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Rapid detection and identification of angiotensin-converting enzyme inhibitors by on-line liquid chromatography–biochemical detection, coupled to electrospray mass spectrometry

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Abstract

An analytical method based on on-line liquid chromatography–biochemical detection (LC–BCD) coupled to electrospray mass spectrometry was developed for the detection and identification of angiotensin-converting enzyme (ACE) inhibitors in complex mixtures, such as hydrolyzed whey proteins. ACE inhibitory activity was detected by coupling a homogeneous, substrate conversion based bioassay on-line to high-performance liquid chromatography (HPLC). Chemical information was obtained by directing part of the HPLC effluent towards a mass spectrometer. After correlating the biochemical and chemical data, the accurate molecular masses of the bioactive peptides were used as search queries in protein databases. Combined with the recorded mass spectrometry (MS)–MS fingerprints, bioactive peptides were selected from the database search results. The results of LC–BCD–MS analyses were verified by establishing a bioactivity balance. Reference samples, containing several peptides at concentration levels similar to those observed in the hydrolyzed milk samples, were analyzed by LC–BCD–MS. High recoveries of biological activity were obtained, indicating that the correct ACE inhibitors were identified and that no co-elution of significantly bioactive molecules had occurred. Approximately, 30 ACE inhibitors were detected and identified. IC_{50} values of ACE inhibitors, reported in literature, ranged between 43 and 580 μM.

Keywords: Biochemical detection; Detection, LC; Enzyme inhibitors; Angiotensine-converting enzyme inhibitors

1. Introduction

High blood pressure is a frequently occurring condition as 15–20% of all adults are estimated to suffer from the disease [1]. Hypertension is currently considered to be one of the most serious chronic illnesses as the risks of obtaining coronary heart diseases are increased significantly [2]. Angiotensin-I converting enzyme (ACE) plays an important role in the regulation of peripheral blood pressure in the human body. The dipeptidylcarboxypeptidase, ACE, catalyzes both

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the production of the potent vasopressor angiotensin-II as well as the deactivation of the vasodepressor peptide bradykinin [3]. During the last decades many synthetic drugs have been developed, which inhibited the enzymatic activity of ACE and proved successful in reducing blood pressure in hypertensive patients [4]. ACE inhibitory compounds, however, are widespread in nature and have been found in many medicinal plants [5–9] and food sources such as red wine [10], sake [11], mushrooms [12] and several types of cheese [13]. In addition, hydrolyzed samples of corn gluten [14], bonito [15], Alaska pollack skin [16], tuna [17], α-zein [18], *Bacillus subtilis* [19], *Antartic krill* [20] and Baker's yeast [21] were all found to exhibit ACE inhibitory activity.

Milk proteins, especially caseins, are commonly known as precursors of a range of biologically active peptides. The effects of these peptides have been correlated to nutrient uptake, immunomodulation, opioid and hypertensive activities [22]. The fact that milk proteins are precursors of a variety of biologically active molecules has been particularly appealing to functional food development. However, the detection and identification of these biologically active peptides has proven to be a challenging task. Hydrolyzed milk samples are known for their complexity and can contain up to hundreds of different molecules. Locating bioactive peptides in these or similar samples has commonly been a time consuming and difficult task. Fractions often still contain multiple compounds and need several additional fractionation, evaporation and biological evaluation cycles in order to identify the bioactive molecules. Typically, loss of bioactivity, due to compound instability or non-specific binding, is considered as one of the complicating factors of such strategies.

In the past we have described analytical techniques where biological assays were coupled on-line to liquid chromatography (LC) and used in parallel to mass spectrometry with the goal to simultaneously generate chemical and biological information. For example, on-line LC–biochemical detection (BCD)–mass spectrometry (MS) systems have been successfully applied in order to detect and characterize biochemically active compounds in Narcissus 'Sir Winston Churchill' [23], pomegranate peel extract [24] and a large natural extract library [25]. Crude extracts were separated by HPLC, after which the presence of biochemically active compounds was detected by means of an on-line biochemical assay. The advantages of on-line LC–BCD–MS over the traditional fractionation approaches were demonstrated in terms of dereplication speed and resources required. The current paper focuses on the applicability of on-line LC–BCD–MS in the characterization of functional foods. Monitoring of ACE inhibitory compounds in hydrolyzed milk samples during several stages of functional food development could increase efficiency of this process, as the appearance of new biochemically active compounds as well as the disappearance of others can accurately be determined.

2. Materials and methods

2.1. Chemicals

Angiotensin-converting enzyme (ACE) was purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Methanol and acetonitrile were obtained from J.T. Baker (Deventer, The Netherlands). Sodium chloride, trifluoroacetic acid (TFA), nitric acid, Tris and Tween 20 were obtained from Merck (Amsterdam, The Netherlands). Reference peptides and substrate, *ortho*-aminobenzoic acid–phenylalanine–arginine–lysine–dinitrophenol–proline (abz-FRK(dnp)P-OH [26]), were synthesized by Bachem (Weil am Rhein, Germany). Phenomenex analytical columns were purchased from *Aurora borealis* (Schoonbeek, The Netherlands). Altima, 250 mm × 2.1 mm, C_{18}, 5 μm, 100 Å analytical columns were obtained from Altech (Amsterdam, The Netherlands).

2.2. Hydrolyzed milk samples

Two commercially available hydrolyzed milk samples, denominated here as hydrolysate I and II, were used during the current studies. Typically, 0.5% (w/v) solutions were used for analysis, unless stated otherwise.

2.3. On-line LC–BCD–MS

Hydrolyzed milk samples were analyzed for ACE inhibitory activity in a high-resolution screening (HRS) instrument (Kiadis, The Netherlands). The

gradient setup consisted of four Agilent 1100 series LC pumps (Waldbronn, Germany). Two LC pumps were used to deliver the solutions for chromatographic separation. The two remaining LC pumps were used to add make-up solutions to the LC column effluent and compensate for changes in organic modifier percentage and HPLC flow rate throughout the chromatographic run. The aqueous and organic modifier solutions, which were used to perform gradient chromatography, consisted of 0 and 95% methanol. Similarly, the aqueous and organic modifier solutions, which acted as makeup phases after chromatographic separation, consisted of 0 and 35% methanol. All solutions contained 0.05% TFA. The total flow rate of the gradient effluent after makeup was kept constant at 1 ml/min and contained 10% methanol. Using a three-way flow splitter, 50 μl/min of the gradient effluent was introduced into the biochemical assay, 200 μl/min was directed towards a Micromass quadrupole time-of-flight (QTOF) micro mass spectrometer (Almere, The Netherlands), whereas 750 μl/min was directed to waste. The substrate and enzyme solution, i.e. abz-FRK(dnp)P-OH (10 μM) and ACE (0.0375 U/ml), were dissolved in 200 mM Tris, 300 mM NaCl, 0.5% Tween at pH 7.5 and transferred into 50 ml Pharmacia superloops (Uppsala, Sweden). Both superloops were positioned in a Spark Mistral oven (Emmen, The Netherlands) and were thermostatted at 4 °C. The superloops were connected to Agilent 1100 series LC pumps, which displaced the bioreagents at a flow rate of 25 μl/min. Affinity reactions were carried out in open tubular, knitted, polytetrafluoroethylene (PTFE), 0.5 mm i.d. reaction coils. The coils were positioned in a Shimadzu CTO-10AC vp oven (Den Bosch, The Netherlands), which was set at a temperature of 50 °C. Fluorescence detection was performed with an Agilent 1100 series fluorescence detector (Waldbronn, Germany). Liquid handling was performed with a Cavro MSP9500 autosampler (Sunnyvale, CA, USA), which was equipped with a Valco six-port injection valve (Schenkon, Switzerland).

2.4. On-line angiotensin-converting enzyme bioassay

ACE inhibition was monitored via a substrate conversion based bioassay format, published previously (Fig. 1) [27]. During the first step of the biochemical assay, compounds eluting from the analytical column were mixed with ACE. The mixture was allowed to interact for 60 s. In a second step, an internally quenched fluorescent substrate, i.e. abz-FRK(dnp)P-OH, was

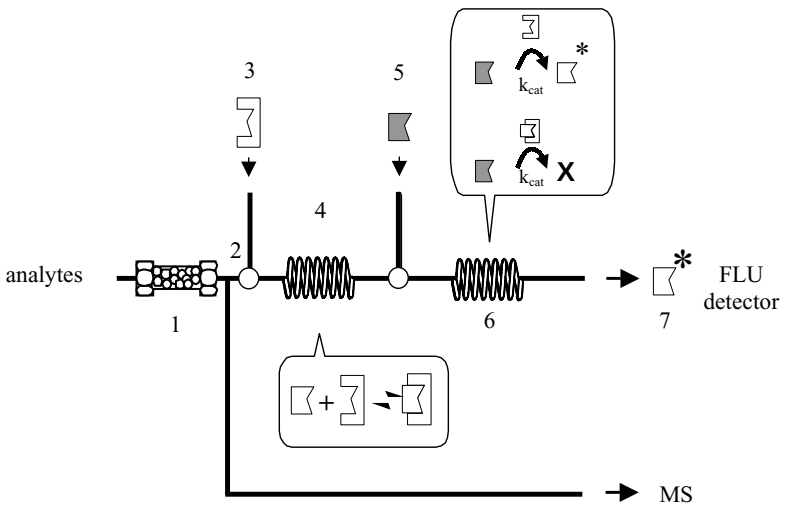

Fig. 1. On-line biochemical assay configuration: (1) separation of analytes by HPLC; (2) HPLC effluent splitted towards biochemical assay and mass spectrometer; (3) addition of ACE (0.0375 U/ml); (4) 60 s affinity interaction between analytes and ACE in reaction coil; (5) addition of substrate, abz-FRK(dnp)P-OH (10 μM); (6) conversion of substrate by ACE (120 s) in 0.5 mm i.d. PTFE reaction coil; (7) detection of reaction product by fluorescence detection.

added to the mixture and was allowed to interact with ACE for 120 s. Subsequent enzymatic conversion of the substrate molecule reduced the internal quenching efficiency as a result of bond cleavage between the fluorescent and quenching moieties, *o*-aminobenzoic acid (abz) and 2,4-dinitrophenyl (dnp). Cleavage of the substrate by ACE occurs between arginine and lysine. The fluorescence enhancement was monitored continuously at an excitation and emission wavelength of 320 and 420 nm, respectively. Compounds exhibiting ACE inhibitory activity temporarily reduce the rate of enzymatic substrate conversion and were detected as negative peaks in the biochemical readout. In addition to the biological activity data, chemical information was obtained in real-time by allowing part of the HPLC effluent to be directed towards a mass spectrometer. This way, both activity information as well as chemical characteristics of the biochemically active compounds, were obtained during a single chromatographic run. The assay conditions applied ensured maximum enzymatic activity by applying substrate saturation (10 μM). Under these conditions, the presence of the potent inhibitor captopril could be detected linearly between 1 and 20 nM, whereas moderately strong ACE inhibitors such as IPP and VPP could be detected with the same bioassay setup and both showed linear ranges of 2–20 μM.

2.5. Liquid chromatography–mass spectrometry (LC–MS)

2.5.1. On-line biochemical assay

Analytical separations were carried out at room temperature on an Altech Ultima, 250 mm × 2.1 mm, C_{18} column, packed with 5 μm, 100 Å particles. The starting flow rate over the analytical column was set at 200 μl/min. The total flow rate after post-column makeup was kept constant at 1 ml/min. In general, bioactivity profiling of the samples was achieved using 2–95% MeOH in 0.05% aq. TFA gradients with run times up to 90 min. After flow splitting, 200 μl/min of the HPLC effluent was introduced into a QTOF-micro mass spectrometer (Micromass, Manchester, UK). The electrospray ionizaion (ESI) QTOF-micro MS detector was predominantly operated in positive ion full scan mode. The desolvation and source temperatures applied were 300 and 80 °C. The capillary, sample cone and extraction voltages were set at 3000, 50 and 2.5 V. The cone and desolvation gas flow equaled 50 and 450 l/h. Under these conditions most of the peptides showed considerable fragmentation, which facilitated structure elucidation. Caffeine was used as a lock mass and was added continuously at a flow rate of 10 μl/min. The mass spectrometer was calibrated daily with a diluted phosphoric acid solution.

2.5.2. Quantification

The actual concentration levels of the active peptides were determined by preparing calibration curves of peptide standards in representative matrices. In order to simulate potential matrix effects, calibration curves of the ACE inhibitors, originating from hydrolysate II were prepared in hydrolysate I and vice versa. For hydrolysate I 10 calibration standards between 0 and 100 μg/ml were prepared. For hydrolysate II 7 calibration standards between 0 and 50 μg/ml were prepared. All standards were measured in triplicate. Reference samples and hydrolysates were separated by an Agilent 1100 series HPLC system using the following gradient settings: 0–3 min MeOH–water (5:95), acetic acid 0.1%, 3–43 min MeOH–water (95:5), acetic acid 0.1%, 43–44 min MeOH–water (95:5), acetic acid 0.1% and 44–45 min MeOH–water (5:95), acetic acid 0.1%. Analytical separations were carried out at room temperature on a Phenomenex Inertsil ODS-3, 150 mm × 2.1 mm, 5 μm column at 200 μl/min. The injection volume equaled 20 μl. The desolvation and source temperatures of the mass spectrometer were set at 350 and 120 °C. The capillary, sample cone and extraction voltages applied were 3000, 30 and 2.0 V. The cone and desolvation gas flow equaled 50 and 600 l/h. Caffeine was used as an internal standard. Leucine enkephalin was used as lock mass and was added continuously at a flow rate of 10 μl/min. The responses of the ACE inhibitors were determined in positive ion full scan MS mode.

2.6. Detection and characterization of biochemically active peptides

Biochemical assay responses were correlated to the recorded MS data by analyzing control samples, containing known ACE inhibitors, prior to the analysis of the digests. This way, the difference in retention time of the response in the biochemical assay and the MS detector (*m*/*z* 1053.2 for the known inhibitor

Pyr–GLPPGPPIPP) was determined and used during further data evaluation. Biochemical responses in the hydrolysates were corrected for the measured time difference, allowing ACE inhibitors to be linked to the corresponding MS spectra. Subsequently, reconstructed ion currents (RIC) of all *m*/*z* traces (relative intensity >5%), which were observed in the MS spectra, were derived. The retention times of the *m*/*z* traces were compared with the expected elution time of the bioactive compound. Compounds, which eluted outside a ±0.2 min range from the expected elution time, were discarded. According to this criterion the majority of the biochemically active peptides was located within the MS spectra. In case of co-elution, the similarity in peak shape between the biochemical response and the RIC of the *m*/*z* trace was used as an additional method to eliminate closely eluting molecules. Similarities between RIC traces allowed the identification of ion fragments, dimers, multiple charged molecules and adducts corresponding to the biochemically active molecules as well. In case a biochemically active peptide could not be selected unambiguously, the run time of the analysis was prolonged in order to enhance chromatographic resolution.

2.7. Structure elucidation of biochemically active peptides

Accurate molecular masses and ion fragments obtained due to in-source fragmentation were used to elucidate the structure of the biochemically active peptides. The accurate masses (±20 ppm) were used as a search query in protein databases. Typically, several peptide sequences were found to match the molecular weight of the bioactive compound. The databases used, contained amino acid sequences of common mature milk proteins, i.e. β-casein, α-s_1-casein, α-s_2-casein, κ-casein, α-lactalbumin and β-lactoglobuline and were obtained from the Swiss Institute for Bioinformatics (Swissprot database). By combining the results of the database searching with interpretation of the fragmentation pattern, biochemically active compounds were identified. The molecular structures were verified by processing the MS spectra via Micromass Proteinlynx software. The spectra of the detected biochemically active peptides were compared with their theoretical fragmentation patterns. The proposed amino acid sequences of the biochemically active peptides were accepted if a substantial number of fragments were identified. Additional, indirect structural confirmation was obtained by comparing the identified peptides with theoretical digests of common milk proteins, i.e. β-casein, α-s_1-casein, α-s_2-casein, κ-casein, α-lactalbumin and β-lactoglobuline (Swissprot database).

2.8. Confirmation identification by HRS-MS analysis of synthetic mixtures

Based on the quantification results, reference samples were prepared containing inhibitors of interest in similar concentrations as those measured in the hydrolysate solutions. By comparing the biochemical activity profiles of the synthetic mixtures and the original sample in terms of retention times and peak area, additional confirmation of identity is obtained.

3. Results and discussion

3.1. LC–BCD–MS analysis

3.1.1. Analysis of reference solution

The ability to accurately correlate HRS and MS data throughout an entire chromatographic run represents one of the most crucial requirements of LC–BCD–MS analysis. Accurate correlation of HRS and MS data was demonstrated by analyzing a reference solution, containing 3 known ACE inhibitory peptides: VPP, IPP and Pyr–GLPPGPPIPP. After LC–BCD–MS analysis, the difference in response time between biochemical and chemical detection was determined for the bioactive molecule Pyr–GLPPGPPIPP. Using this value, the retention times of the biochemical responses of VPP and IPP were corrected for the measured difference in response times and were subsequently linked to the recorded MS data (Fig. 2). Maximum biochemical responses of VPP and IPP were detected at t_R 10.68 and 12.57 min, whereas the corresponding mass traces were observed at t_R 10.62 and 12.50 min. Based on the determined difference in response times within the control sample, the retention times of the biochemical responses in additional positive reference samples were corrected. Similarly, the difference in retention time correlation was found to be below 0.1 min.

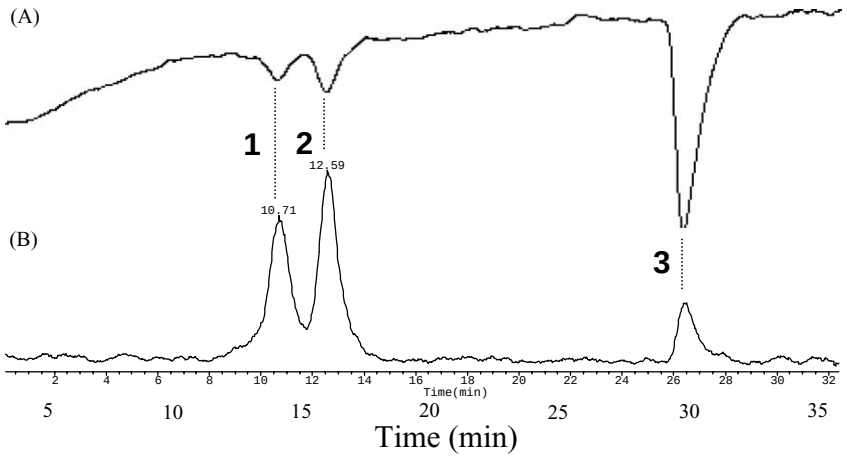

Fig. 2. LC–BCD–MS analysis of reference sample: (A) response on-line biochemical assay; (B) reconstructed ion current of *m*/*z* 312.2, 326.4, 1053.2. Reference compounds: (1) 2 μM VPP (*m*/*z* 312.2); (2) 2 μM IPP (*m*/*z* 326.4); (3) 0.5 μM Pyr–GLPPGPPIPP (*m*/*z* 1053.2). Chromatographic conditions: 2–95% MeOH gradient in 30 min followed by 2 min postgradient at 0.2 ml/min (start flow rate). Analytical column: Phenomenex, 150 mm × 2.1 mm, ODS (2) packed with 5 μm particles.

3.2. LC–BCD–MS analysis of hydrolyzed samples

The hydrolyzed milk samples, I and II, were analyzed by LC–BCD–MS using a 2–95% MeOH in 0.05% aq. TFA gradient of respectively 70 and 60 min, followed by a 5 min isocratic postgradient period. The biochemical chromatograms (Fig. 3I(A) and II(A)) each show the presence of a multitude of biologically active compounds. The retention times of all the bioactive compounds were determined at maximum intensity, corrected for the difference in response time between biochemical and MS readout and subsequently, matched with the recorded MS data. Typically, as a result of closely eluting compounds, fragmentation and background interferences the MS spectra of the bioactive compounds were characterized by the presence of a multitude of additional mass traces, besides the molecular ion ($[M + H]^+$). Fig. 4 shows a typical example of such a MS spectrum (bioactive peak 10, hydrolysate II). Ions are distributed over a large mass range and differ significantly in intensity. In order to deselect co-eluting compounds and trace the bioactive molecule(s), reconstructed ion currents of all mass traces (relative intensity >5%) were derived. Based on the retention time of the bioactive peak ($t_R = 41.54$ min) and the difference in response time between biochemical and MS detection (2.59 min), an ion current maximum of the bioactive compound was expected at 38.95 min. As was demonstrated previously by LC–BCD–MS analysis of the reference solution, biochemical and chemical data could be correlated within 0.1 min. By applying this correlation accuracy, molecules, which did not show a peak maximum within a 38.95 ± 0.1 min time window, were identified and ignored during further data interpretation (Fig. 5). By processing each mass trace in a similar manner, only a few ions remained as potential biologically active compounds. Typically, the majority of these ions corresponded to fragments or adducts of the bioactive peptide, which were identified by their almost identical retention times and peak shapes. In this case, several mass traces were found to match accurately with the biochemical response. These ions showed similar elution profiles, in terms of retention time and peak shape, which indicated significant in-source fragmentation of a single peptide. In order to locate the molecular ion of the bioactive peptide, the selected mass traces were scanned for the presence of multiple charged ions and dimers first. The presence of a double charged ion at *m*/*z* 490.7 and a dimer at *m*/*z* 1859.6, strongly indicated *m*/*z* 979.6 as the molecular ion. The remaining mass traces were found to correspond to the sodium adduct of *m*/*z* 979.6 and a series of fragments. After correcting the molecular ion for mass shifting, the accurate mass, 979.5123,

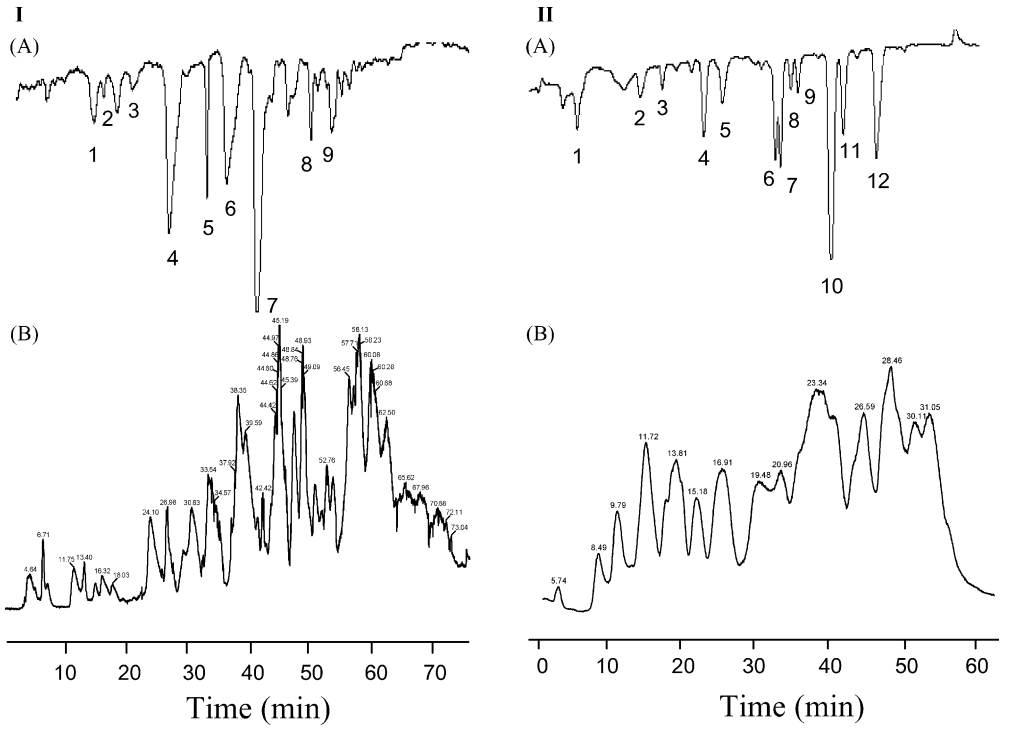

Fig. 3. LC–BCD–MS analysis of hydrolyzed milk samples I and II: I(A) response on-line biochemical assay; (B) total ion current (TIC). II(A) Response on-line biochemical assay; (B) total ion current. Chromatographic conditions. (I): 2–95% MeOH gradient in 70 min followed by 5 min postgradient. (II): 2–95% MeOH gradient in 60 min followed by 5 min postgradient. Maximum flow rate over analytical column 0.2 ml/min. Analytical column: Phenomenex, 150 mm × 2.1 mm, ODS (2) packed with 5 μm particles.

was used as a database search query. The databases, which contained the milk precursor proteins of interest, were searched for amino acid sequences with similar molecular masses (±0.050). Several possible amino acid sequences were found. LDAYPSGAW and NLLRFFVA were found in α-s1-casein; SSEESIISQ, FALPQYLK and KFALPQYL were present in α-s2-casein; TESQSLTLT, ELQDKIHP, PIPNSLPQN, NSLPQNIPP and PNSLPQNIP were found in β-casein, whereas PYYAKPAAV was found in κ-casein. Subsequent selection of the correct bioactive peptide was achieved by interpretation of the fragmentation pattern. The settings of the mass spectrometer were chosen in such a way as to enhance partial fragmentation of the bioactive peptide. This way, low intensity bioactive peptides, which could potentially be obscured by higher intensity ions during survey scanning, could be elucidated during a single LC–BCD–MS run as well. The MS spectrum of bioactive compound 10 (Fig. 4) showed several characteristic fragment ions, which could be correlated to the amino acid sequence FALPQYLK (y_5 at *m*/*z* 648.5 and y_6 at *m*/*z* 761.6). The theoretical digests (pepsin/trypsin treatment) of several common milk proteins confirmed the presence of this peptide in the hydrolyzed sample. The identity of the ACE inhibitor was verified by matching the ion fragments found with the theoretical fragmentation pattern of FALPQYLK using ProteinLynx software (Fig. 6). A substantial number of y″ (6) and b″ (4) ions were found, which confirmed the identity of the biologically active compound. The detection and identification procedure, which is described here, was applied to identify all other bioactive compounds in hydrolysates I and II as well (Tables 1 and 2). The measured monoisotopic masses of the bioactive peptides were in good agreement with the theoretical values. Typically, mass differences below 30 ppm were observed.

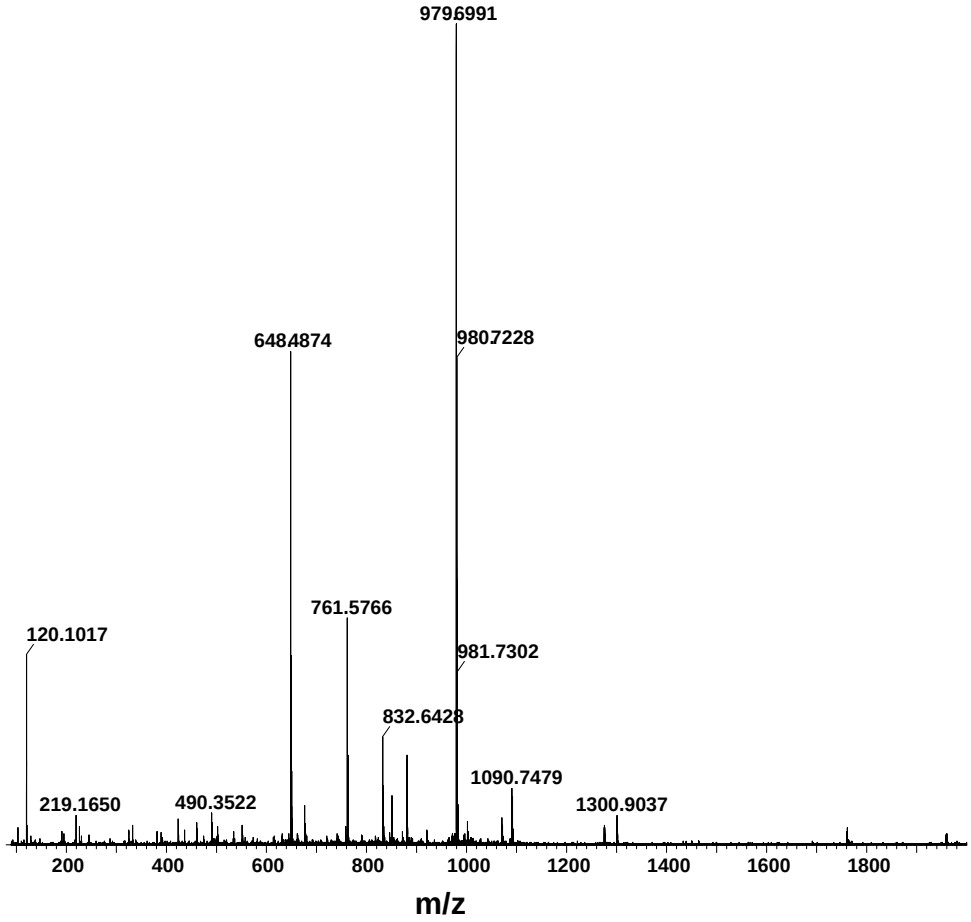

Fig. 4. MS spectrum of bioactive peak 10 originating from hydrolysate II.

Table 1
Structures of LC–BCD–MS identified bioactive peptides in hydrolysate I

Bioactive peptide	M_r		Error (ppm)	Structure	Source	Position	Theoretical digest[a]
	Measured	Calculated					
1	409.2043	409.2087	11	FDK	β-Lactoglobulin	136–138	+
2	618.3443	618.3463	10	EQLTK	α-Lactalbumin	20–24	−
3	425.2699	425.2625	17	HIR	β-Lactoglobulin	146–148	+
4	ntbd[b]	ntbd[b]	–	Peptide A	β-Lactoglobulin	ntbd[b]	+
5	654.3978	654.3728	38	WLAHK	α-Lactalbumin	123–127	+
6	673.3813	673.3885	11	GLDIQK	β-Lactoglobulin	9–14	+
7	837.4757	837.4769	1	ALPMHIR	β-Lactoglobulin	142–148	+
8	ntbd[b]	ntbd[b]	–	Peptide B	β-Lactoglobulin	ntbd[b]	+
9	ntbd[b]	ntbd[b]	–	Peptide C	β-Lactoglobulin	ntbd[b]	+

[a] Amino acid sequences are matched with the theoretical digests (pepsin and trypsin) of β-lactoglobuline and α-lactalbumin.
[b] For patent reasons the masses and positions can not be disclosed.

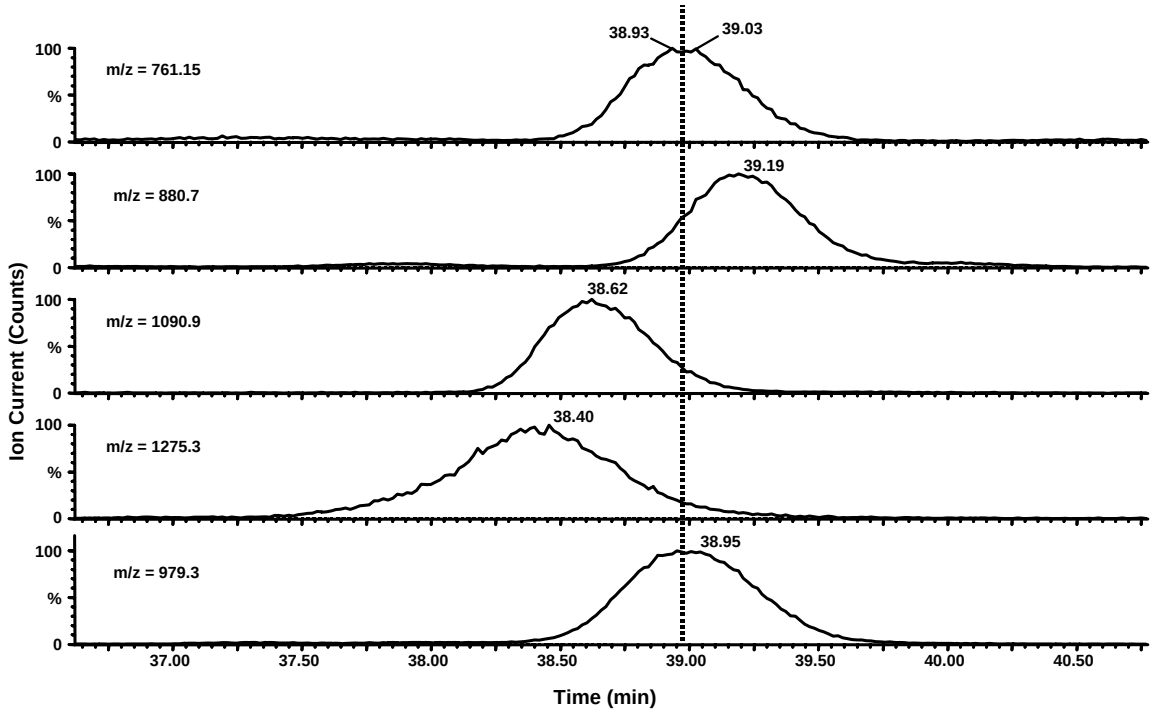

Fig. 5. Reconstructed ion currents of mass traces correlating to bioactive peak 10 originating from hydrolysate II (*m/z* 761.2, 880.7, 979.3, 1090, and 1275.3). The calculated retention time is marked with a black line, 38.94 min. Chromatographic conditions: 2–95% MeOH gradient in 60 min followed by 5 min postgradient at 0.2 ml/min (start flow rate). Analytical column: Alltima, 150 mm × 2.1 mm, C_{18}, packed with 5 μm particles, 100 Å.

Table 2
Structures of LC–BCD–MS identified peptides in hydrolysate II

Bioactive peptide	M_r		Error (ppm)	Structure	Source	Position	Theoretical digest[a]
	Measured	Calculated					
1	374.2332	374.2398	18	INK	β-Casein	26–28	+
2	903.4524	903.4660	15	TVYQHQK	α-s2-Casein	197–203	+
3	689.3749	689.3834	12	VNELSK	α-s1-Casein	37–42	+
4	830.4355	830.4525	20	AVPYPQR	β-Casein	177–183	+
5	780.4739	780.4983	31	VLPVPQK	β-Casein	170–176	+
6	971.4808	971.4852	5	HPHPHLSF	k-Casein	98–105	+
7	737.3708	737.3735	4	HPHLSF	k-Casein	100–105	−
8	1102.5244	1102.5256	1	HPHPHLSFM	k-Casein	98–106	−
9	868.4058	868.4140	9	HPHLSFM	k-Casein	100–106	−
10	979.5383	979.5611	23	FALPQYLK	α-s2-Casein	189–196	+
11	748.3639	748.3698	8	TTMPLW	α-s1-Casein	194–199	+
12	1384.6890	1384.7305	30	FFVAPFPFVFGK	α-s1-Casein	23–34	+

[a] Amino acid sequences are matched with the theoretical digests (pepsin and trypsin) of α-s1-casein, α-s2-casein, β-casein, k-casein and lactoferine.

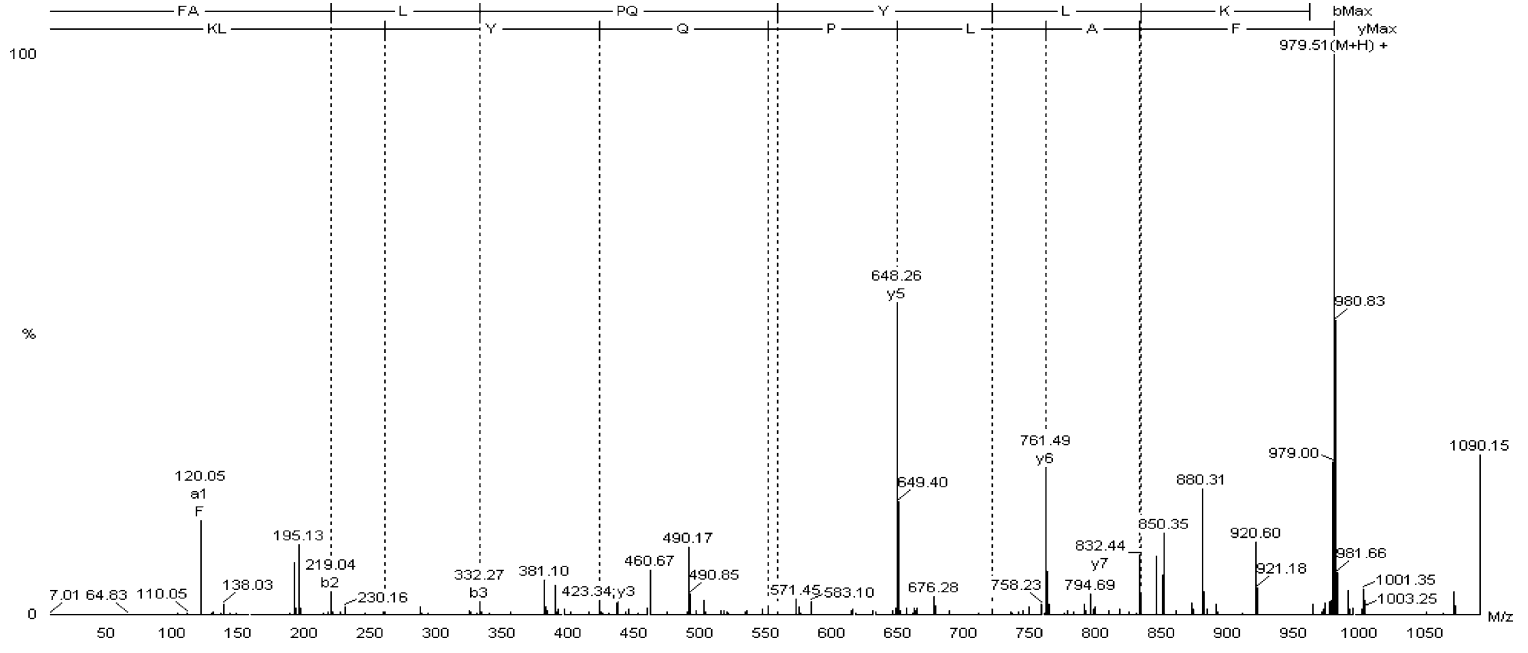

Fig. 6. Confirmation of peptide sequence FALPQYLK ($[M + H]^+$ 979.51) using Masslynx, PepSequence software. Dotted lines represent identified y and b ions of the ACE inhibitor.

Table 3
Summary quantification results hydrolysates I (1.0%, w/v) and II (1.0%, w/v)

Hydrolysate	Peptide	Correlation coefficient	[a]Concentration (μM)	[b]ACE inhibition (%)
I	Peptide A	0.9953	432.9	5.7
	WLAHK	0.9994	19.7	59.2
	ALPMHIR	0.9907	337.7	12.2
	GLDIQK	0.9929	449.2	4.4
	Peptide C	0.9946	16.3	18.6
II	AVPYPQR	0.9964	428.5	15.7
	FALPQYLK	0.9911	236.7	66.7
	FFVAPFPEVFGK	0.9947	628.7	17.6

[a] Concentration of hydrolysates used during quantification 1.0% (w/v).
[b] Normalized ACE inhibition, i.e. inhibition percentage at equimolar concentrations.

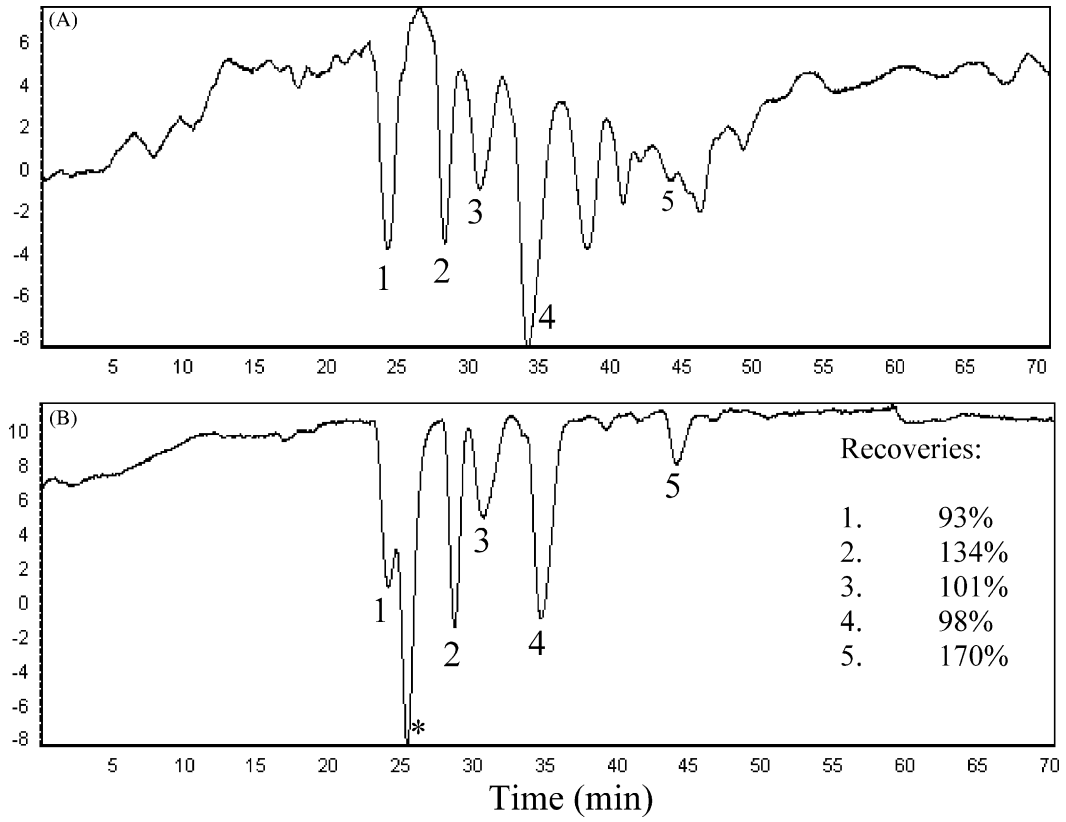

Fig. 7. Bioactivity balance of hydrolysate I. (A) LC–BCD–MS analysis of 0.5% hydrolysate I, 0.5% (w/v). (B) Response on-line biochemical assay, reference solution of ACE inhibitory compounds. (1) Peptide A (216 μM); (2) WLAHK (98 μM); (3) GLDIQK (224 μM); (4) ALPMHIR (119 μM); (5) peptide C (78 μM). Chromatographic conditions: 2–95% MeOH gradient in 60 min at 0.2 ml/min (start flow rate), 10 min post-gradient. Analytical column: Altima (Altech), 250 mm × 2.1 mm, C_{18}, 5 μm particles, 100 Å. (∗) Impurity in reference solution (*m/z* 873.5).

3.3. Normalization of ACE inhibition percentage

The relative contribution of the individual peptides to the total ACE inhibitory activity is calculated by comparing the peak areas with the summed areas of all inhibitors (Fig. 3). However, as the concentrations of the peptides differ significantly, relative bioactivities merely based on peak areas, do not provide a realistic representation of the relative potency of these inhibitors. Combining concentration levels and peak areas on the other hand, enables ACE inhibition to be normalized in terms of biochemical response per concentration unit. Several of the identified ACE inhibitors were synthesized in order to accurately determine their concentration in the milk hydrolysates. For these quantification purposes the reference compounds were spiked to representative hydrolysate milk samples. At the concentration levels used and under the chromatographic conditions applied, no interferences were observed from co-eluting compounds. The ion currents of the reference compounds spiked to milk hydrolysates were comparable to those measured in aqueous solution (data not shown). Correlation coefficients of all reference compounds exceeded 0.99 under the conditions applied. The effect of normalizing ACE inhibition, based on peak areas and concentration levels, is demonstrated clearly (Table 3). Peptide WLAHK, which showed a moderately large biochemical response in the bioaffinity chromatogram (Fig. 3(I), peak 5), appeared to be one of the most potent inhibitors present in the hydrolysate. In contrast, ALPMHIR, which generated the highest biochemical

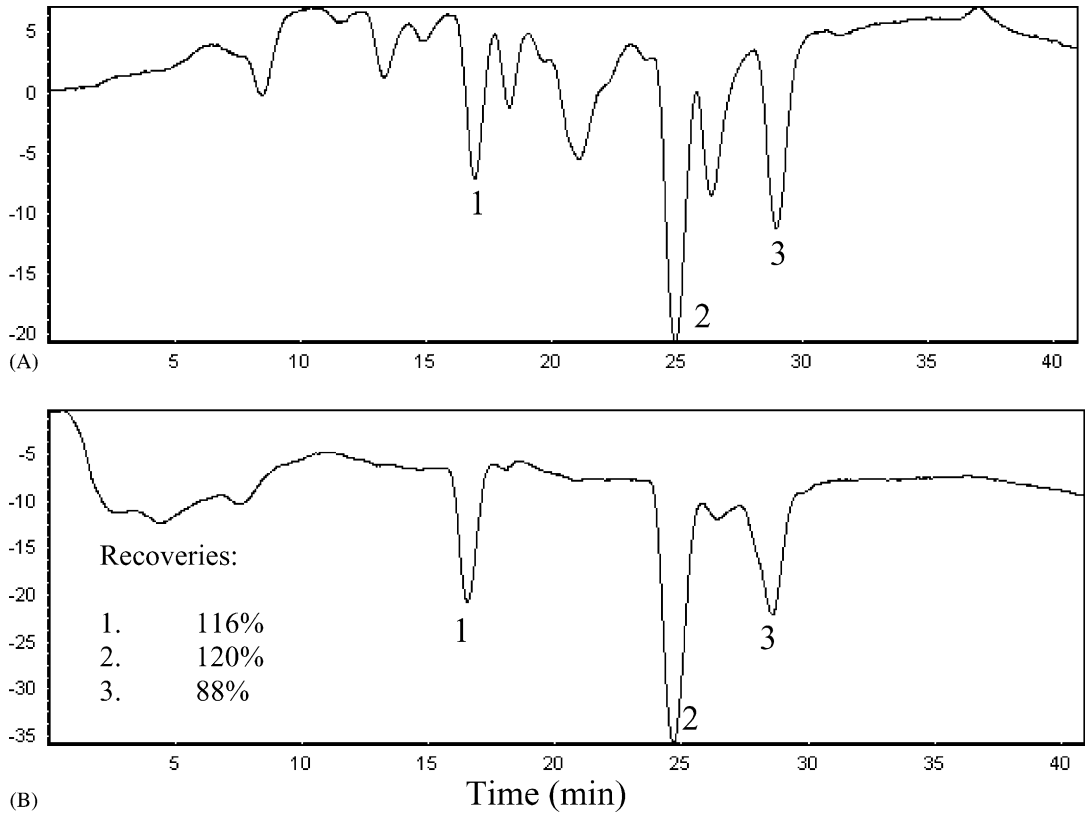

Fig. 8. Bioactivity balance of hydrolysate II. (A) LC–BCD–MS analysis of hydrolysate II, 0.5% (w/v). (B) Response on-line biochemical assay, reference solution of ACE inhibitory compounds. (1) AVPYPQR (214 μM); (2) FALPQYLK (118 μM); (3) FFVAPFPFVGK (315 μM). Chromatographic conditions: 2–95% MeOH gradient in 30 min at 0.2 ml/min (start flow rate). Analytical column: Altima (Altech), 250 mm × 2.1 mm, C_{18}, 5 μm particles, 100 Å.

response, was determined to exhibit considerably less ACE inhibitory activity when expressed as activity per concentration unit. The potency order of the quantified bioactive peptides in hydrolysate I was determined as: WLAHK > peptide C > ALPMHIR > peptide A > GLDIQK. The difference in potency between WLAHK and GLDIQK, as detected by LC–BCD–MS, is well reflected by the reported IC_{50} values of respectively, 77 and 580 μM [22]. Similarly, the potency order in hydrolysate II was found to be: FALPQYLK > FFVAPFPEVFGK > AVPYPQR.

3.4. Confirmation identification by HRS-MS analysis of synthetic mixtures

Several inhibitors of interest were synthesized and analyzed by HRS-MS in order to obtain additional identity confirmation by comparing the biochemical activity profiles with that of the original samples. The synthetic mixture contained reference inhibitors at concentration levels similar to those observed in the original sample. The biochemical activity chromatograms of the hydrolyzed and reference samples were found to be in good agreement (Figs. 7 and 8). All reference compounds showed similar retention times with those present in the original samples whereas the peak areas showed a high degree of similarity as well, thus providing additional evidence that: (1) the correct inhibitors were identified in the hydrolyzed milk samples, (2) the larger part of the biochemical responses of interest could almost completely be assigned to the identified inhibitors. The observed variations in recoveries, as displayed in Figs. 7 and 8, are mainly caused by differences in baselines of the biochemical activity chromatograms of the original and reference samples. Nevertheless, the similar profiles demonstrate limited co-elution of additional biochemically active compounds.

4. Conclusion

Approximately 20 ACE inhibitory compounds were identified in hydrolyzed milk protein samples by applying LC–BCD–MS analysis. Compared to the traditional analytical and biochemical methods, which are typically applied in functional food analysis, LC–BCD–MS was shown to significantly reduce the time needed for detection and identification of bioactive peptides. The implementation of a QTOF mass spectrometer, which generated accurate masses, proved to be a valuable tool in reducing the number of possible molecular formulae. Combined with database searching and interpretation of fragmentation patterns, structure elucidation of the biologically active peptides was straightforward. With the biochemical assay employed, it was possible to detect weak affinity ACE inhibitors ($K_i > 10^{-4}$ M).

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