Journal of Chromatography A, 1229 (2012) 121–128

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Identification of bioactive peptides in a functional yogurt by micro liquid chromatography time-of-flight mass spectrometry assisted by retention time prediction

Pradeep B. Kunda, Fernando Benavente*, Sergio Catalá-Clariana, Estela Giménez, José Barbosa, Victoria Sanz-Nebot

Department of Analytical Chemistry, Nutrition and Food Safety Research Institute (INSA-UB), University of Barcelona, Diagonal 647, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 27 July 2011
Received in revised form
22 December 2011
Accepted 26 December 2011
Available online 8 January 2012

Keywords:
Bioactive peptides
Prediction
Retention
Solid-phase extraction
Time-of-flight
Yogurt

ABSTRACT

In this study we used micro liquid chromatography coupled to time-of-flight mass spectrometry (microLC–TOF-MS) for separation and identification of bioactive peptides in a yogurt marketed as an antihypertensive functional food. An appropriate sample clean-up using solid-phase extraction (SPE) allowed detection of a large number of low-molecular-mass bioactive peptides by reversed-phase microLC–TOF-MS. The preliminary identification was solely based on the experimental monoisotopic molecular mass values (M_{exp}). Later, we evaluated the correlations between predicted normalized elution time (NET) and experimental normalized retention times (t'_r) values to describe the retention behavior of the proposed sequences. The assistance of retention prediction proved to be useful to improve reliability of the identification, avoiding misinterpretations and solving some identity conflicts. After revision, the identity of only fifty bioactive peptides was confirmed. Significant number of these peptides was reported as angiotensin converting enzyme (ACE) inhibitors and nine of them were antihypertensive. The presence of peptide sequences with other biological activities such as antibacterial, antithrombotic, antioxidant, cell modulation, immune or phagocytosis stimulation, epitopes of B cells and opioid agonists was also confirmed.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

In recent years, there is an increasing commercial interest in the production of bioactive peptides from various sources to use them as ingredients in functional foods, drugs or cosmetics. Milk proteins are precursors of many different bioactive peptides that may remain latent until being released by enzymatic proteolysis during gastrointestinal digestion or food processing. Immunostimulating, antimicrobial, opioid, angiotensin-converting enzyme (ACE) inhibition, mineral binding, antithrombotic and allergenic are some of the described bioactivities [1–7]. Identification and quantification of bioactive peptides in milk protein hydrolysates, fermented dairy products or, in general, in functional dairy foods with health-promoting or disease-preventing peptide ingredients is a challenging task because these highly complex samples can contain up to hundreds of different peptides at different levels of concentration [8–15]. This is the case of some antihypertensive functional yogurts that are produced by fermentation of milk with starter cultures containing specific bacterial strains [16,17].

Liquid chromatography–mass spectrometry (LC–MS) using conventional columns has been extensively used to analyze intact milk proteins or peptides released from milk proteins in a wide variety of food products (e.g. fermented dairy products, hydrolysates from simulated gastrointestinal digestion of milk protein fractions, hypoallergenic infant milk formulas, etc.) [8–15]. Micro- and nanoliquid chromatography (microLC and nanoLC, respectively) have been applied to a lower extent, despite their advantages over conventional LC. The smaller diameter columns (0.2–0.8 mm) and lower mobile phase flow rates (1–20 μL/min) allow for less solvent, reagents and packing materials consumption, as well as samples of limited availability. Furthermore, it is possible the on-line coupling to mass spectrometry (microLC–MS) without the need of flow splitting [18].

In the present work, microLC–MS was applied to identify the presence of bioactive peptides in a commercial antihypertensive yogurt. A time-of-flight (TOF) analyzer providing high resolution and mass accuracy was used for molecular mass measurements [19,20]. Yogurt samples were cleaned up using SPE and analyzed by microLC–TOF-MS. Database search allowed the tentative

* Corresponding author. Tel.: +34 934039123; fax: +34 934021233.
E-mail address: fbenavente@ub.edu (F. Benavente).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.12.093

identification of a large set of low-molecular-mass bioactive peptides. This preliminary identification was later revised considering retention behavior of the detected sequences. Several authors have regarded prediction of chromatographic retention or electrophoretic migration of peptides in complex samples, as an excellent tool to refine the structural assignments made on the basis of molecular mass measured by CE-MS or LC–MS [21–36]. We evaluated the correlations between predicted normalized elution time (NET [25,27–31]) and experimental normalized retention time (t'_r) values to check the accuracy of the proposed peptide sequences, in order to improve reliability of the characterization procedure.

2. Materials and methods

2.1. Chemicals, reagents and samples

All chemicals used in the preparation of buffers and solutions, unless otherwise indicated, were analytical reagent grade. Formic acid, acetonitrile (LC–MS grade), methanol, sodium hydroxide, trisodium citrate dihydrate and urea were purchased from Merck (Darmstadt, Germany). DL-Dithiothreitol (DTT) was supplied by Sigma (St. Louis, MO, USA). Water with a conductivity value lower than 0.05 $\mu S\,cm^{-1}$ was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). ESI Low Concentration (ESI-L) tuning mix for tuning and calibration of the TOF mass spectrometer was supplied by Agilent Technologies (Waldbronn, Germany). The antihypertensive yogurt was purchased at a local market and was stored at 4 °C when not in use.

2.2. Sample preparation

Yogurt sample (0.5 mL) was diluted with 2.5 mL of reduction buffer and incubated for 1 h at room temperature. Reduction buffer was prepared by dissolving 73 mg of trisodium citrate dihydrate and 38 mg of DTT in 37.5 mL of 8 M urea. pH was adjusted to 8 with a dilute solution of sodium hydroxide and the volume was made up to 50 mL with water. After incubation, the samples were centrifuged at 4600 r.p.m. for 30 min. Fat layer was removed and clear solutions were filtered through 0.22 μm nylon filters (MSI, Westboro, MA, USA) before solid-phase extraction (SPE) with 1 mL Sep-Pack® C18 (C18) (50 mg of sorbent) (Waters, Milford, MA, USA) and polymeric StrataX™ (STX) (30 mg of sorbent) (Phenomenex, Torrance, CA, USA) cartridges [19]. The SPE cartridges were first conditioned with 2 mL of methanol and 2 mL of water. After loading 2 mL of sample, the retained compounds were eluted with 200 μL of a solution containing 80:20 (v/v) methanol:water and 0.1% (v/v) of formic acid. Flow rate was maintained at approximately 1 mL/min during all these steps. Eluate was evaporated to dryness under a stream of air at room temperature and the final residue was reconstituted with 200 μL of water. To prevent injector or column clogging, samples were filtered through Ultrafree® centrifugal filter devices (Durapore PVDF 0.22 μm) (Millipore) before microLC–TOF-MS experiments. All solutions were stored at 4 °C when not in use.

pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52-03 (Crison instruments, Barcelona, Spain). Centrifugation procedures were carried out in an Avanti TM J-25 centrifuge (Beckman Coulter, Fullerton, CA, USA).

2.3. microLC–TOF-MS

MicroLC–TOF-MS experiments were performed using a 1200 series capillary liquid chromatography system (Agilent Technologies) coupled to a 6220 oa-TOF mass spectrometer (Agilent Technologies) with a dual-nebulizer ESI source. Separation conditions were adapted from several reversed-phase methods described in the literature for the analysis of dairy products [8–15]. Separations were performed in a C8 column (150 mm × 0.3 mm Zorbax 300SB-C8, 3.5 μm stationary phase particles, Agilent Technologies). Experiments were performed at room temperature under gradient elution at a flow rate of 4 μL/min and injecting 0.15 μL of yogurt extract. Eluting solvents were water and acetonitrile, both with 0.1%, v/v of formic acid. The optimum linear gradient, as acetonitrile proportion (v/v), was as follows: 0–45 min, 4–45%; 45–50 min, 45%; 50–55 min, 100%; 55–60, 100%; 60–65, 0%; 65–70, 4%. The orthogonal nebulizer of the dual-nebulizer ESI source was used for MS measurements. The other source, typically used for introduction of mass calibration solution, was disabled to avoid interferences with microLC–TOF-MS experiments [37]. TOF mass spectrometer was tuned and calibrated following the manufacturer's instructions. Later the measurement parameters were finely tuned for the analysis of low-molecular-mass peptides as described elsewhere [37]. The optimum operational conditions in positive mode were as follows: capillary voltage 4000 V, drying gas (N_2) temperature 200 °C, drying gas flow rate 4 L/min, nebulizer gas (N_2) 7 psig, fragmentor voltage 215 V, skimmer voltage 60 V, OCT 1 RF Vpp voltage 300 V. Data was collected in profile (continuum) at 1 spectrum/s (approx. 10,000 transients/spectrum) between m/z 250 and 1250 working in the extended dynamic range (2 GHz) mode with the mass range set to standard (3200 m/z). microLC–TOF-MS control, data acquisition and analysis were performed using the MassHunter workstation software (Agilent Technologies).

2.4. Data analysis

Compounds were identified in the smoothed base peak chromatograms (BPCs) using Masshunter workstation software (Agilent Technologies). The software tool, "find compounds by molecular feature" determines the experimental molecular mass (M_{exp}) and abundance of the most relevant low-molecular-mass components after application of several constraints related to the intensity threshold of the chromatographic peaks or molecular ions, as well as with their type (charge and adduct). For comparison of different analyses, retention time and abundance of the detected compounds were normalized considering the components with M_{exp} 855.4338 and 1150.6860 eluting at 18.10 and 31.93 min, respectively. Bioactive peptides were identified using a database containing accurate monoisotopic molecular mass values (M_{teo}), location in the sequence of the protein precursor and type of biological activity from about 300 bioactive peptides from bovine milk proteins, which was built from more than one hundred bibliographic references [19]. Some other free protein databases available on the Web were useful to confirm some of the identifications, especially BIOPEP, which is a database of bioactive peptides from a variety of origins [5]. Mass accuracy was calculated in ppm as $|M_{exp} - M_{teo}|/M_{teo} \times 10^6$ [20].

2.5. Retention time prediction using the NET approach

Retention behavior of bioactive peptides tentatively identified only by their M_{exp} and database search was evaluated using NET prediction software (omics.pnl.gov/software/NETPredictionUtility.php, Pacific Northwest National Laboratory, Richland, USA) to calculate their NET values [27,28]. NET prediction software is a small utility designed to calculate predicted NET values for a list of peptide sequences. Its mathematical algorithms were established from NET values obtained considering the hydrophobicity and the retention behavior in reversed-phase LC of a large set of peptide

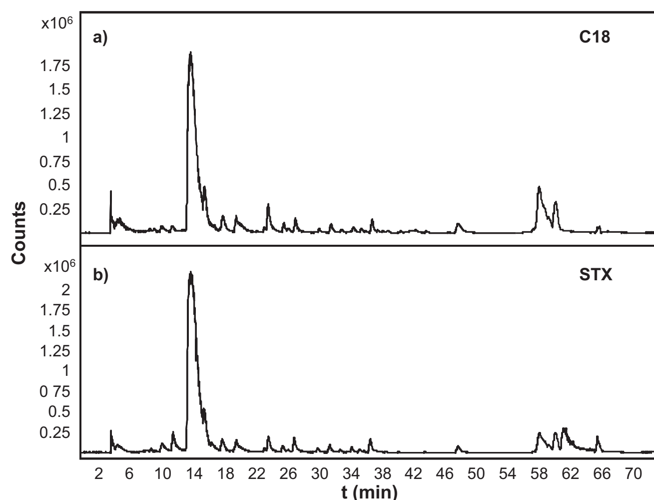

Fig. 1. Base peak chromatograms (BPCs) of yogurt using (a) C18 and (b) STX cartridges.

sequences obtained from digestion of different proteins. Three algorithms were used to predict NET values namely Kangas/Petritis LC retention time [27], Krokhin hydrophobicity [29] and Mant hydrophobicity [25] models. In order to obtain NET values with Krokhin model, NET software normalizes to a scale of 0–1 the "hydrophobicity" (H) values obtained with the model algorithm described by Krokhin et al. [29] and referred as Version 1 in their Web site (hs2.proteome.ca/SSRCalc/SSRCalcX.html, Manitoba Centre for Proteomics and Systems Biology, Manitoba, Canada). This H value is referred as "hydrophobicity" because it is related to the real hydrophobicity of the separated species [29–31]. NET values of the bioactive peptides were useful to confirm the sequences proposed by microLC–TOF-MS after studying their correlation with t_r' obtained with both cartridges. We also tested the model described by Gorshkov et al. [33,34] (theorchromo.ru, Institute for Energy Problems of Chemical Physics, Moscow, Russia). In order to compare C18 and STX SPE selectivities, the average hydrophobicity values of the confirmed peptides were calculated using the scale for the amino acids of J.L. Fauchere and V.E. Pliska [38].

3. Results and discussion

Yogurt is a complex sample which contains proteins, carbohydrates, fats and other essential nutrients that could interfere in detection of low-molecular-mass peptides. In a previous work, we demonstrated that the use of a reduction buffer with an appropriate composition allowed obtaining a transparent solution from hypoallergenic infant milk formulas, which are also milk-like emulsions, ready to be preconcentrated by SPE using C18 and STX cartridges before capillary electrophoresis–mass spectrometry [19]. A similar sample clean-up was applied for yogurt sample before microLC–TOF-MS. Fig. 1a and b shows the BPCs obtained by microLC–TOF-MS for yogurt samples processed with C18 (a) and STX (b) cartridges. Both yogurt samples appeared as a complex mixture of components coeluting in several chromatographic peaks of different intensities within 70 min. Only subtle differences due to the characteristic selectivity of each SPE cartridge could be observed by visual inspection of both chromatographic profiles. Following the procedure described in Section 2 a total of 1544 and 1676 low-molecular-mass components were detected in samples processed with C18 and STX cartridges, respectively, confirming the great complexity of yogurt and the excellent sensitivity and selectivity of the MS detector. Both lists of low-molecular-mass components were filtered to identify only bioactive peptides using our homemade database [19]. Table 1 shows the amino acid sequence, protein precursor and biological activity of the identified peptides. As can be observed, a total of 85 peptides from the main bovine milk proteins with different bioactivities were identified in samples processed with C18 and STX cartridges. Only 5 of these peptides were specifically detected in samples processed with C18 (n 47, 48, 53, 61 and 69 in Table 1) or STX (n 18, 26, 30, 31 and 33 in Table 1), suggesting that one of the cartridges would be enough to achieve a good coverage of low-molecular-mass bioactive peptides of the studied yogurt. With regard to the confidence of the identification, this was a tentative identification solely based on the agreement between M_{exp} and M_{teo} (accuracy was always lower than 5 ppm). Table 1 shows that identification of some bioactive peptides was not unequivocal. Two types of retention time conflicts were noticed that we named as Type 1 and Type 2. In Type 1 conflict a certain bioactive peptide was identified eluting at significantly different times (e.g. n 1–3 in Table 1). In Type 2 conflict two or more isobaric bioactive peptides were associated with the same retention time because of the agreement of M_{teo} with M_{exp} (e.g. n 6 and 7 in Table 1). As it is summarized in Table 1, it was also possible to find both conflicts together (e.g. n 10–15).

In order to improve reliability of the identification, avoiding misinterpretations and solving such identity conflicts, we evaluated the use of retention prediction. We tested some of the prediction models with the greatest potential [25,27–34], which were freely available online as easy-to-use calculator tools. However, none of them was developed for reversed-phase chromatographic retention of peptides in 300-Å pore size C8 columns using linear gradients of acetonitrile–water mobile phases with formic acid. This limitation could have important implications in accuracy of retention prediction. The algorithm described by Gorshkov et al. [33,34] was developed from very basic physical assumptions about peptides, chromatographic systems and linear gradient chromatography. It allowed selection of some parameters related to the separation (C18 column diameter, pore size, gradient slope, delay time, flow, ionic modifier and linear acetonitrile–water gradient), but results in our case were less satisfactory. The rest of the studied approaches were established from the study of retention of a large set of peptides at certain chromatographic conditions in C18 columns and linear gradients of acetonitrile–water, in general, with trifluoroacetic acid as ion pairing agent [27–32]. In our case, the Krokhin and Kangas/Petritis models provided with NET utility allowed acceptable results [28,29]. It is worth mentioning that together with chromatographic conditions, the set of peptides used for training the models may also influence prediction accuracy. In this case, both models were developed with large sets of tryptic peptides and the shortest peptides detected after trypsin digestion are in general longer than 4 or 5 amino acids, 11–12 on average, terminated by R or K and sometimes reduced and alkylated. In order to refine our identity assignments, predicted NET values of the bioactive peptides detected in samples processed with C18 and STX were correlated with their t_r' values. Peptide n 85 in Table 1 was excluded from the study because of a post-translational modification, which could not be taken into account to calculate NET values [28]. Fig. 2a and b shows the plots of NET values for the bioactive peptides tentatively identified in yogurt processed with C18 and STX cartridges against their t_r' values, together with the parameters resulting from a linear least squares regression, using Krokhin and Kangas/Petritis models, respectively. As can be observed, linear correlation coefficients were low (R^2 = 0.27 and 0.25 respectively), when the typical values for these semiempirical relationships are above 0.80 [27–30]. Poor linear correlation may not be only a matter of prediction inaccuracy due to the specificities of our chromatographic conditions or non-tryptic peptide set. As NET calculations were based on the amino acid sequence proposed from comparison of M_{teo} with M_{exp}, an erroneous sequence assignment could

Table 1
Bioactive peptides identified in yogurt using C18 and STX cartridges.

n	Conflict[a]	t'_r (min)	M_{exp} (Da)	M_{teo} (Da)	Error (ppm)	SPE	Sequence	Protein precursor	Activity	NET	Refs.
1	Type 1	13.0	262.1299	262.1317	6.9	C18,STX	FP	BCN A1–A2 (62–63)(111–112)(157–158)(205–206)	ACE-inhibitor, antihypertensive	YES	[5,39–41]
2	Type 1	14.5	262.1301	262.1317	6.1	C18,STX	FP	BCN A1–A2 (62–63)(111–112)(157–158)(205–206)	ACE-inhibitor, antihypertensive		[5,39–41]
3	Type 1	15.7	262.1302	262.1317	5.9	C18,STX	FP	BCN A1–A2 (62–63)(111–112)(157–158)(205–206)	ACE-inhibitor, antihypertensive		[5,39–41]
4		8.1	278.1255	278.1267	4.3	C18,STX	YP	BCN A1–A2 (60–61)//AS1CN (146–147) (159–160)//KCN (35–36) (58–59)//KCN (58–59)//BCN A1–A2 (58–59)//BCN A2 (114–115)	ACE-inhibitor, antihypertensive	YES	[39–41,13]
5		16.8	278.1622	278.1630	3.1	C18,STX	LF	BLG (104–105)//ALA (52–53)	ACE-inhibitor	YES	[41]
6	Type 2	11.7	294.1568	294.1579	3.7	C18,STX	LY	BCN A1–A2 (192–193)	ACE inhibitor	YES	[41]
7	Type 2	11.7	294.1568	294.1579	3.7	C18,STX	YL	AS1CN (91–92)//AS2CN (206–207)//BLG (102–103)	ACE inhibitor		[5,13]
8		6.1	311.1830	311.1845	4.8	C18,STX	VPP	BCN A1–A2 (84–86)	ACE-inhibitor, antihypertensive	YES	[13,39–49]
9		19.1	312.1467	312.1474	2.4	C18,STX	FF	AS1CN (23–24)	Antihypertensive	YES	[44,47]
10	Type 1 &2	18.6	317.1728	317.1739	3.6	C18,STX	LW	AS1CN (198–199)	ACE-inhibitor		[39,40,47]
11	Type 1 &2	26.1	317.1729	317.1739	3.2	C18,STX	LW	AS1CN (198–199)	ACE-inhibitor		[39,40,47]
12	Type 1 &2	18.6	317.1728	317.1739	3.6	C18,STX	IW	ALA (59–60)	ACE-inhibitor		[13]
13	Type 1 &2	26.1	317.1729	317.1739	3.2	C18,STX	IW	ALA (59–60)	ACE-inhibitor		[13]
14	Type 1 &2	18.6	317.1728	317.1739	3.6	C18,STX	WL	ALA (104–105) (118–119)	ACE-inhibitor	YES	[13]
15	Type 1 &2	26.1	317.1729	317.1739	3.2	C18,STX	WL	ALA (104–105) (118–119)	ACE-inhibitor		[13]
16		8.6	325.1995	325.2001	2.0	C18,STX	IPP	BCN A1–A2 (74–76)//KCN (108–110)	ACE-inhibitor, antihypertensive	YES	[8,39–44,46–49]
17		14.0	333.1315	333.1325	3.0	C18,STX	EW	ALA (25–26)	ACE-inhibitor	YES	[14]
18		58.8	340.2087	340.2110	6.8	STX	PPK	KCN (109–111)	Antithrombotic		[45]
19	Type 1	14.8	351.1770	351.1794	7.0	C18,STX	YGL	ALA (50–52)	ACE-inhibitor, opiod agonist	YES	[39,41,48]
20	Type 1	9.3	351.1780	351.1794	4.0	C18,STX	YGL	ALA (50–52)	ACE-inhibitor, opiod agonist		[39,41,48]
21		15.8	356.2045	356.2060	4.2	C18,STX	LPQ	AS1CN (11–13)	ACE-inhibitor		[50]
22		11.9	386.2624	386.2641	4.4	C18,STX	LVR	BLG (122–124)	ACE-inhibitor	YES	[48,50]
23		26.6	391.2468	391.2471	0.9	C18,STX	LLF	BLG (103–105)	ACE-inhibitor	YES	[13]
24		18.4	393.2251	393.2264	3.3	C18,STX	YVL	KCN (30–32)	Antimicrobial	YES	[42,51,52]
25		18.7	407.2403	407.2420	4.2	C18,STX	LLY	BCN A1–A2 (191–193)	Immunostimulator (+), phagocytosis stimulator	YES	[43,44,47,49,51]
26	Type 1	21.7	414.2198	414.2267	16.7	STX	PLW	AS1CN (197–199)	ACE-inhibitor	YES	[39,40,47,48]
27	Type 1	26.4	414.2261	414.2267	1.6	C18,STX	PLW	AS1CN (197–199)	ACE-inhibitor		[39,40,47,48]
28		16.2	432.2362	432.2372	2.3	C18,STX	FVAP	AS1CN (24–27)	ACE-inhibitor	YES	[39,40,47,48]
29		20.1	438.2832	438.2842	2.3	C18,STX	LPLP	BCN A2 (135–138)	ACE-inhibitor	YES	[9]
30	Type 2	12.8	503.2370	503.2340	6.0	STX	NQDK	KCN (113–116)	Antithrombotic		[43,44]
31	Type 2	12.8	503.2370	503.2380	2.0	STX	PYPQ	BCN A1–A2 (179–182)	Cell modulation	YES	[8]
32		15.7	512.2730	512.2747	3.4	C18,STX	IHPF	BCN A1–A2 (49–52)	ACE-inhibitor	YES	[8]
33		59.8	556.3631	556.3584	8.4	STX	TKVIP	AS2CN (198–202)	ACE-inhibitor, antihypertensive		[44]
34		20.4	567.2889	567.2839	8.8	C18,STX	ALPMH	BLG (142–146)	ACE-inhibitor,hypocholesterolemic effect		[40,42]
35		9.4	576.3098	576.3119	3.6	C18,STX	VTSTAV	KCN (164–169)	ACE-inhibitor	YES	[39]
36		15.6	590.3257	590.3275	3.0	C18,STX	STVATL	KCN (141–146)	Antibacterial	YES	[53]
37		16.4	627.3207	627.3268	9.7	C18,STX	YLGYL	AS1CN (91–95)	Opioid agonist		[44]
38		16.3	632.3150	632.3129	3.3	C18,STX	KNQDK	KCN (112–116)	Antithrombotic		[43]
39		10.0	646.3246	646.3286	6.3	C18,STX	AQTQSL	BCN A1–A2 (53–58)	ACE-inhibitor	YES	[52]
40		17.8	651.3928	651.3955	4.1	C18,STX	VLPVPQ	BCN A2 (170–175)	ACE inhibitor, antihypertensive	YES	[8,9]

Table 1 (*Continued*)

n	Conflict[a]	t'_r (min)	M_{exp} (Da)	M_{teo} (Da)	Error (ppm)	SPE	Sequence	Protein precursor	Activity	NET	Refs.
41		17.0	653.3673	653.3649	3.7	C18,STX	WLAHK	ALA (104–108)	ACE-inhibitor, opiod agonist	YES	[39,41,46,48]
42		26.4	671.3666	671.3715	7.4	C18,STX	VVRNAN	AS2CN (43–48)	Mineral binding		[49]
43		14.7	673.3410	673.3435	3.7	C18,STX	AVPYPQ	BCN A1–A2 (177–182)	Cell modulation	YES	[8]
44	Type 2	27.2	688.4254	688.4272	2.6	C18,STX	LHLPLP	BCN A2 (133–138)	ACE-inhibitor		[48]
45	Type 2	27.2	688.4254	688.4272	2.6	C18,STX	HLPLPL	BCN A2 (134–139)	ACE-inhibitor, antioxidant	YES	[8,9,45,48]
46		10.8	702.3995	702.4064	9.8	C18,STX	KFALPQ	AS2CN (173–178)	ACE-inhibitor		[45]
47	Type 2	28.8	747.3565	747.3625	8.0	C18	EMPFPK	BCN A1–A2 (108–113)	ACE-inhibitor		[8,9,42,44,47]
48	Type 2	28.8	747.3565	747.3625	8.0	C18	TTMPLW	AS1CN (194–199)	ACE-inhibitor, immunomodula-tor,antihypertensive	YES	[8,40,42–46,48–50,54]
49		20.9	747.3764	747.3803	5.2	C18,STX	YQEPVL	BCN A1–A2 (193–198)L	ACE-inhibitor, immunomodulator	YES	[8,41,42,44,47]
50		24.4	750.3577	750.3588	1.5	C18,STX	YPVEPF	BCN A2 (114–119)	Opioid agonist	YES	[55]
51		15.8	755.3941	755.3966	3.4	C18,STX	DKIHPF	BCN A1–A2 (47–52)	ACE-inhibitor	YES	[8,9,42]
52		13.7	756.3652	756.3694	5.6	C18,STX	YLGYLE	AS1CN (91–96)	Opioid agonist		[43,44]
53		8.9	771.3625	771.3552	9.5	C18	SRYPSY	KCN (33–38)	Opioid antagonist	YES	[43,47]
54	Type 2	16.4	779.4866	779.4905	5.0	C18,STX	KVLPVPQ	BCN A2 (169–175)	ACE-inhibitor, antihypertensive		[8,45–47]
55	Type 2	16.4	779.4866	779.4905	5.0	C18,STX	VLPVPQK	BCN A2 (170–176)	Antioxidant, ACE-inhibitor	YES	[8,45]
56		16.1	787.3999	787.4076	9.8	C18,STX	RELEEL	BCN A1–A2 (1–6)	ACE-inhibitor		[46]
57		21.9	788.3376	788.3381	0.6	C18,STX	AYFYPE	AS1CN (143–148)	ACE-inhibitor	YES	[39,47]
58		26.4	796.4454	796.4483	3.6	C18,STX	VPPFLQP	BCN A1–A2 (84–90)	ACE-inhibitor	YES	[45]
59		12.7	801.4346	801.4385	4.9	C18,STX	KAVPYPQ	BCN A1–A2 (176–182)	Cell modulation	YES	[8]
60		18.1	855.4325	855.4338	1.5	C18,STX	NVPGEIVE	BCN A1–A2 (7–14)	ACE-inhibitor	YES	[54]
61		4.3	874.5425	874.5501	8.7	C18	RPKHPIK	AS1CN (1–7)	ACE inhibitor		[42]
62		26.4	875.4547	875.4575	3.3	C18,STX	KTTMPLW	AS1CN (193–199)	ACE-inhibitor	YES	[8,45,50]
63		30.2	897.5385	897.5436	5.7	C18,STX	RGPFPIIV	BCN A1–A2 (202–209)	ACE-inhibitor	YES	[8,9]
64		16.1	904.4824	904.4866	4.6	C18,STX	TVQVTSTAV	KCN (161–169)	Antibacterial	YES	[53]
65		23.2	968.5143	968.5179	3.7	C18,STX	LNVPGEIVE	BCN A1–A2 (6–14)	ACE-inhibitor	YES	[54]
66		23.6	976.4807	976.4800	0.7	C18,STX	RDMPIQAF	BCN A1–A2 (183–190)	ACE-inhibitor	YES	[39]
67		31.4	996.6050	996.6120	7.0	C18,STX	VRGPFPIIV	BCN A1–A2 (201–209)	ACE-inhibitor	YES	[50]
68	Type 1	23.7	1078.5966	1078.6022	5.2	C18,STX	NIPPLTQTPV	BCN A1–A2 (73–82)	ACE-inhibitor	YES	[41,45,47,54]
69	Type 1	35.7	1078.5981	1078.6022	3.8	C18	NIPPLTQTPV	BCN A1–A2 (73–82)	ACE-inhibitor		[41,45,47,54]
70		27.8	1099.5651	1099.5702	4.6	C18,STX	VYPFPGPIPN	BCN A2 (59–68)	Opioid agonist, ACE inhibitor, antihypertensive	YES	[55]
71		14.2	1110.5253	1110.5346	8.4	C18,STX	ALNEINQFY	AS2CN (81–89)	ACE-inhibitor		[48]
72		30.6	1123.6208	1123.6277	6.1	C18,STX	VVPPFLQPEV	BCN A1–A2 (83–92)	Epitopes B cells	YES	[7]
73		31.9	1150.6817	1150.6862	3.9	C18,STX	GPVRGP	BCN A1–A2 (199–204)	Antihypertensive		[42,44,45]
74		32.7	1192.6795	1192.6856	5.1	C18,STX	TPVVVPPFLQP	BCN A1–A2 (80–90)	ACE-inhibitor, antihypertensive		[39–41,45,56]
75	Type 2	33.8	1196.6744	1196.6628	9.7	C18,STX	LPLPLLQSWM	BCN A1–A2 (135–144)	Epitopes B cells		[7]
76	Type 2	33.8	1196.6744	1196.6805	5.1	C18,STX	LTQTPVVVPPF	BCN A1–A2 (77–87)	ACE-inhibitor	YES	[41]
77		31.0	1299.6831	1299.6863	2.5	C18,STX	VYPFPGPIPNSL	BCN A2 (59–70)	Opioid agonist	YES	[56]
78		26.0	1328.6748	1328.6560	14.1	C18,STX	ARHPHPHLSFM	KCN (96–106)	Antioxidant	YES	[46]
79		32.7	1421.7846	1421.7918	5.1	C18,STX	FSDKIAKYIPIQ	KCN (18–29)	Antibacterial	YES	[53]
80		25.5	1465.7284	1465.7354	4.8	C18,STX	YPFPGPIHNSLPQ	BCN A1 (60–72)	Opioid agonist	YES	[55]
81		8.4	1534.8748	1534.8844	6.3	C18,STX	RPKHPIKHQGLPQ	AS1CN (1–13)	ACE-inhibitor		[39,45,50]
82		32.3	1637.8745	1637.8817	4.4	C18,STX	LVYPFPGPIPNSLPQ	BCN A1–A2 (58–72)	ACE-inhibitor		[41,46,54]
83		36.3	1880.0514	1880.0559	2.4	C18,STX	YQEPVLGPVRGPFPIIV	BCN A1–A2 (193–209)	Antimicrobial, ACE-inhibitor, immunomodulator		[41,43,44,49,54]
84		36.5	1956.1038	1956.1084	2.4	C18,STX	NIPPLTQTPVVVPPFLQP	BCN A1–A2 (73–90)	ACE-inhibitor		[56]
85	PTM	15.6	514.2058	514.2063	1.1	C18,STX	YPSY·OCH3	KCN (35–38)	Opioid antagonist		[7]

α-lactoalbumin (ALA); β-lactoglobulin (BLG); αs1-casein (AS1CN); αs2-casein (AS2CN); β-casein (BCN) and κ-casein (KCN); M_{exp}, M accuracy and t'_r values are average values.

[a] Type 1: peptide identified at different retention times; Type 2: isobaric peptides and PTM: peptide with post-translational modifications.

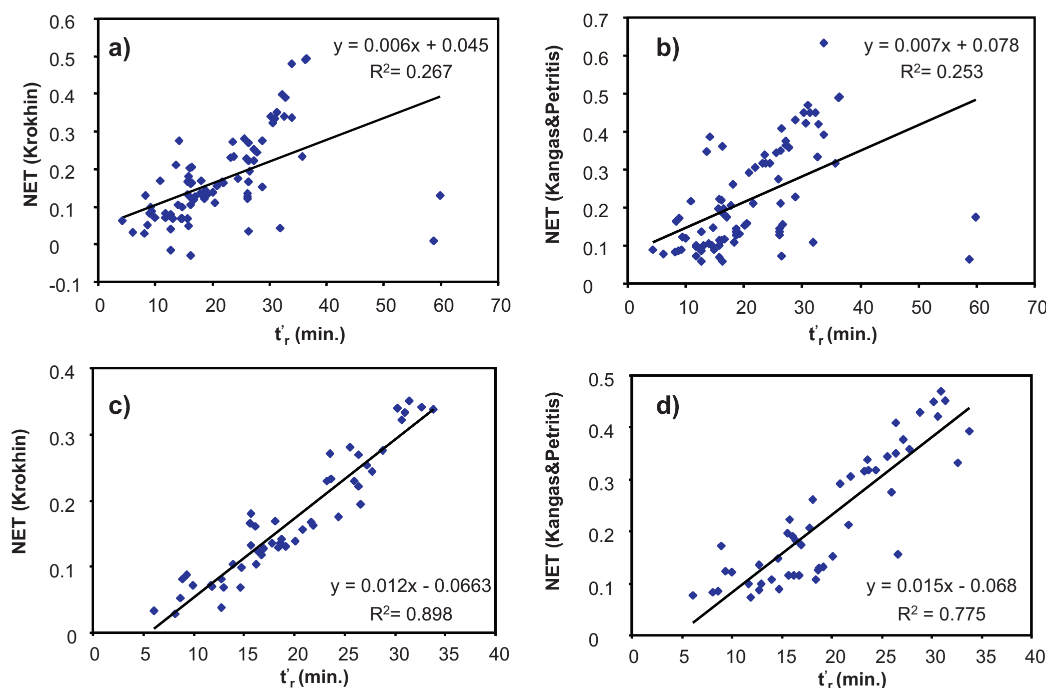

Fig. 2. Plots of NET values for the bioactive peptides of yogurt processed with C18 and STX cartridges against their t'_r values using Krokhin and Kangas/Petritis models. (a) and (b) Before revision and (c) and (d) after revision. Parameters resulting from linear least squares regression are given as insets.

lead to an erroneous NET prediction and hence to a deviation from the expected linear trend. The Krokhin model result, which was slightly better, was used to refine the linear correlations taking into account the absolute value of the standardized residuals from the dependent variable ($r_i = (\hat{y}_i - y_i)/s$, where y_i are the raw NET values, $\hat{y}_i$ the NET values obtained from linear regression, and s the standard deviation of the residuals, $\hat{y}_i - y_i$). It is widely accepted that any of the observation with an absolute r_i value ($|r_i|$) higher than 2 can be considered an outlier [57]. In our case, these outliers were removed from the set of values used to build a new linear model and the process was repeated until $|r_i|$ values for all the observations with the rebuilt linear model were lower than 2. A number of procedures have been proposed for detecting outliers in linear regression [58–60]. However, there is not a unique final solution yet and their detection still challenging and influenced by the specificities of the data set, especially when there are multiple outliers in the data. This type of forward sequential procedures for identifying and removing outliers after fixing a certain critical value are commonly used because of its simplicity and ease of programming. However, they are not free of errors such as masking (an outlier obscure the existence of another) or swamping (a non-outlier is wrongly considered as an outlier). Anyway, in our case this process was proposed to refine the identity assignments, not to claim the validity of the retention models which were demonstrated by Krokhin and Kangas/Petritis [28,29]. Fig. 2c and d shows the new plots after removing the misinterpreted components with Krokhin and Kangas/Petritis models, respectively. In both cases, R^2 values were significantly improved while the $|r_i|$ values for all the observations were lower than 2. Krokhin model gave the best result with a R^2 value of 0.90. The advanced versions of the Krokhin algorithm [30,31], which were established also with tryptic peptides and C18 columns, but in some cases, with formic acid in the acetonitrile–water mobile phases, produced only slightly improved R^2. The 50 bioactive peptides confirmed following this approach are indicated in Table 1 with a “YES” in the NET column. Only 2 of these peptides were specifically detected in samples processed with C18 (n 48 and 53 in Table 1) or STX (n 26 and 31 in Table 1), reinforcing that one of the cartridges would be enough to obtain a good map of low-molecular-mass bioactive peptides of the studied yogurt. In order to have a deeper understanding of the selectivity of both SPE cartridges, the binary logarithms of the abundance ratios ($Log_2(A_{C18}/A_{STX})$) for the confirmed bioactive peptides in samples processed with C18 and STX cartridges were plotted against their average hydrophobicity values (Fig. 3). As can be observed, the extraction efficiency of C18 cartridges was higher, because positive values of $Log_2(A_{C18}/A_{STX})$ predominated over negative values. In general, the extracted bioactive peptides showed between low and average hydrophobicity taking into account that Arginine and Tryptophan are the most hydrophilic and hydrophobic amino acids

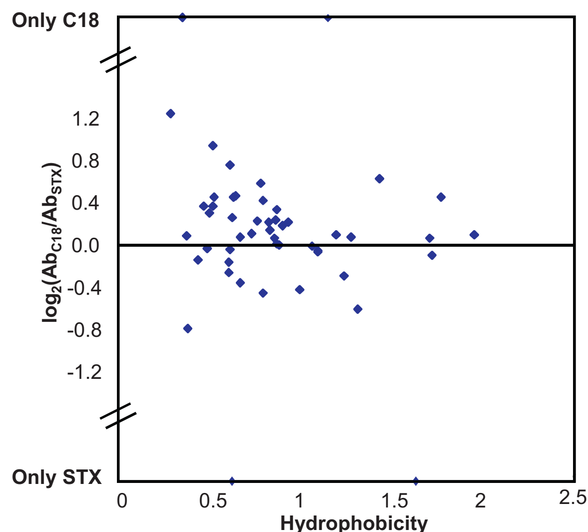

Fig. 3. Plots of binary logarithms of abundance ratios ($Log_2(A_{C18}/A_{STX})$) for confirmed bioactive peptides in samples processed with C18 and STX cartridges against their average hydrophobicity values.

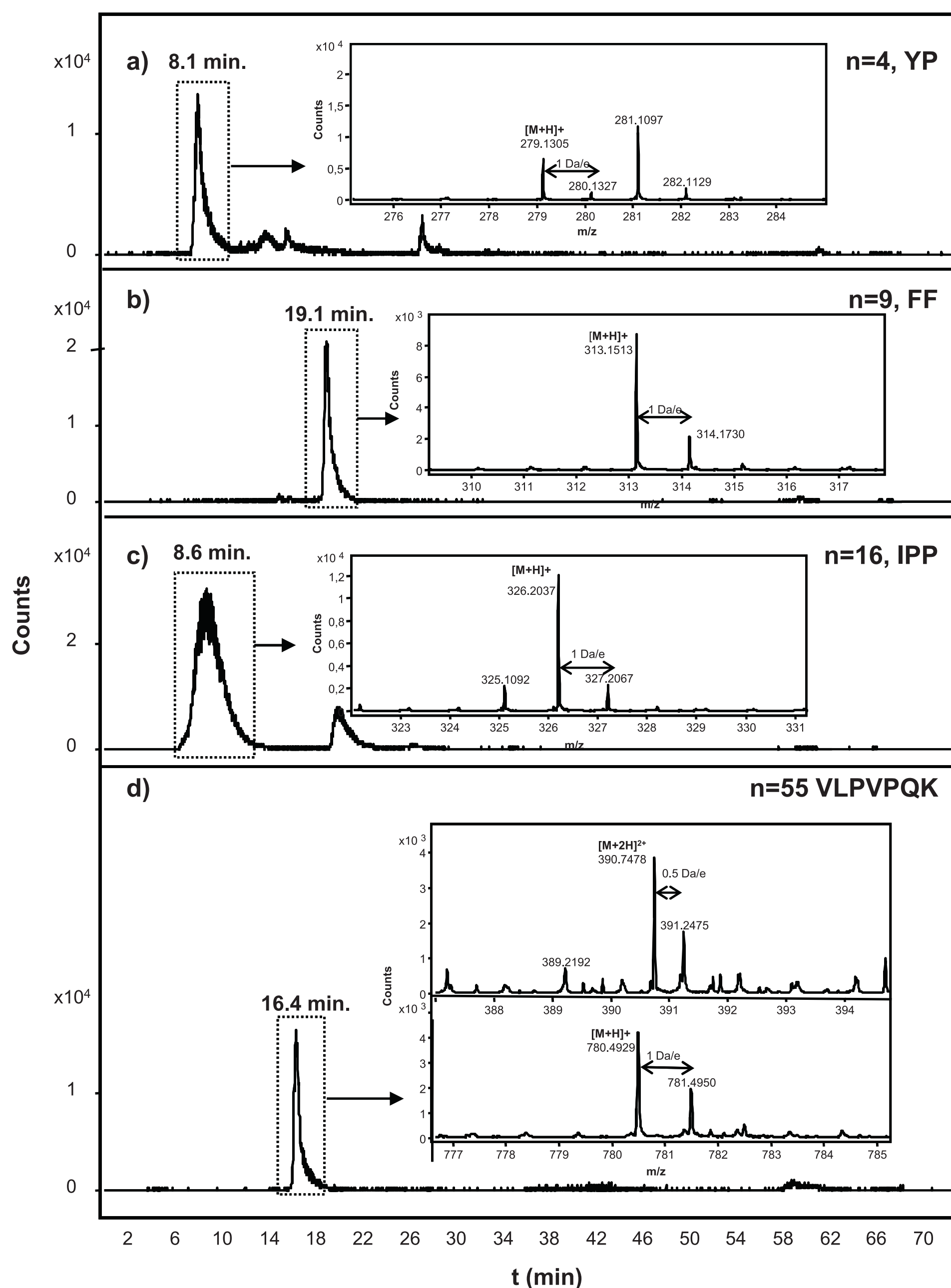

Fig. 4. Extracted ion chromatograms (XICs) for ions $[M+H]^+$ or $[M+2H]^{2+}$ of bioactive peptides labeled as (a) *n* 4, (b) *n* 9, (c) *n* 16 and (d) *n* 55 in Table 1. *m*/*z* window was 10 ppm.

(hydrophobicity = −1.010 and 2.250, respectively) in accordance to the hydrophobicity scale defined by Fauchere and Pliska [38].

The presence of the 50 bioactive peptides was further confirmed obtaining their extracted ion chromatograms (XICs), and revising the mass spectrum at the appropriate retention time to check the characteristic isotope distribution for $[M+H]^+$ or $[M+2H]^{2+}$ ions of the target peptides. As an example, Fig. 4 a–d shows the XICs of four antihypertensive peptides detected in samples processed with both cartridges, together with the molecular mass spectra of each chromatographic peak. The molecular mass spectra show the typical isotopic distribution of single or double charged molecular ions of the target peptides that can be resolved with a TOF analyzer, as well as molecular ions of other components eluting at the same retention time. As can be observed in Table 1, a large amount of the bioactive peptides in the final revised list of 50 were casein fragments and were reported to be inhibitors of the ACE, which is an enzyme involved in regulation of blood pressure. Some of these ACE inhibitors have been also reported to be antihypertensive in vivo. Between peptide sequences reported to be antihypertensive: FP, YP, VPP, FF, IPP, VLPVPQ, TTMPLW, VLPVPQK and VYPFPGPIPN (*n* 1, 4, 8, 9, 16, 40, 48, 55 and 70), it is worth mentioning that VPP and IPP were declared by the manufacturer to be the main active ingredients of this yogurt [16,17]. The presence of peptide sequences with other biological activities such as antibacterial, antithrombotic, antioxidant, cell modulation, immune or phagocytosis stimulation, epitopes of B cells and opioid agonists was also confirmed (Table 1).

4. Concluding remarks

MicroLC–TOF-MS can be regarded as an excellent choice for separation and identification of bioactive peptides in milk derived

products with a reduced consumption of solvents, reagents and samples. The proposed sample preparation method using C18 and STX SPE cartridges followed by microLC–TOF-MS was useful for identification of bioactive peptides in yogurt. However, mass accuracy and resolution of the TOF analyzer were not enough to achieve an unequivocal identification. We demonstrated that prediction of retention behavior provided a simple way to confirm the identities of some of the proposed peptide sequences that were mainly reported to be ACE inhibitors and antihypertensive. In general, prediction of retention behavior may be regarded as a simple, rapid, inexpensive and efficient tool to improve the identification potential of LC–TOF-MS. This is especially interesting when no standards are available or access to mass analyzers of improved selectivity is limited, such as state-of-the-art quadrupole time-of-flight (Q-TOF) or Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometers that allow a deeper characterization through MS–MS or ultra high resolution experiments, respectively. However, due to the complexity of the retention mechanisms in LC, it is not always easy to find or develop models that accurately describe retention behavior, even of a family or specific set of structurally related compounds, such as in this case peptides.

Acknowledgement

Part of this work was supported by the Spanish Ministry of Education and Science (CTQ2008-00507/BQU).

References

[1] H. Meisel, Curr. Med. Chem. 12 (2005) 1905.
[2] R. Hartmann, H. Meisel, Curr. Opin. Biotechnol. 18 (2007) 163.
[3] H. Korhonen, A. Pihlanto, Int. Dairy J. 16 (2006) 945.
[4] H. Korhonen, J. Funct. Foods 1 (2009) 177.
[5] M. Dziuba, M. Darewicz, Food Sci. Technol. Int. 13 (2007) 393.
[6] K. Erdmann, B.W.Y. Cheung, H. Schröder, J. Nutr. Biochem. 19 (2008) 643.
[7] L. Monaci, V. Tregoat, A.J. van Hengel, E. Anklam, Eur. Food Res. Technol. 223 (2006) 149.
[8] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, J. Chromatogr. A 1049 (2004) 107.
[9] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, J. Agric. Food Chem. 52 (2004) 1504.
[10] D. Weber, P. Raymond, S. Ben-Rejeb, B. Lau, J. Agric. Food Chem. 54 (2006) 1604.
[11] I. de Noni, Food Chem. 110 (2008) 897.
[12] B. Hernández-Ledesma, A. Quirós, L. Amigo, I. Recio, Int. Dairy J. 17 (2007) 42.
[13] M. Martin, A. Wellner, I. Ossowski, T. Henle, J. Agric. Food Chem. 56 (2008) 6333.
[14] C.J. van Platerink, H.G.M. Janssen, J. Haverkamp, Anal. Bioanal. Chem. 391 (2008) 299.
[15] J.A. Gómez-Ruiz, M. Ramos, I. Recio, Electrophoresis 28 (2007) 4202.
[16] http://www.kaikuvitaten.com/.
[17] http://www.valio.com/portal/page/portal/valiocom/R_D/Nutritional_research.
[18] T. Takeuchi, Chromatography 26 (2005) 1.
[19] S. Catalá-Clariana, F. Benavente, E. Giménez, J. Barbosa, V. Sanz-Nebot, Anal. Chim. Acta 683 (2010) 119.
[20] K. Web, T. Bristow, M. Sargent, B. Stein, Methodology for Accurate Mass Measurement of Small Molecules. Best Practice Guide, 1st ed., LGC Limited, Teddintong, UK, 2004.
[21] J.L. Meek, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 1632.
[22] S.J. Su, B. Grego, B. Niven, M.T.W. Hearn, J. Liq. Chromatogr. 4 (1981) 1745.
[23] C.A. Browne, H.P.J. Bennett, S. Solomon, Anal. Biochem. 124 (1982) 201.
[24] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 359 (1986) 499.
[25] R.S. Hodges, J.M. Parker, C.T. Mant, R.R. Sharma, J. Chromatogr. 458 (1988) 147.
[26] M. Palmblad, M. Ramström, K.E. Markides, P. Håkansson, J. Bergquist, Anal. Chem. 74 (2002) 5826.
[27] A.D. Norbeck, M.E. Monroe, J.N. Adkins, R.D. Smith, J. Am. Soc. Mass Spectrom. 16 (2005) 1239.
[28] K. Petritis, L.J. Kangas, B. Yan, M.E. Monroe, E.F. Strittmatter, W. Qian, Anal. Chem. 78 (2006) 5026.
[29] O.V. Krokhin, R. Craig, V. Spicer, W. Ens, K.G. Standing, R.C. Beavis, Mol. Cell. Proteomics 3 (2004) 909.
[30] O.V. Krokhin, Anal. Chem. 78 (2006) 7785.
[31] O.V. Krokhin, V. Spicer, Anal. Chem. 81 (2009) 9522.
[32] M. Gilar, H. Xie, A. Jaworski, Anal. Chem. 82 (2010) 265.
[33] A.V. Gorshkov, I.A. Tarasova, V.V. Evreinov, M.M. Savitski, M.L. Nielsen, R.A. Zubarev, M.V. Gorshkov, Anal. Chem. 78 (2006) 7770.
[34] I.A. Tarasova, V. Guryca, M.L. Pridatchenko, A.V. Gorshkov, S. Kieffer-Jaquinod, V.V. Evreinov, C.D. Masselon, M.V. Gorshkov, J. Chromatogr. B 877 (2009) 433.
[35] V. Sanz-Nebot, F. Benavente, J. Barbosa, J. Chromatogr. A 950 (2002) 99.
[36] B.J. Williams, W.K. Russell, D.H. Russell, Anal. Chem. 79 (2007) 3850.
[37] M. Borges-Alvarez, F. Benavente, E. Giménez, J. Barbosa, V. Sanz-Nebot, J. Sep. Sci. 33 (2010) 2489.
[38] J.L. Fauchere, V.E. Pliska, Eur. J. Med. Chem. 18 (1983) 369.
[39] T. Saito, in: Z. Bösze (Ed.), Bioactive Components of Milk, Springer, New York, 2008, 295 pp.
[40] N. Yamamoto, M. Ejiri, S. Mizuno, Curr. Pharm. Des. 9 (2003) 1345.
[41] J. Otte, S.M.A. Shalaby, M. Zakora, M.S. Nielsen, Int. Dairy J. 17 (2007) 1460.
[42] E. Haque, R. Chand, Eur. Food Res. Technol. 227 (2008) 7.
[43] H. Meisel, Int. Dairy J. 8 (1998) 363.
[44] S.V. Silva, F.X. Malcata, Int. Dairy J. 15 (2005) 1.
[45] B. Hernández-Ledesma, B. Miralles, L. Amigo, M. Ramos, I. Recio, J. Sci. Food Agric. 85 (2005) 1041.
[46] H. Korhonen, A. Pihlanto, Curr. Pharm. Des. 9 (2003) 1297.
[47] M. Gobbetti, F. Minervini, C. Grizzello, Int. J. Dairy Technol. 57 (2004) 173.
[48] B.A. Murray, R.J. FitzGerald, Curr. Pharm. Des. 13 (2007) 773.
[49] D.D. Kitts, K. Weiler, Curr. Pharm. Des. 9 (2003) 1309.
[50] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, Int. Dairy J. 14 (2004) 889.
[51] D.A. Clare, H.E. Swaisgood, J. Dairy Sci. 83 (2000) 1187.
[52] I. López-Expósito, I. Recio, Int. Dairy J. 16 (2006) 1294.
[53] I. López-Expósito, F. Minervini, L. Amigo, I. Recio, J. Food Prot. 69 (2006) 2992.
[54] M. Goggetti, L. Stepaniak, M. De Angelis, A. Corsetti, R. Di Cagno, Crit. Rev. Food Sci. Nutr. 42 (2002) 223.
[55] Y. Jinsmaa, M. Yoshikawa, Peptides 20 (1999) 957.
[56] C.E.H. Schmelzer, R. Schöps, L. Reynell, R. Ulbrich-Hofmann, R.H.H. Neubert, K. Raith, J. Chromatogr. A 1166 (2007) 108.
[57] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 5th ed., Pearson Prentice Hall, Harlow, UK, 2005, 145 pp.
[58] F. Kianifard, W.H. Swallow, NC State Department of Statistics Technical Reports, 1906 (1987) 1.
[59] F. Kianifard, W.H. Swallow, Commun. Stat. -Theor. Meth. 19 (1990) 1913.
[60] A.S. Hadi, J.S. Simonoff, J. Am. Stat. Assoc. 88 (1993) 1264.