subsequently 4 mL of permeate were loaded on devices with 3000 NMWL. The devices were centrifuged for 40 min at 6000 rpm at 25 °C, using a centrifuge with fixed angle rotor (35°). In order to recover the peptides, the devices were washed with 4 mL of acidified water at pH 3.0 by formic acid. A peptide fraction, roughly 3,5 mL, with molecular weight <3000 Da was collected, filtered through a 0.45 μm pore cellulose membrane (Millipore©) and lyophilized for 24 h (LyoQuest-55, Telstar Technologies, Spain). This fraction was solubilized in 0.1% (*v/v*) aqueous TFA, and purified from salts and sugars with a Strata C18-E cartridge (Phenomenex®), previously equilibrated in 0.1% TFA, then eluted with 70/30/0.1 ACN/water/TFA (*v/v/v*) and finally re-lyophilized and stored at −20 °C. Twenty milligram of the lyophilized were solubilized in 1 mL of water and injected.

2.4. Columns

For high efficiency monodimensional (1D) comparative analysis two Titan™ 100 mm × 2.1 mm 1.9 μm (80 Å) (Supelco, Bellefonte, PA, USA), and two Kinetex™ 100 + 50 × 2.1 mm, 1.7 μm (Phenomenex®) were employed; the columns were placed in series by a 6 cm × 0.100 mm I.D. viper capillary (Thermo Fisher Scientific, Milan, Italy). In LC × UHPLC analysis a Discovery® C18 Bio Wide pore 150 mm × 1.0 mm, 5 μm (300 Å) from Supelco was used in ^{1}D of the LC × UHPLC system. In ^{2}D two different columns were tested: the Kinetex™ C18 50 mm × 3.0 mm, 1.7 μm (100 Å) from Phenomenex® and a Titan™ C18 50 mm × 3.0 mm, 1.9 μm (80 Å) from Supelco. Moreover a Kinetex™ C18 50 mm × 3.0 mm, 2.6 μm

Please cite this article in press as: E. Sommella, et al., Evaluation of two sub-2 μm stationary phases, core–shell and totally porous monodisperse, in the second dimension of on-line comprehensive two dimensional liquid chromatography, a case study: Separation of milk peptides after expiration date, J. Chromatogr. A (2014), http://dx.doi.org/10.1016/j.chroma.2014.11.072