Application of mass spectrometry to the characterization and quantification of food-derived bioactive peptides

María del Mar Contreras[a], Iván López-Expósito[ab], Blanca Hernández-Ledesma[c], Mercedes Ramos, Isidra Recio*

Instituto de Fermentaciones Industriales (CSIC). Juan de la Cierva 3, 28006 Madrid. Spain.

* Corresponding author: Dr. I. Recio
Tel.: +34 91 5622900
Fax: +34 91 5644853
E-mail address: recio@ifi.csic.es

[a] Present address: Mount Sinai School of Medicine. New York, USA.
[b] Both authors contributed equally to this work.
[c] Present address: College of Natural Resources. University of Berkeley, USA.

ABSTRACT

Biologically active peptides are of particular interest in food science and nutrition because they have shown to play different physiological roles, including antihypertensive, opioid, antimicrobial and immunostimulating activities. Because these peptides are generated by protein hydrolysis or fermentation, they can represent only minor constituents in a highly complex matrix, and therefore, identification of biologically active peptides in food matrices is a challenging task in food technology. In this context, mass spectrometry has developed into a necessary tool to assess quality and safety of food, and more recently, to determine the presence and the behavior of functional components, such as, these bioactive peptides. This review highlights the existing methods based on mass spectrometry to identify, characterize and quantify food-derived biologically active peptides. The paper is organized taking in account the different ionization sources used for the analysis of these high-value food components. Other section specifically deals with the quantitative determination of bioactive peptides in food products or biological fluids.

INTRODUCTION

Proteins play an extremely important role in determining nutritional and functional properties of food products. Further, proteins are potential health-promoting ingredients as a consequence of the biological value ascribed to the presence of bioactive peptides in their primary sequences. Bioactivities of peptides encrypted in major milk proteins are latent until released and activated during gastrointestinal digestion or food processing (1 & 2). Recent reviews have described peptides with different biological activities such as opioid, antihypertensive, antimicrobial, antithrombotic, immunomodulating, and metal-binding activities (2-7). Many of these peptides represent only minor constituents in a highly complex matrix, and therefore, identification of biologically active peptides in food matrices is a challenging task in food technology. Milk protein hydrolysates are known for their complexity and can contain up to hundreds of different peptide sequences. Identification of bioactive peptides in fermented dairy products or milk protein hydrolysates generated by the action of unspecific enzymes is a labor-intensive and difficult task.

During the last three decades, many research efforts have been done to develop techniques and methods for the separation, purification and characterization of food peptides and proteins. HPLC has been the most widely used technique for analytic and preparative separations, commonly coupled with conventional UV and fluorescence detectors. Structural characterization of protein was gained by alternative methods such as amino acid composition, molecular spectroscopy, whereas SDS-electrophoresis and/or ultracentrifugation were used for mass determination (8 & 9). Most recently, since the introduction of “soft” ionization techniques (FAB, ESI and MALDI), mass spectrometry (MS) has emerged as an important tool for protein identification and characterization. The application of MS to characterization of food proteins has been outlined in recent reviews (8-12). The use of MS in food products has allowed the accurate determination of molecular mass and protein sequences, the detection of post-translational modifications (phosphorylation and glycosylation), or chemical modifications of primary sequence, detection of new genetic variants, identification of protein degradation products and the study of protein conformations. The MS analysis of food-derived peptides has also been considered in several publications and reviews (8 & 10).

Current nutrition research focuses on the link between diet constituents and their physiological effects. Among different functional ingredients present in food, bioactive

peptides have received special attention due to their versatility. Development of functional foods containing these functional peptides requires not only identification of the active form, but also quantitative analysis and study of the peptide stability in complex food and biological matrices. For these purposes, mass spectrometry has become the analytical method of choice, offering selectivity and sensitivity for peptide identification, characterization and quantification.

This review deals with the application of MS to the identification and quantification of bioactive peptides (antihypertensive, antioxidant, mineral carriers, antimicrobial, opioid, immunomodulators, etc) derived from food protein, especially from dairy products, eggs and cereals. Furthermore, recent MS and MS^n methods applied to peptide quantification and monitorization, in both, foods and in biological fluids will be also illustrated.

2. Identification of bioactive peptides by plasma desorption, secondary ion mass spectrometry and fast atom bombardment.

Until the early 1980s, biomolecules were usually ionized by electron-impact (EI) or chemical-ionization (CI) methods. However, polar thermally labile samples cannot be analyzed by EI- or CI-MS without prior derivation (13). The development of desorption techniques represented the first major breakthrough for the formation of gaseous protein or large peptides ions without fragmentation. These desorption techniques include field desorption (FD), plasma desorption (PD), laser desorption (LD), secondary ion (SI) and fast atom bombardment (FAB) (10). In this manner, Kinoshita and co-workers (14) have characterized a potent ACE (angiotensin-converting enzyme)-inhibitory peptide derived from soy sauce by combining of SI-MS, NMR and infrared spectrum, elemental analysis. Singh et al. (15) identified several phosphopeptides originated from α_{S1}-, α_{S2}- y β-casein (α_{S1}-, α_{S2}- y β-CN) in the diafiltration retentate of the water-soluble extract of Cheddar cheese. In this case, the peptides were sequenced by Edman degradation analysis and masses were determined by PD-MS. This MS technique was also applied to characterize a fraction from β-lactoglobulin (β-Lg) tryptic digest where an ACE-inhibitory peptide, β-Lg f(142-148), was identified (16).

FAB ionization is one of the pioneering desorption techniques that has contributed to the substantial progress in the mass determination of peptides and small proteins and has permitted MS to expand into new areas of chemistry and biology (17).

This technique has successfully been used to identify biologically active peptides derived from milk and milk products by combining the molecular mass value with the N-terminal sequence obtained by Edman degradation. By using this strategy, numerous phosphopeptides have been identified in Grana Padano cheese (18 & 19), Parmigiano-Reggiano cheese (20 & 21), Comté cheese (22) and cheese whey (23). This combination of structural techniques has also been applied to identify the antihypertensive peptide β-Lactosin B (β-Lg f(142-145) from a commercial whey product (24) and antihypertensive peptides that naturally exists in Gouda cheese (25). Other applications of the FAB-MS, in combination with other techniques, includes the detection of previously reported immunostimulating and opioid peptides from UHT milk fermented with *Lactobacillus* GG and consecutively digested by pepsin and trypsin (26). A peptide having strong free radical scavenging activity, corresponding to α_{S1}-CN f(144-149), was separated from a peptic digest of casein by chromatographic analyses and the peptide was characterized by amino acid analysis, Edman degradation and FAB-MS performed on a double-focusing spectrometer (27). Minkiewicz and co-workers (28) studied the susceptibility of some different glycosylated caseinmacropeptide (CMP) to enzymatic attack by the Glu-specific endopeptidase from *S. aureus* V8. Hydrolytic products were successfully identified, included peptides contained phosphate, glycosidic moieties and methionine sulfoxide, by FAB-MS carried out on a four-sector instrument and electrospray (ESI)-MS performed on a quadrupole mass spectrometer.

There have been relatively fewer studies with other food peptides different than those derived from dairy proteins. Miyoshi and co-workers (29) isolated ACE-inhibitory peptides from a thermolysin hydrolysate of α-zein (a major component of maize endosperm protein) that had previously showed hypotensive activity on spontaneously hypertensive rats. The peptides were sequenced by amino acid analysis and Edman degradation analysis combined with FAB-MS performed on a double focusing mass spectrometer. The most potent ACE-inhibitor, peptide LRP, showed a slight hypotensive effect on spontaneously hypertensive rats. Suetsuna and Nakano (30) identified four ACE-inhibitory and antihypertensive tetrapeptides derived from the peptic digest of protein prepared from wakame algae by amino acid and Edman degradation analysis and FAB-MS performed on double focusing mass spectrometer. More recently, this research group has identified four antihypertensive dipeptides

among 10 peptides with ACE-inhibitory activity derived from hot water extract of this alga (31).

In some reports, FAB coupled to a four-sector mass spectrometer was used to directly sequence biologically active peptides from fermented milk products or protein hydrolysates. Gobetti et al. (32) studied the ACE-inhibitory activity of RP-FPLC fractions from two fermented milks with *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. Most active fractions were subsequently purified by several chromatographic steps and the peptides were sequenced by FAB-MS/MS using a four-sector mass spectrometer. The peptides produced in the first fermented milk corresponded to sequences of β-CN f(6-14), f(7-14), f(73-82), f(74-82) and f(75-82) and peptides derived from second fermented milk were β-CN f(7-14), f(47-52) and f(169-175) and κ-CN f(152-160) and f(155-160). More recently, an enzymatic hydrolysate of wheat gluten was further digested with porcine pepsin and pancreatin to obtain an indigestible pyroglutamyl peptides mixture. A total of 13 peptides were identified and some of them were sequenced by FAB-MS/MS using four-sector mass spectrometer (33). All these applications of FAB-MS to the identification of bioactive peptides are summarized in Table 1.

3. Identification of bioactive peptides by electrospray ionization mass spectrometry (ESI-MS)

Today, atmospherically pressure ionization (API) interfaces like ESI and the atmospheric pressure chemical ionization (APCI) have displaced more antique soft ionization methods like FAB (34). In this manner, ESI has become the predominant ionization method for HPLC-MS in biomolecular analysis (35).

Electrospray has been used in conjunction with all common mass analyzers (see Table 2). When it was firstly introduced, ESI was most used in conjunction with a quadrupole mass analyzer. In this mode, numerous bioactive peptides have been characterized by combining mass determination with the amino acid composition or Edman degradation. For instance, HPLC and ESI-MS on a single quadrupole was useful to monitor the production of an immunomodulatory peptide, β-CN f(193-199), during continuous hydrolysis of β-casein with chymosin in a membrane reactor (36). Matar and Goulet (37) detected an opioid peptide, β-casomorphin 1-4 (60-63), in a peptide extract derived from milk fermented with an X-prolyl-dipeptidyl amino peptidase-deficient

mutant strain of *Lactobacillus helveticus* L89. Using RP-HPLC off-line with ESI-MS, N-terminal amino acid sequencing, and amino acid analysis allowed to Dionysius and Milne (38) the identification of peptides with antimicrobial activity from a peptic digest of bovine lactoferrin. Several ACE-inhibitory peptides from milk proteins fermented with different lactic acid starters and hydrolysed with digestive enzymes were identified by amino acid and MS-analysis (39). This analytical strategy was also used to identify two potent ACE-inhibitory peptides, (α_{s2}-CN f(174-181) and α_{s2}-CN f(174-179), from a tryptic α_{s2}-CN hydrolystate (40). In addition, Xiao and co-workers have developed a sensitive and rapid method to determine ACE-inhibitory activity based on a combination of enzymatic reaction followed by HPLC-ESI-MS determination (41). Chen and co-workers have isolated 6 antioxidant peptides from soybean β-conglycinin hydrolysate with protease S from *Bacillus sp* by SEC and RP-HPLC fractionation steps. Their amino acid sequences were determined by amino acid analysis and gas-phase sequenciation and mass were obtained using triple quadrupole mass spectrometer (42). More recently, antioxidant peptides (FD, WV, LW and WL) from a royal jelly hydrolysate with protease N have been identified (43). These peptides showed potent activity against lipid peroxidation and three of them (FD, WV and LW) protected against oxidative stress-induced cell death in human cultured cells.

Nevertheless, at the present time, the vast majority of peptide sequencing experiments is carried out by ESI-MS/MS, for instance by using a triple quadrupole mass analyzer. Recio and Visser (44) identified and sequenced two potent antibacterial peptides derived from a peptic hydrolysate of bovine α_{S2}-casein, f(183-207) and f(166-179). The release of bioactive bovine albutensin and human albutensin has been reported by De Noni and Floris (45) by tryptic hydrolysis of bovine and human serum albumin, respectively. Other biologically active peptides, such as ACE-inhibitory peptides and antithrombotic peptides from fermented soy products (46) and peptides from digests of durum wheat gliadin with potential positive effects in celiac disease (47) have been identified by using this technique. This mass spectrometer has also been applied to develop a multidimensional electrospray MS-based strategy for phosphopeptides mapping of a tryptic digested of bovine α_S-casein (48). This technique has been used to evaluate the digestibility of the caseinphosphopeptide β-CN f(1-25), recognized as mineral carrier, during its duodenal transit in rats (49).

Nowadays, the relative easy combination of ESI with an ion trap (IT) mass spectrometer (for a review of recent developments in IT-MS, see 50) has permitted many investigations of polar substances over a wide range of interests (51), including food proteins and peptides. This MS technique has permitted the characterization of a large number of peptides with antimicrobial, ACE-inhibitory and antioxidant activities and phosphopeptides. Concerning antimicrobial peptides, McCann and co-workers (52) characterized a chymosin digest of bovine sodium caseinate, which showed antibacterial activity against *Listeria innocua*. Five α_{s2}-CN-derived fragments were identified by ESI-IT and N-terminal amino acid sequencing. Lately, this research group applied this characterization strategy and tandem MS for the identification of two antibacterial peptides from pepsin digest of bovine casein. The sequences corresponded to α_{S1}-CN f(99-109) and α_{S2}-CN f(183-207) (53). Figure 1 shows the identification of an antibacterial peptide from a ovine α_{S2}-casein hydrolysate through ion exchange chromatography followed by RP-HPLC and *off-line* and *on-line* HPLC-ESI-MS/MS (54). Ten different peptide sequences were identified. After chemical synthesis to check their antibacterial potency, α_{S2}-CN f(165-170) was found the most active peptide. Recently, it has been reported that this peptide sequence also exerts potent ACE-inhibitory activity (55). Following a similar strategy, 21 peptides were identified in the active HPLC fractions of a pepsin hydrolysate of bovine κ-casein. Some of them were chemically synthesized and showed antibacterial effects. Among of them, the synthesized peptides, κ-CN f(18-24), f(30-32), and f(139-146) were most effective (56). Antimicrobial peptides can also been found in cheese extracts. The WSE of 9 Italian cheeses were fractioned by RP-FPLC and active fractions were analyzed by HPLC-ESI-MS/MS. Fractions of Pecorino Romano, Canestrato Pugliese, Crescenza, Caprino del Piemonte, Caciocavallo and Mozzarella had peptides showed high levels of homology with previously reported antimicrobial and multifunctional peptides (57). In a parallel study, 45 peptides were identified in the cheese extracts using HPLC-ESI-MS/MS and MS^3 (58).

ESI-IT has also been extensively used to follow the formation of antihypertensive peptides derived from milk proteins. In this case, ESI-IT-MS analysis has allowed the elucidation of a great number of peptides with ACE-inhibitory activity released during milk fermentation, for instance, in commercial kefir (59) or by using certain selected starters, such as, *Lactobacillus helveticus* NCC 2765 (60), *Lactobacillus*

animalis DPC6134 (61) and *Enterococcus faecalis* CECT 5727(62). In this latter fermented milk, eight peptides showing different ACE-inhibitory activity were identified by ESI-MS/MS and their presence in fermented milk with other selected strains of *E. faecalis* (CECT 5728, 5826 and 5827) were confirmed by HPLC-ESI-MS. Among these peptides, two of them corresponding to β-CN f(133-138) and f(58-76) were potent ACE inhibitors and showed antihypertensive effects in model animals (63). Didelot and co-workers (64) have studied the ACE-inhibitory activity of caprine whey fermented by 25 different cheese extracts. The highest ACE-inhibitory activity was obtained after whey fermentation by the microflora from 18 months ripened Comté cheese and a potent ACE-inhibitoroy peptide was characterized as α–La f(104-108) by amino acid analysis and LC-ESI-MS. In a complex food matrix as ripened cheese, Gómez-Ruiz and co-workers (65) could identified 22 peptides in several chromatographic fractions that exhibited potent ACE-inhibitory activity obtained from an 8-months-aged Manchego cheese. Lately, 75 peptides were found in the fraction with molecular mass below 3000 Da from this cheese, without a previously HPLC fractionation step, by HPLC-ESI-MS/MS. Some of them corresponded or present high homology with previously described peptides with antihypertensive and/or ACE-inhibitory activity (66). Recently, 41 peptides were identified in the water-soluble extract<1000 Da from different Spanish cheeses (Cabrales, Idiazábal, Roncal, Manchego, Mahón and goat's milk cheese) by HPLC-MS/MS and off-line MS/MS. Several of them showed moderate or low ACE-inhibitory activity (67). Not only milk fermentation, but also enzymatic hydrolysis of milk protein has a great potential to produce ACE-inhibitory peptides, and their formation has been monitored by using an ESI-IT mass spectrometer. Thus, tryptic hydrolysis of κ-casein macropeptide (CMP) (68) and thermolysin hydrolysis of caprine β-Lg (69), bovine β-Lg A (70) and α-La and β-CN A2 (71 & 72) are good examples of the application of this technique to search for bioactive sequences in food protein hydrolysates. Egg proteins are a very important source of bioactive peptides but have received less attention than milk proteins. In this via, Miguel and co-workers (2004) have found three novel peptides with potent ACE-inhibitory activity from egg white proteins hydrolysed by pepsin corresponding to ovoalbumin f(106-114), f(359-365) and f(178-180). These peptides showed antihypertensive effect in spontaneously hypertensive rats but their administration did not affect the arterial blood pressure of normotensive Wistar Kyoto rats (73-75). Quiros

and co-workers (76) showed hydrolysis of ovalbumin under high-pressure increase the formation of these ovoalbumin-derived antihypertensive peptides. IT-MS has also been applied for peptide identification in soy protein hydrolysed by protease D3 (originated from soybean). A potent ACE inhibitor corresponding to NWGLP derived from glycinin G3 was identified (77). Combining SDS-PAGE and gel permeation chromatography-ESI-MS/MS has been successfully applied for the peptide characterization during fermentation cocoa bean (78). Although HPLC-MS is a powerful technique for peptide sequencing, RP separations has some difficulties as for instance, the identification of small hydrophilic peptides eluting in the first part of the chromatogram, which is usually discarded to avoid salts entrance into the MS system. Via CE-MS/MS Gómez-Ruiz and co-workers (79) have identified four novel potent ACE-inhibitory peptides from *k*-CN hydrolysed with digestive enzymes.

A number of casein phosphopeptides (CPPs) has currently been characterized in milk-based infant formulas and casein hydrolysates by RP-HPLC-ESI-IT-MS/MS. Most of them contained the cluster sequence SpSpSpEE, a mineral binding site (80-83). Miquel et al. (84) carried out a speciation of calcium, iron and zinc in CPPs fractions from *in vitro* digested toddler milk-based formula. CPPs were separated by anion-exchange high-performance liquid chromatography and peptides were identified by RP-HPLC-IT-MS/MS. Antioxidant peptides derived from egg proteins (73 & 85) and from milk proteins (86 & 87) have also been currently identified by RP-HPLC-ESI-IT-MS/MS. These bioactive peptides could be generated during gastrointestinal digestion of human breast milk and they might contribute to the high biological value attributed to human milk. A total of 23 peptides with different potential bioactivities were identified by RP-HPLC-ESI-IT-MS/MS in an ultrafiltration-permeate of an *in vitro* digested human milk (88).

Sensitivity of ESI is quite good, with most practitioners obtaining 100 fmol detection levels for many peptides. In one case, a microESI source has been developed to operate at low-flow-rates (about 10-100 nL/min) (13). Micro-ESI has been used for high sensitive analysis of peptides in zeptomole/attomole per microlitre (89). Other version of ESI, known as nanoESI, is optimized for very small amounts of sample uses flow rate as low as 20-50 nL/min (35 & 90). Micro-ESI and nano-ESI have also been reported for the investigation of bioactive peptides (for more examples see section 5). In this mode, Carr and co-workers (91) identified and sequenced 7 phosphopeptides from a

tryptic digest of α-casein at the fentomol levels by nano-ESI coupled to a triple quadrupole mass spectrometer.

In recent papers, the characterization of bioactive peptides has been performed by hybrid MS instruments. These instruments combine the advantages of different mass spectrometer types into one instrument (12). Basically, it consists of two mass analyzers in serie and a mean for fragmenting ions. A typical example for this was quadrupole-orthogonal time-of-flight (QTOF). This mass spectrometer type has been used to characterize four novel antimicrobial peptides (jelleines) purified from royal jelly of honeybees by RP-HPLC (92). A total of 21 peptides derived from tryptic hydrolysates of ovine β-Lactoglobulin were sequenced by nanoscale capillary LC-MS/MS, being most of them were derived from the N-terminal region (93). Van Platerink and co-workers (94) have developed an at-line method for the identification of ACE-inhibitory peptides. The assay consisted of a two-dimensional approach using RP-HPLC. In the first analysis an activity profile is obtained and different fractions collected, while in the second analysis these fractions are analyzed in detail. Then, peptides fractions are sequenced by HPLC coupled to QTOF. In this manner, two new ACE-inhibitory peptides derived from commercial hydrolysed caseinate were identified, α_{S2}-casein f(119-121) and β-casein f(102-104). Pohlentz and co-workers (95) have developed an easy and fast method to avoid the loss of proteolytic peptides during extraction and/or purification procedures. It is based on "in-capillary" proteolysis of proteins and identification by means of CE-nanoESI-MS/MS on a QTOF mass spectrometer. The method was successfully applied to elephant milk proteins. In this manner, different peptides were sequenced from lactoferrin, α_{S1}-CN, β-CN, δ-CN and κ-CN.

4. Identification of bioactive peptides by matrix-assisted liquid desorption/ ionization mass spectrometry (MALDI-MS)

MALDI is a solid state sputtering method that produces ions by laser bombardment of crystals containing a small amount of analyte dispersed in a large amount of matrix. This one acts as a chromophore for the laser radiation and protects even thermally sensitive analyte molecules. Because the samples are solids, MALDI is less easily adapted to chromatographic interfacing.

Because of the longer length of their sequences, several milk derived antimicrobial peptides have been identified with MALDI-TOF instruments. Liepke et al. (96) isolated a new antimicrobial peptide derived from human milk hydrolysed with pepsin simulating the digestion in infant stomachs, which inhibited the growth of Gram-positive, Gram-negative bacteria and yeasts. Analysis by MALDI-MS revealed a mass of 6430 Da in the most active fraction. Edman degradation analysis showed that the mass corresponded to κ-casein f(63-117). The same year, Malkoski et al. (97) fractionated CMP by RP-HPLC, and each fraction was tested for activity against *Streptococcus mutans*. Fractions were characterized by N-terminal sequence and mass spectrometry using a MALDI-TOF spectrometer equipped with delayed extraction. By this analysis, the active form of CMP was shown to be the nonglycosylated, phosphorylated κ-casein f(106 to 169), which was designated as kappacin. Three antimicrobial peptides produced by fermentation of sodium caseinate with *Lactobacillus acidophilus* DPC 6026 with activity against pathogenic strains of *Enterobacter sakazakii* and *Escherichia coli* have been recently identified (98). The sequences corresponded all of them to bovine α_{S1}-CN fragments (residues 21-29, 30-37 and 195-208). MALDI-TOF has also been employed to follow purification of lactoferricin, a potent antimicrobial milk-derived peptide, on an industrial grade cation exchange resin (99). In addition to the described milk-derived antimicrobial peptides, a recent report described the isolation and identification of 24 novel antifungal peptides derived from seeds of a wheat variant (*Triticum kiharae* Dorof. et Migusch.) by MALDI-TOF mass spectrometry and Edman degradation (100). Although in a lesser extent, other biologically active food-derived peptides have been described by means of MALDI-MS. Antioxidant peptides in the mass range of m/z 4000-7000 have been detected in the water extract of Mungoong, a traditional Thai fishery product produced from the cephalotorax of shrimp (101). Lunasin, a promising chemopreventive peptide from soybean was characterized by MALDI mass spectrometry (102). Throughout these studies, they found that during seed development, lunasin peptide appears 5 weeks after flowering and persists in the mature seed.

MALDI as ionization source has also been employed for the identification and characterization of food-derived antihypertensive peptides. Minervini et al. (103) characterized various ACE-inhibitory peptides in sodium caseinate hydrolysed with a proteinase from a food-grade microorganism (*Lactobacillus helveticus* PR4). Identified

peptides corresponded to bovine α_{S1}-casein f(24-47), α_{S1}-casein f(169-193) and β-casein f(58-76); ovine α_{S1}-casein f(1-6), α_{S2}-casein f(182-185) and α_{S2}-casein (186-188); caprine β-casein f(58-65) and α_{S2}-casein f(182-187); buffalo β-casein f(58-66); and a mixture of three peptides originated from human β-casein. Bean proteins have also been a source of ACE-inhibitory peptides after hydrolysis with alcalase. Li et al. (104) have isolated three ACE-inhibitory peptides from mung bean protein hydrolysate with alcalase. More recently, this group using a similar procedure (MALDI-TOF MS/MS) identified an antihypertensive peptide (TQVT), with activity in model animals, in a rice protein hydrolysate with alcalase (105).

Besides to their role in the identification of antihypertensive peptides, MALDI techniques have contributed as well to the bioavailability studies of antihypertensive peptides. Most ACE-inhibitory peptides exert their effect if they reach the bloodstream in an active form. Therefore, they have to show some resistance to gastrointestinal proteases and brush border peptidases and they have to be absorbed through the intestinal wall with preservation of their physiological activity. Using MALDI-TOF mass spectrometry, it was demonstrated the presence of the antihypertensive peptide, ALPMHIR, derived from a tryptic digestion of β-lactoglobulin, in both, the mucosal and serosal sides of a Caco-2 cell monolayer (106). They found out that the heptapeptide was transported intact through the monolayer, but in concentrations too low to exert an ACE-inhibitory activity.

Phosphorylated peptides are usually in low concentrations in hydrolysis products in such a way that the presence of nonphosphorylated peptides can suppress the ion signal of the phosphorylated species in mass spectrum (107). Furthermore, electronegativity of phosphate groups usually reduces the ionization efficiency during positive mode MS analysis (108). Thus, enrichment or purification methods are necessary previous to MS analysis. Zhou and co-workers (109) showed the successful identification of α- and β-casein derived phosphopeptides applying immobilized metal ion affinity chromatography (IMAC) and direct analysis with MALDI-TOF-MS helped by phosphatase and carboxypeptidase Y treatment. Recently, other techniques have been successfully applied to selectively concentrate phosphopeptides prior to MALDI-TOF-MS analysis, such as titanium dioxide micro columns (110), alumina- and zirconia-coated magnetic particles (107 & 111), and porous anodic alumina membrane (108). A recent promising technique for food-derived phosphopeptides involves the

direct detection and sequenciation of IMAC-enriched peptides through MALDI-MS/MS on an orthogonal injection quadrupole time-of-flight (QqTOF) mass spectrometer (112). Bennet and coworkers (113) employed this MS technique with or without IMAC purification step for phosphopeptides identification from β-casein and egg ovalbumin.

5. MALDI-MS combined with ESI-MS

In some cases, a full structural characterization of the peptides in the samples requires the combination of various MS techniques. Sometimes, the results of MALDI measurements compared to those of ESI are remarkably different, so that only a combination of both ionization techniques allows a comprehensive analysis of the protein samples (114). Mamone and co-workers (115) have reported a combined procedure to analyze ovine casein components to define phosphorylated sites. It is based on two-dimensional gel electrophoresis for protein separation and MS analysis using MALDI-TOF-MS and HPLC-ESI-MS on single quadrupole mass spectrometer and applying nanoESI-TOF-MS/MS to the sequenciation of tryptic phosphopepides.

In addition to the task of identification of bioactive peptides, MALDI-TOF mass spectrometry has been employed to better understand the digestion processes that lead to the formation of bioactive peptides. In this way, Schmelzer et al. have investigated the peptic proteolysis of bovine β-casein under gastro-analogous conditions. Sequence coverage was 75% and 41 peptides (2-36 amino acids) were characterized by MALDI-TOF with post-source decay/collision-induced dissociation (PSD/CID), as well as, by HPLC-ESI-MS/MS performed on an IT mass spectrometer. The small peptides (di- and tri-peptides) identified in this study could be promising candidates for intestinal absorption and exerts biological effects (116). In continuation of this study, this research group has analyzed this peptic digest using HPLC followed by MALDI-TOF-PSD/CID and nanoESI-QTOF-MS/MS (117). A combined sequencing approach using *de novo* interpretation and databases was employed. The combination of these ionization techniques allowed a comprehensive analysis of the complete digest. Overall, 100% of β-casein was covered by identifying 125 peptides of 4-84 residues of length, including 3 phosphorylated species. Between those peptides they identified the opioid agonist peptide β-casein (59-70) and the ACE-inhibitory peptides β-CN f(75-83) and β-CN f(177-183). The results showed that the peptic hydrolysis starts at the C-terminus of

the protein. Ferranti et al. (118) studied the protein and peptide fraction of human milk samples by use of RP-HPLC-ESI-MS carry out on single-quadrupole and/or MALDI-TOF-MS and sequenced by automatic Edman degradation and/or tandem ESI-MS. The authors defined the pathway of casein hydrolysis, which leaded to the formation of small peptides through intermediate oligopeptides. It was found that the action of a plasmin-like enzyme acting on specific lysine residues is the primary step in casein degradation. This is followed by endopeptidases-mediated cleavage of oligopeptides, which, in turn, produces a wide range of short peptides, being some of them bioactive.

6. Quantification of bioactive peptides by mass spectrometry

The ability to monitor bioactive peptides concentration in food or biological fluids is crucial and can be achieved by mass spectrometric approaches. Although a remarkable progress in protein analysis as a consequence of proteomic research has been achieved, the field of absolute quantification of peptides by mass spectrometry has not grown as rapidly.

The well-established selectivity and sensitivity advantages offered by mass spectrometry for protein and peptide analysis suggest that peptide quantification can be achieved by techniques such as HPLC-MS or HPLC-MS/MS (for a review see 119). However, it seems that immunological techniques such as a competitive ELISA and a radio immunoassay are still widely employed for peptide quantification and that the potential of mass spectrometry has not been profusely explored for peptide quantification. Methods based on MS demand extensive validation to meet defined criteria such as accuracy, precision, recovery, selectivity, stability and robustness. Special attention should be directed to matrix effects because they can suppress ionization misleading calculations (120). In other hand, the addition of an internal standard can improve accuracy and aid to discover deviations in the analytical process (121).

The antihypertensive activity of ACE-inhibitory peptides has been shown to be highly dependent on the peptide dosage (122), therefore, quantification of these peptides in functional foods is essential to ensure the activity of the final product. Several methods based on HPLC coupled with MS have recently been reported to quantify antihypertensive peptides in protein hydrolysates or fermented products. Curtis et al. (123) developed a straightforward, quantitative method for measuring the

antihypertensive peptide LKPNM in bonito muscle hydrolysate using one step of solid-phase extraction (SPE) followed by quantification using HPLC-MS/MS with a hybrid QTOF spectrometer. Van Platerink (124) developed an HPLC-MRM-MS on triple quadrupole instrument for the quantification of 17 ACE-inhibitory peptides, including IPP, VPP and HLPLP, in plasma samples from human volunteers that previously consumed peptide-enriched drinks. The internal standard used was [U^{13} C]IPP (fully ^{13}C labeled in I only). The limit of the detection was below 0.01 ng/mL and the limit of the quantification was between 0.05 and 0.2 ng/mL. The antihypertensive peptide LHLPLP was quantified in fermented milk products by HPLC-MS/MS with an ion trap instrument (125). The determination by HPLC-MS/MS was based on the peak area of the most abundant product ions from fragmentation of the molecular ion of LHLPLP and did not use internal standard. The use of MS or MS/MS produced very clean chromatograms for this peptide, with a negligible contribution from the fermented milk background, as it is shown in figures 2 and 3. Lately, this method was applied to study the transepithelial transport of the peptide LHLPLP using Caco-2 cells (126). Recently, Geerlings et al. (127) quantified the caprine ACE-inhibitory sequences TGPIPN, SLPQ and SQPK from a goat milk hydrolysate. Peptides were quantified by mass spectrometry using calibration curves prepared with synthetic peptides but they did not report any validation criteria for this method.

In several human studies, a dose dependant blood pressure lowering effect of the two tripeptides IPP and VPP has been proven (128-130). In order to achieve quantification of these two peptides, several approaches has been developed. Matsuura et al (131) described an HPLC-MS approach to quantify these peptides in a casein hydrolysate using the peptide APP as an internal standard. The method required a previous SPE stage as a cleanup procedure and showed reproducibility between 3.9-4.1%, linearity in the range from 0 to 48 μg/mL and good accuracy. Recently, Bütikofer et al. (132) employed a HPLC with subsequent triple mass spectrometry (MS^3) procedure for the quantitative determination of VPP and IPP in the water-soluble extracts of several traditional cheeses. Quantification of VPP and IPP was carried out in a linear ion trap mass spectrometer with the sum of the most intense ions in the MS^3 experiments. The use of PPPP as an internal standard for quantification substantially improved the repeatability of the method. The limit of determination was 0.22 mg/kg for VPP and 0.03 mg/kg for IPP. The repeatability and recovery obtained showed that method as a suitable method for the detection of IPP and VPP in cheese.

CMP is a polypeptide of 64 amino acid residues, derived from the C-terminal part of bovine κ-casein. CMP exhibits several biological activities such as antimicrobial, antiviral, immunomodulant and antithrombotic. CMP is a complex mixture of non-glycosylated and variously glycosylated forms. Mollé et al. (133) by using HPLC-ESI-MS reported several reliable conditions to unable unequivocal determination of aglyco-CMP variant A, aglyco-CMP variant B, and total CMP. A high degree of selectivity was obtained using multiple reaction monitoring detection and the limit of quantification was 10 pmol within the standard curve range of 10-1000 pmol. Several commercial products were analyzed and data obtained were in agreement with those indicated in the literature.

Bioactive peptides have not only been quantified in food products, there are a few attempts dealing with quantification of such peptides in biological fluids employing HPLC-MS. Kuwata et al. (134) evaluated the generation, in vivo, of lactoferricin after ingestion of bovine lactoferrin by surface-enhanced laser desorption/ionization (SELDI). SELDI was used in the affinity mass spectrometry operational mode to detect and quantify lactoferricin directly from unfractionated gastric contents. The recovery of the method was near to 100% and the amount of lactoferricin found in the gastric contents was 16.9±2.7 μg/mL. Recently, Fanciulli et al. (135 & 136) developed a method for the quantification of wheat gluten derived opioid peptides (gluten-exorphin A5 and B5) in cerebrospinal fluid. Mass spectrometric detection was performed by using a single stage quadrupole instrument equipped with an ESI source.

Although almost all the approaches for quantifying bioactive peptides in food described to date are based in HPLC, Arias et al. (137) applied capillary zone electrophoresis with mass spectrometry (CE-MS) to the quantitative determination of γ-glutamil-S-ethenyl-cysteine (GEC), a bioactive and unstable compound present in *Vicia carbonensis* L. seeds. GEC dipeptide is responsible for palatability reduction in grain and some of the toxic properties for nonruminants. A comparative study was carried out between the quantitative capabilities of CE-MS vs. CE-UV. They found that both techniques provided similar limit of detection and can be applied with confidence within the same linear dynamic range. Nevertheless, reproducibility and speed were better using CE-UV. Although from the results obtained it was deducted that CE-UV seems better suited for GEC analysis than CE-MS, there is an evident advantage of CE-

MS over CE-UV is that the detection of GEC can be done directly from the MS spectra, while availability of the purified GEC is mandatory for its CE-UV analysis.

7. Conclusions

The role of food-derived peptides as physiologically active components in the diet is being increasingly acknowledged. Due to their physiological and physio-chemical versatility, food-derived bioactive peptides are regarded as highly prominent components for health promoting foods or pharmaceutical applications. In addition to the necessary verification of the efficacy in vivo, main current challenges in the exploitation of bioactive peptides and hydrolysates are establishment of the concentration of the active compound in food and stability in the food matrix. Intensive efforts are now focused on identification and quantification of these bioactive peptides in the developed food ingredients (protein hydrolysates, fermentates), in the final functional food product, and in the organism to determine absorption, degradation and metabolism. These bioactive peptides may be present in complex food matrices or biological fluids as minor constituents. Due to the specificity and sensitivity of mass spectrometry, especially in combination with HPLC, it has become the indispensable analytical tool for identification, quantification and bioavailability of these health-promoting peptides. The instrumental advances, the development of interfaces and ionization technologies and the reduced prices of the equipments will allow further advances in the analysis of these compounds. Particularly, the improved resolution and sensitivity of the equipments, MALDI, nano-ESI, and the introduction of hybrid mass spectrometer types into one instrument will suppose even better sensitivity and a simplification in sample preparation due the high selectivity.

Acknowledgements

This work was financially supported by projects AGL2007-65035 and Consolider-Ingenio 20010.CSD2007-0063 from the Spanish Ministry of Education and Science (MEC) and S-505/AGR/0153-01 from the Autonomous Community of Madrid.

References

(1) Meisel, H. (2004) *Biofactors* **21**, 55–61

(2) Korhonen, H., & Pihlanto, A. (2006) *Int. Diary J.* **16**, 945-960

(3) Li, G-H., Li, G-W., Shi, Y-H., & Shrestha, S. (2004) *Nut. Res.* **24**, 469-486

(4) Pihlanto, A., & Korhonen, H. (2003) in *Adv. Food Nutr. Res.*, vol. 47, Taylor, S. L. (Ed.), Elsevier Academic Press, London, U.K., pp 175–276

(5) Séverin, S., & Wenshui, W. (2005) *Crit. Rev. Food Sci. Nutr.* **45**, 645-656

(6) Silva, S.V., & Malcata, X.M. (2005) *Int. Dairy J.* **15**, 1-15

(7) FitzGerald, R., & Murray, B. A. (2006) *Int. J. Diary Technol.* **59**, 118-125

(8) Careri, M., & Mangia, A. (2003) *J. Chromatogr. A.* **1000**, 609-635

(9) Léonil, J., Gagnaire, V., Mollé, D., Pezennec, S., &, Bouhallab, S. (2000) *J. Chromatogr. A.* **881**, 1-21

(10) Alomirah, H.F., Alli, I., & Konishi, Y. (2000) *J. Chromatogr. A*. **893**, 1-21

(11) Ferranti, P. (2004) *Eur. J. Mass Spectrom.* **10**(3), 349-358

(12) Kussmann, M., Affolter, M., Nagy, K., Holst, B., & Fay, L.B. (2007) *Mass Spectrom.* **26**, 727-750

(13) Griffiths, W.J., Jonsson, A.P., Liu, S., Rai, D.K., & Wang, Y. (2001) *Biochem. J.* **355**, 545-561

(14) Kinoshita, E., Yamakoshi, J., & Kikuchi, M. (1993) *Biosci. Biotech. Biochem.* **57**, 1107-1110.

(15) Singh, T.K., Fox, P.F., & Healy, A. (1997) *J. Dairy Res.* **64**, 433-443

(16) Mullally, M.M., Meisel, H., FitzGerald, R.J. (1997) *FEBS Lett.* **402**, 99-101

(17) Beranová-Giorgianni, S., & Desiderio, D.M. (1997) *Methods Enzymol.* **289**, 478-499

(18) Ferranti, P., Barone, F., Chianese, L., Addeo, F., Scaloni, A., Pellegrino, L., & Pierpaolo, R. (1997) *J. Dairy Res.* **64**, 601-615

(19) Ferranti, P., Itolli, E., Barone, F., Malorni, A., Garro, G., Laezza, P., Chianese, L., Migliaccio, F., Stingo, V., & Addeo, F. (1997) *Lait* **77**, 683-697

(20) Addeo, F., Chianese, L., Salzano, A., Sacchi, R., Cappuccio, U., Ferranti, P., & Malorni, A. (1992) *J. Dairy Res.* **59**, 401-411

(21) Addeo, F., Chianese, L., Sacchi, R., Musso, S.S., Ferranti, P., & Malorni, A. (1994) *J. Dairy Res.* **61**, 365-374

(22) Roudot-Algaron, F., Le Bars, D., Kerhoas, L., Einhorn, J., & Gripon, J.C. (1994) *J. Food Sci.*, **59**, 544-547

(23) Chianese, L., Caira, S., Ferranti, P., Laezza, P., Malorni, A., Mucchetti, G., Garro, G., & Addeo, F. (1997) *Lait*, **77**, 699-715

(24) Murakami, M., Tonouchi, H., Takahashi, R., Kitazawa, H., Kawai, Y., Negishi, H., & Saito, T. (2004) *J. Dairy Res.* **87**, 1967-1974

(25) Saito, T., Nakamura, T., Kitazawa, H., Kawai, Y., & Itoh, T. (2000) *J. Dairy Sci.* **83**, 1434-1440

(26) Rokka, T., Syvaoja, E.L., Tuominen, J., & Korhonen, H. (1997) *Milchwissenschaft* **52**, 675-678

(27) Suetsuna, K., Ukeda, H., & Ochi, H. (2000) *J. Nutr. Biochem.* **11**, 128-131

(28) Minkiewicz, P., Slagen, C.J., Lagerwerf, F.M., Haverkamp, J., Rollema, H.S., & Visser, S. (1996) *J. Chromatogr. A* **743**, 123-135

(29) Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H., & Maruyama, S. (1991) *Agric. Biol. Chem.* **55**, 1313-1318

(30) Suetsuna, K., & Nakano, T. (2000) *J. Nutr. Biochem.* **11**, 450-454

(31) Suetsuna, K., Maekawa, K., & Chen, J.R. (2004) *J. Nutr. Biochem.* **15**, 267-272

(32) Gobbetti, M., Ferranti, P., Smacchi, E., Goffredi, F., & Addeo, F. (2000) *Appl. Environ. Microbiol.* **66**, 3898-3904

(33) Higaki-Sato, N., Sato, K., Esumi, Y., Okumura, T., Yoshikawa, H., Tanaka-Kuwajima, C., Kurata, A., Kotaru, M., Kawabata, M., Nakamura, Y., & Ohtsuki, K. (2003) *J. Agric. Food Chem.* **51**, 8-13

(34) Klampfl, C. W. (2004) *J. Chromatogr. A*. **1044**, 131-144

(35) Baldwin, M. A. (2005) *Methods Enzymol.* **402**, 3-48

(36) Bouhallab, S., Mollé, D., & Léonil, J. (1993) *Biotechnol. Lett.* **15**, 697-702

(37) Matar, C. & Goulet, J. (1996) *Int. Dairy J.*, **6**, 383-397

(38) Dionysius, D.A., & Milne, J.M. (1997) *J. Dairy Sci.* **80**, 667-674

(39) Pihlanto-Leppälä, A., Rokka, T. and Korhonen, H. (1998) *Int. Dairy J.* **8**, 325–331

(40) Tauzin, J., Miclo, L., & Gaillar, J-L. (2002) *FEBS Lett.* **531**, 369-374

(41) Xiao, X., Luo, X., Chen, B., & Yao, S. (2006) *J. Chromatogr. B* **834**, 48-54

(42) Chen, H-M., Muramoto, K., & Yamauchi, F. (1995) *J. Agric. Food Chem.* **43**, 574-578

(43) Guo, H., Kouzuma, Y., & Masami, Y. (2005) *Food Sci. Technol. Res.* **11**, 222-230

(44) Recio, I., & Visser, S. (1999) *Biochim. Byophis. Acta* **1428**, 314-326

(45) De Noni, I., & Floris, R. (2007) *Int Dairy J.* **17**, 504-512

(46) Gibbs, B.F., Zougman, A., Masse, R., & Mulligan, C. (2004) *Food Res. Int.* **37**, 123-131

(47) De Vincenzi, M., Gasbarrini, G., & Silano, V. (1997) *Toxicolgy* **120**, 207-213

(48) Annan, R.S., Huddleston, M.J., Verma, R., Deshaies, R.J., & Carr, S.A. (2001) *Anal. Chem.* **73**, 393-404

(49) Bouhallab, S., Aït Oukhatar, N., Mollé, D., Henry, G., Maubois, J-L., Arhan, P., & Bouglé, D.L.J. (1999) *Nutr. Biochem.* **10**, 723-727

(50) Brancia, F. L. (2006) *Expert Rev. Proteomics* **3**, 143-151

(51) Hao, C., & March, R. (2001) *Int. J. Mass Spectrom.* **212**, 337-357

(52) McCann, K.B., Shiell, B.J., Michalski, W.P., Lee, A., Wan, J., Roginski, H., & Coventry, M.J. (2005) *Int. Dairy J.* **15**, 133-143

(53) McCann, K.B., Shiell, B.J., Michalski, W.P., Lee, A., Wan, J., Roginski, H., & Coventry, M.J. (2006) *Int. Dairy J.* **16**, 316-323

(54) López-Expósito, I., Gómez-Ruiz, J.A., Amigo, L., & Recio, I. (2006) *Int. Dairy J.* **16**, 1072-1080

(55) López-Expósito, I., Quirós, A., Amigo, L., & Recio, I. (2007) *Lait* **87**, 241-249

(56) López-Expósito, I., Minervini, F., Amigo, L., & Recio, I. (2006) *J. Food Prot.* **69**, 2992-2997

(57) Rizzello, C.G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M.D., Zambonin, P.G. (2005) *J. Dairy Sci.* **88**, 2348-2360

(58) Losito, I., Carbonara, T., Domenica De Bari, M., Gobbetti, M., Palmesano, F., Rizzello, C.G., Zambonin, P.G. (2006) *Rapid Común. Mass Spectrom.* **20**, 447-455

(59) Quirós, A., Hernández-Ledesma, B., Ramos, M., Amigo, L., & Recio, I. (2005) *J. Dairy Sci.* **88**, 3480-3487

(60) Robert, M.C., Razaname, A., Mutter, M., & Juillerat, M.A. (2004) *J. Agric. Food Chem.* **52**, 6923-6931

(61) Hayes, M., Stanton, C., Slattery, H., O'Sullivan, O., Hill, C., Fitzgerald, G.F., & Ross, R.P. (2007) *Appl. Enviromen. Microbiol.* **73**, 4658-4667

(62) Quirós, A., Ramos, M., Muguerza, B., Delgado, M.A., Miguel, M., Aleixandre, A., & Recio, I. (2007) *Int. Dairy J.* **17**, 33-41

(63) Miguel, M., Recio, I., Ramos, M., Delgado, M.A., & Aleixandre, M.A. (2006) *J. Dairy Sci.*, **89**, 3352-3359

(64) Didelot, S., Bordenave-Juchereau, S., Rosenfeld, E., Fruitier-Arnaudin, I., Piot, J-M., & Sannier, F. (2006) *Int. Dairy J.* **16**, 976-983

(65) Gómez-Ruiz, J.A., Ramos, M., & Recio, I. (2002) *Int. Dairy J.* **12**, 697-706

(66) Gómez-Ruiz, J.A., Ramos, M., & Recio, I. (2004) *J. Chromatogr. A.* **1054**, 269-277

(67) Gómez-Ruiz, J.A., Taborda, G., Amigo, L., Recio, I., & Ramos, M. (2006) *Eur. Food Res. Technol.*, **223**, 595-601

(68) Manso, M.A., & Lopez-Fandino, R. (2003) *J. Food Prot.*, **66**, 1686-1692

(69) Hernández-Ledesma, B., Recio, I., Ramos, M., & Amigo, L. (2002) *Int. Dairy J.* **12**, 805-812

(70) Hernández-Ledesma, B., Ramos, M., Recio, I., & Amigo, L. (2006) *J. Chromatogr. A* **1116**, 31-37

(71) Otte, J., Shalaby, S.M., Zakora, M., Pripp, A.H., Sayed, A., & El-Shabrawy, A. (2007) *Int. Dairy J.* **17**, 488-503

(72) Otte, J., Shalaby, S.M.A., Zakora, M., & Nielsen, M.S. (2007) *Int. Dairy J.* **17**, 1460-1472

(73) Miguel, M., Recio, I., Gómez-Ruiz, J.A., Ramos, M., & López-Fandino, R. (2004) *J. Food Prot.* **67**, 1914-1920

(74) Miguel, M., López-Fandino, R., Ramos, M., & Aleixandre, A. (2005) *Br. J. Nutr.* **94**, 731-737

(75) Miguel, M., Aleixandre, M.A., Ramos, M., & Lopez-Fandino, R. (2006) *J. Agric. Food Chem.* **54**, 726-731

(76) Quirós, A., Chichón, R., Recio, I., & López-Fandiño, R. (2007). *Food Chem.* **104**, 1734-1739

(77) Kodera, T., & Nio, N. (2006) *J. Food Sci.* **71,** C164-C172

(78) Buyukpamukcu, E., Goodall, D.M., Hansen, C.E., Keely, B.J., Kochhar, S., & Wille, H. (2001) *J. Agric. Food Chem.* **49**, 5822-5827

(79) Gómez-Ruiz, J.A., Ramos, M., & Recio, I. (2007) *Electrophoresis* **28**, 4202-4211

(80) Miquel, E., Gómez, J.A., Alegría, A., Barberá, R., Farré, R., & Recio, I. (2005) *J. Agric. Food Chem.* **53**, 3426-3433

(81) Miquel, E., Alegría, A., Barberá, R., & Farré, R. (2006) *Food Sci. Tech. Int.* **12**, 531-537

(82) Miquel, E., Gómez, J.A., Alegría, A., Barberá, R., Farré, R., & Recio, I. (2006) *Food Sci. Tech. Int.* **12**, 379-384

(83) Miquel, E., Gómez, J.A., Alegría, A., Barberá, R., Farré, R., & Recio, I. (2006) *Eur. Food Res. Technol.* **222**, 48-53

(84) Miquel, E., Alegría, A., Barberá, R., & Farré, R. (2005) *Anal. Bioanal. Chem.* **381**, 1082-1088

(85) Davalos, A., Miguel, M., Bartolomé, B., & Lopez-Fandino, R. (2004) *J. Food Prot.* **67**, 1939-1944

(86) Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005) *J. Agric. Food Chem.* **53**, 588-593

(87) Hernández-Ledesma, B., Miralles, B., Amigo, L., Ramos, M., & Recio, I. (2005) *J. Sci. Food Agric.* **85**, 1041-1048

(88) Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, I. (2007) *Int. Dairy J.* **17**, 42-49

(89) Andrén, P.E., Emmett, M.R., & Caprioli, R.M. (1994) *J. Am. Soc. Mass Spectrom.* **5**, 867-869

(90) Wilm, M.S., & Mann, M. (1994) *Int. J. Mass. Spectrom. Ion Processes* **136**, 167-180

(91) Carr, S.A., Huddleston, M.J., & Annan, R.S. (1996) *Anal. Biochem.* **239**, 180-192

(92) Fontana, R., Mendes, M.A., Monson de Souza, B., Konno, K., Marcondes César, L.M., Malaspina, O., & Palma, M.S. (2004) *Peptides* **25**, 919-928

(93) Chobert, J-M., El-Zahar, K., Sitohy, M., Dalgalarrondo, M., Métro, F., Choiset, Y, & Haertlé, T. (2005) *Lait* 141-152

(94) Van Platerink, C.J., Hans-Gerd, M.J., & Haverkamp, J. (2007) *J. Chromatogr. B* **846**, 147-154

(95) Pohlentz, G., Kölbl, S., & Peter-Katalinic, J. (2005) *Proteomics*, **5**, 1758-1763

(96) Liepke, C., Zucht, H.D., Forssmann, W.G., & Ständker, L. (2001) *J. Chromatogr. B* **752**, 369-377

(97) Malkoski, M., Dashper, S.G., O´Brien-Simpson, N.M., Talbo, G.H., Macris, M., Cross, K.J., & Reynolds, E. (2001) *Antimicrob. Agents Chemoth.* ***45***, 2309-2315

(98) Hayes, M., Ross, R.P., Fitzgerald, G.F., Hill, C., & Stanton, C. (2006) *Appl. Envirom. Microbiol.* ***72***, 2260-2264

(99) Chan, J.C.K., & Li-Chan, E.C.Y. (2007) *J. Agric. Food Chem.* ***55***, 493-501

(100) Egorov, T.A., Odintsova, T.I., Pukhalsky, V.A., & Grishin, E.V. (2005) *Peptides* ***26***, 2064-2073

(101) Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M., & Kishimura, H. (2008) *Food Chem.* ***106***, 185-193

(102) Jeong, H.J., Park, J.H., Lam, Y., & De Lumen, B. O. (2003) *J. Agric. Food Chem.* ***51***, 7901-7906

(103) Minervini, F., Algaron, F., Rizzello, C.G., Fox, P.F., Monnet, V., & Gobetti, M. (2003) *Appl. Envirom. Microbiol.* ***69***, 5297-5305

(104) Li, G-H., Wan, J-Z., Le, G-W., & Shi, Y-H. (2006) *J. Pept. Sci.* 12, 509-514

(105) Li, G-H., Qu, M-R., Wan, J-Z., & You, J-M. (2007) *Asia Pac. J. Clin. Nutr.* **16**, 275-280

(106) Vermeirssen, V., Deplancke B., Tappenden, K.A., Van Camp, J., Gaskins, H.R., & Verstraete, W. (2002) *J. Pept. Sci.* **8**, 95-100

(107) Lo, C.Y., Chen, W.Y., Chen, C.T., & Chen, Y.C. (2007) *J. Proteome Res.* **6**, 887-893

(108) Wang, Y., Chen, W., Wu, J., Guo, Y., & Xia, X. (2007) *J. Am. Soc. Mass Spectrom.* **18**, 1387-1395

(109) Zhou, W., Merrick, B.A., Khaledi, M.G., & Tomer, K.B. (2000) *J. Am. Soc. Mass Spectrom.* **11**, 273-282

(110) Larsen, M.R., Thingholm, T.E., Jensen, O.N., Roepstorff, P., & Jorgensen, T.J.D. (2005) *Mol. Cell Proteomics* **4**, 873-886

(111) Chen, C.T., & Chen, Y.C. (2005) *J. Chromatogr. A* **77**, 5912-5919.

(112) Raska, C.S., Parker, C.E., Dominski, Z., Marzluff, W.F., Glish, G.L., Pope, R.M., & Borchers, C.H. (2002) *Anal. Chem.* **74**, 3429-3433

(113) Bennet, K.L., Stensballe, A., Podtelejnikov, A.V., Moniatte, M., & Jensen, O.N. (2002) *J. Mass Spectrom*. **37**, 179-190

(114) Stapels, M.D., Cho, J-C., Giovannoni, S.J., & Barofsky, D.F. (2004) *J. Biomol. Tech.* 15, 191-198

(115) Mamone, G., Caira, S., Garro, G., Nicolai, A., Ferranti, P., Picariello, G., Malorni, A., Chianase, L., & Addeo, F. (2003) *Electrophoresis* **24**, 2824-2837

(116) Schmelzer C.E., Schöps, R., Ulbrich-Hofmann, R., Neubert, R.H., Raith, K. (2004) *J. Chromatogr. A* **1055**, 87-92.

(117) Schmelzer, C.E.H., Schöps, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R. H.H, & Raith, K. (2007) *J. Chromatogr. A* **1166**, 108-115

(118) Ferranti, P., Traisci, M.V., Picariello, G., Nasi, A., Boschi, V., Siervo, M., Falconi, C., Chianese, L., & Addeo, F. (2004) *J. Dairy Res.* **71**, 74-87

(119) Tamvakopoulos, C. (2007) *Mass Spectrom. Reviews* **26**, 389-402

(120) John, H. (2005) *Anal. Bioanal. Chem.* **381**, 51-53

(121) Harald, J., Walden M., Schäfer, S., Genz, S., & Forssmann, W-G. (2004) *Anal. Bioanal. Chem.* **378**, 883-897

(122) Mizuno, S., Matsuura, K., Gotou, T., Nishimura, S., Kajimoto, O., Yabune, M., Kajimoto, Y., & Yamamoto, N. (2005) *Br. J. Nutr.* **94**, 84-91

(123) Curtis, J.M., Dennis, D., Waddell, D.S., MacGillivray, T., & Ewart, H.S. (2002) *J. Agric. Food Chem.* **50,** 3919-3925

(124) Van Platerink, C.J., Janssen, H-G.M., Horsten, R., & Haverkamp, J. (2006) *J. Chromatogr. B* **830**, 151-157

(125) Quirós, A., Ramos, M., Muguerza, B., Delgado, M.A., Martin-Alvarez, P.J., Aleixandre, A., & Recio, I. (2006) *J. Dairy Sci.* **89**, 4527-4535

(126) Quirós, A., Dávalos, A., Lasunción, M.A., Ramos, M., & Recio, I. (2008) *Int. Dairy J.* **18**, 279-286

(127) Geerlings, A., Villar, I.C., Hidalgo Zarco, F., Sánchez, M., Vera, R., & Zafra Gómez, A. (2006) *J. Dairy Sci.* **89**, 3326-3335

(128) Hata, Y., Yamamoto, M., Ohni, Nakajima, K., Nakamura Y., & Takano, T. (1996) *Am. J. Clin. Nutr.* **64**, 767-771

(129) Seppo, L., Kerojoki, O., Suomalainen, T., & Korpela, R. (2002) *Milchwissenschaft* **57**, 124–127

(130) Seppo, L., Jauhiainen, T., Poussa, T., & Korpela, R. (2003) *Am. J. Clin. Nutr.* **77**, 326-330

(131) Matsuura, K., Mizuno, S., Nishimura, S., Gotou, T., & Yamamoto, N. (2005) *Milchwissenschaft* **60**, 24–27

(132) Bütikofer, U., Meyer, J., Sieber, R., & Wechsler, D. (2007) *Int. Dairy J.*, **17**, 968-975

(133) Mollé, D., & Leonil, J. (2005) *Int. Dairy J.* **15,** 419-428

(134) Kuwata, H., Yip, T-T., Yip, C.L., Tomita, M., & Hutchens, W. (1998) *Biochem. Biophys. Res. Commun*. **245**, 764-773

(135) Fanciulli, G., Azara, E., Wood, T.D., Delitala, G., & Marchetti, M. (2007) *J. Chromatogr. B* **852**, 485-490

(136) Fanciulli, G., Azara, E., Wood, T.D., Dettori, A., Delitala, G., & Marchetti, M. (2006) *J. Chromatogr. B* **833**, 204-209

(137) Arias, M., Simó, C., Ortiz, L.T., de los Mozos-Pascual, M., Barbas, C., & Cifuentes, A. (2005) *Electrophoresis* **26**, 2351-2359

(138) Gómez-Ruiz, J.A., Ramos, M., & Recio, I. (2004) *Int. Dairy J.* **14**, 1075-1080

(139) Tauzin, J., Miclo, L., Roth, S., Mollé, D., & Gaillar, J-L. (2003) *Int. Dairy J.* **13**, 15-27

(140) Adamson, N.J., & Reynolds, E.C. (1997) *J. Dairy Res.* **64,** 505-514

(141) Ellegard, K.H., Gammelgard-Larsen, C., Sorensen, E.S., & Fedosov, S. (1999) *Int. Dairy J.* **9**, 639-652

Figure captions

Figure 1. (A) Separation of the peptic digest of ovine α_{S2}-CN by ion-exchange chromatography; (B and C) Fractionation by RP-HPLC at semipreparative scale of fractions C and D, respectively. (D) MS/MS spectrum of the triply charged ion *m/z* 1119.3 derived from fraction D obtained by on-line HPLC-ESI-IT mass spectrometry. Following sequence interpretation and database searching, the peptide was identified as α_{S2}-CN f(181-208). The sequence of this peptide is displayed with the fragment ions observed in the spectrum. Fragments ions are labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984). Reproduced from ref. 54, with permission from Elsevier, copyright 2006.

Figure 2. Analysis of the water-soluble extract of milk fermented with *Enterococcus faecalis* CECT 5728 obtained by (A) HPLC with UV detection at 214 nm; (B) HPLC-ion trap mass spectrometry, showing the total ion current chromatogram; (C) HPLC–ion trap mass spectrometry, showing the extracted ion chromatogram of the molecular ion of peptide LHLPLP with m/z 689.4; and (D) HPLC–ion trap mass spectrometry, showing the extracted ion chromatogram of fragment ions with m/z 574.3, 439.2, 364.2, and 251.0. Reproduced from ref. 125, with permission from the American Dairy Science Association ®, copyright 2006).

Figure 3. (A) HPLC–ion trap spectrum of the peptides eluting between 44.3 and 47.3 min. (B) MS/MS spectrum of the singly charged ion *m/z* 689.4. The sequence of this peptide is displayed as LHLPLP with the fragment ions observed in the spectrum. Fragment ions are labeled according to the nomenclature proposed by Roepstorff and Fohlman (1984). Reproduced from ref. 125, with permission from the American Dairy Science Association ®, copyright 2006).

Table 1. Bioactive peptides identified by using fast atom bombardment as ionization source. The food product of origin, the proposed bioactivity and the mass analyzer employed in the detection are specified.

Food origin and production	Activity[1]	Mass analyser	Ref.
Milk proteins[2]			
Milk fermented by *Lactobacillus* GG and digested by pepsin and trypsin	Immunostimulating Opioid	Double-focusing	26
Milk fermented by *Lactobacillus* sp. or *Lactococcus* sp.	ACE-inhibitory	Four-sector	32
Gouda cheese	ACE-inhibitory Antihypertensive		25
Italian cheeses and cheese whey	Phosphopeptides	Double-focusing	18-21; 23
Comté cheese	Phosphopeptides	Triple quadrupole	22
Commercial whey milk product	ACE-inhibitory Antihypertensive		24
Peptic digest of casein	Antioxidant	Double-focusing	27
Cereal proteins			
Thermolysin hydrolysate of α-zein (maize)	ACE-inhibitory Antihypertensive	Double-focusing	29
Pepsin and pancreatin digest of wheat gluten	Indigestible pyroglutamyl peptides	Four-sector	33
Algae proteins			
Peptic digest of Wakame	ACE-inhibitory Antihypertensive	Double-focusing	30
Hot water extract of Wakame	ACE-inhibitory Antihypertensive	Double-focusing	31

[1] The term activity makes reference to the bioactivity of the food product and/or their derived peptides.
[2] Unless indicated, it corresponds to milk proteins of bovine origin.
ACE, angiotensin-converting enzyme; Ref., reference

Table 2. Bioactive peptides identified by using electrospray as ionization source. The food product of origin, the proposed bioactivity and the mass analyzer employed in the detection are specified.

Food origin and production	Activity[1]	Mass analyser	Ref.
Milk proteins[2]			
Milk fermented with a mutant strain of *Lactobacillus helveticus* L89	Opioid	Triple quadrupole	37
Milk proteins fermented and digested with pepsin and trypsin	ACE-inhibitory		39
Sodium caseinate fermented by *Lactobacillus helveticus* NCC 2765	ACE-inhibitory	Ion trap	60
Sodium caseinate fermented by *Lactobacillus animalis* DPC6134	ACE-inhibitory	Ion trap	61
Milk fermented with *Enterococcus faecalis*	ACE-inhibitory Antihypertensive	Ion trap	62; 63
Caprine whey fermented with microflora of different cheeses	ACE-inhibitory	Ion trap	64
Commercial caprine kefir	ACE-inhibitory Antimicrobial Antioxidant	Ion trap	54; 55; 59
Different Spanish cheeses	ACE-inhibitory	Ion trap	65-67; 138
Different Italian cheeses	Antimicrobial	Ion trap	57; 58
Hydrolysis of β-CN with chymosin	Immunomodulatory	Quadrupole	36
Tryptic hydrolysis of α_{S2}-CN	ACE-inhibitory	Triple quadrupole	40; 139
Tryptic hydrolysis of BSA	ACE-inhibitory	Triple quadrupole	45
Tryptic hydrolysis of CMP	ACE-inhibitory	Ion trap	68
Tryptic hydrolysis of ovine β-Lg	ACE-inhibitory	QTOF	93
Tryptic hydrolysis of α_S-CN	Phosphopeptides	Triple quadrupole	48; 91
Chymosin hydrolysis of sodium caseinate	Antibacterial	Ion trap	52
Peptic hydrolysis of casein	Antibacterial	Ion trap	53
Peptic hydrolysis of ovine α_{S2}-CN	Antibacterial Antioxidant ACE-inhibitory	Ion trap	54; 55
Peptic hydrolysis of κ-CN	Antibacterial Antioxidant ACE-inhibitory	Ion trap	55; 56
Peptic hydrolysis of lactoferrin	Antimicrobial	Triple quadrupole	38
Peptic hydrolysis of α_{S2}-CN	Antibacterial	Triple quadrupole	44
Hydrolysis of α-La and β-CN with thermolysin	ACE-inhibitory	Ion trap	71; 72
Hydrolysis of α-La and β-Lg with Corolase PP®	Antioxidant	Ion trap	86
Commercial hydrolysed caseinate	ACE-inhibitory	QTOF	94

Ovine κ-CN and whole casein hydrolysed with digestive enzymes	ACE-inhibitory	Ion trap	79
In vitro gastrointestinal digestion of human milk	ACE-inhibitory Antioxidant	Ion trap	88
In vitro gastrointestinal digestion of milk-based infant formulas, casein and casein fractions	Phosphopeptides	Ion trap	80-84
Egg proteins			
Peptic hydrolysis of egg white proteins	ACE-inhibitory Antihypertensive Antioxidant	Ion trap	73- 76; 85
Cereals			
Peptic and tryptic hydrolysis of durum wheat gliadin	Prevention of agglutination of K562(S) cells	Triple quadrupole	47
Soy proteins			
Soybean protein isolate hydrolysed with protease D3	ACE-inhibitory Antihypertensive	Ion trap	77
Soy-fermented foods hydrolysed with different proteolytic enzymes	ACE-inhibitory Antithrombotic Antioxidant	Triple quadrupole	46
Royal jelly			
Royal jelly	Antimicrobial	QTOF	92
Royal jelly hydrolysed with protease N	Antioxidant	Triple quadrupole	43

[1] The term activity makes reference to the bioactivity of the food product and/or their derived peptides.

[2] Unless indicated, it corresponds to milk proteins of bovine origin.

ACE, angiotensin-converting enzyme; Ref., reference; CN, casein; β-Lg, β-lactoglobulin; CMP, caseinmacropeptide

Table 3. Bioactive peptides identified by using matrix-assisted liquid desorption ionization, as ionization source. The food product of origin, the proposed bioactivity and the mass analyzer employed in the detection are specified.

Food origin and production	Bioactivity[1]	Mass analyser	Ref.
Milk proteins			
CMP	Antimicrobial	TOF	97
Sodium caseinate fermented with *Lactobacillus acidophilus* DPC 6026	Antimicrobial	TOF	98
Sodium caseinate hydrolysated with a proteinase from *Lactobacillus helveticus* PR4	ACE-inhibitory	TOF	103
Tryptic and pancreatic hydrolysis of casein	Phosphopeptides	TOF	140
Tryptic hydrolysis of sodium caseinate	Phosphopeptides	TOF	141
Tryptic hydrolysis of milk proteins	Phosphopeptides	TOF; QqTOF	107-111; 113
Peptic hydrolysis of human milk	Antimicrobial	TOF	96
Peptic hydrolysis of lactoferrin	Antimicrobial	TOF	99
Crustacean			
Water extract of Mungoong (from cephalotorax of shrimp)	Antioxidant	TOF	101
Cereals			
Seeds *of Triticum kiharae* Dorof. et Migusch	Antimicrobial	TOF	100
Rice protein hydrolysed with alcalase	Antihypertensive	TOF-TOF	105
Leguminous plants			
Soybean seeds	Chemopreventive		102
Mung bean proteins hydrolysed with alcalase	ACE-inhibitory	TOF-TOF	104

1 The term bioactivity makes reference to the bioactivity of the food product and/or their derived peptides.

[2] Unless indicated, it corresponds to milk proteins of bovine origin.

ACE, angiotensin-converting enzyme; Ref., reference; CN, casein; β-Lg, β-lactoglobulin; CMP, caseinmacropeptide

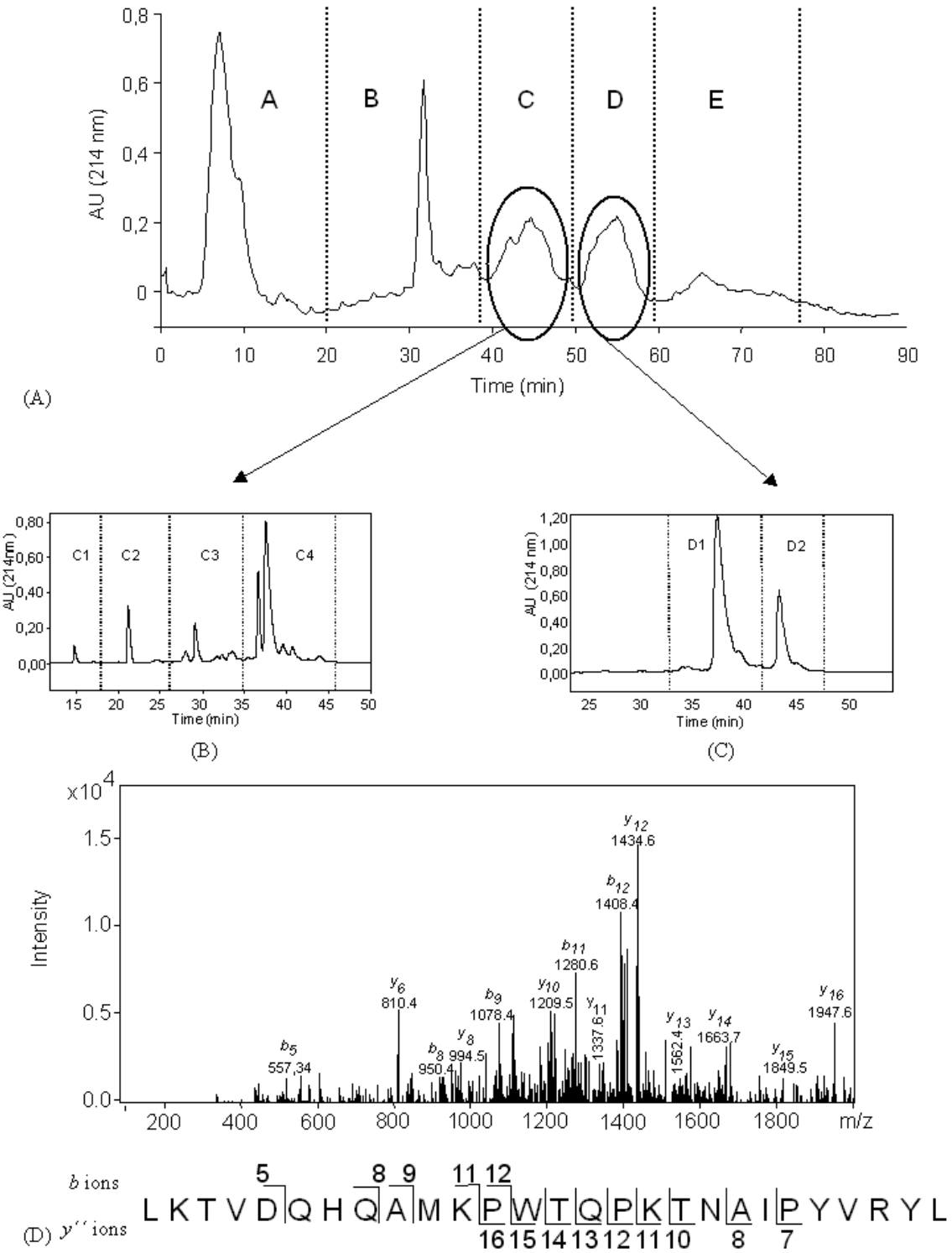
AU (214 nm)
Time (min)
A
B
C
D
E
(A)
C1
C2
C3
C4
(B)
D1
D2
(C)
x10^4
Intensity
m/z
b5 557,34
y6 810.4
b8 950.4
y8 994.5
b9 1078.4
y10 1209.5
y11 1337.6
b11 1280.6
b12 1408.4
y12 1434.6
y13 1562.4
y14 1663.7
y15 1849.5
y16 1947.6
b ions
y'' ions
L K T V D Q H Q A M K P W T Q P K T N A I P Y V R Y L
(D)

Fig. 1

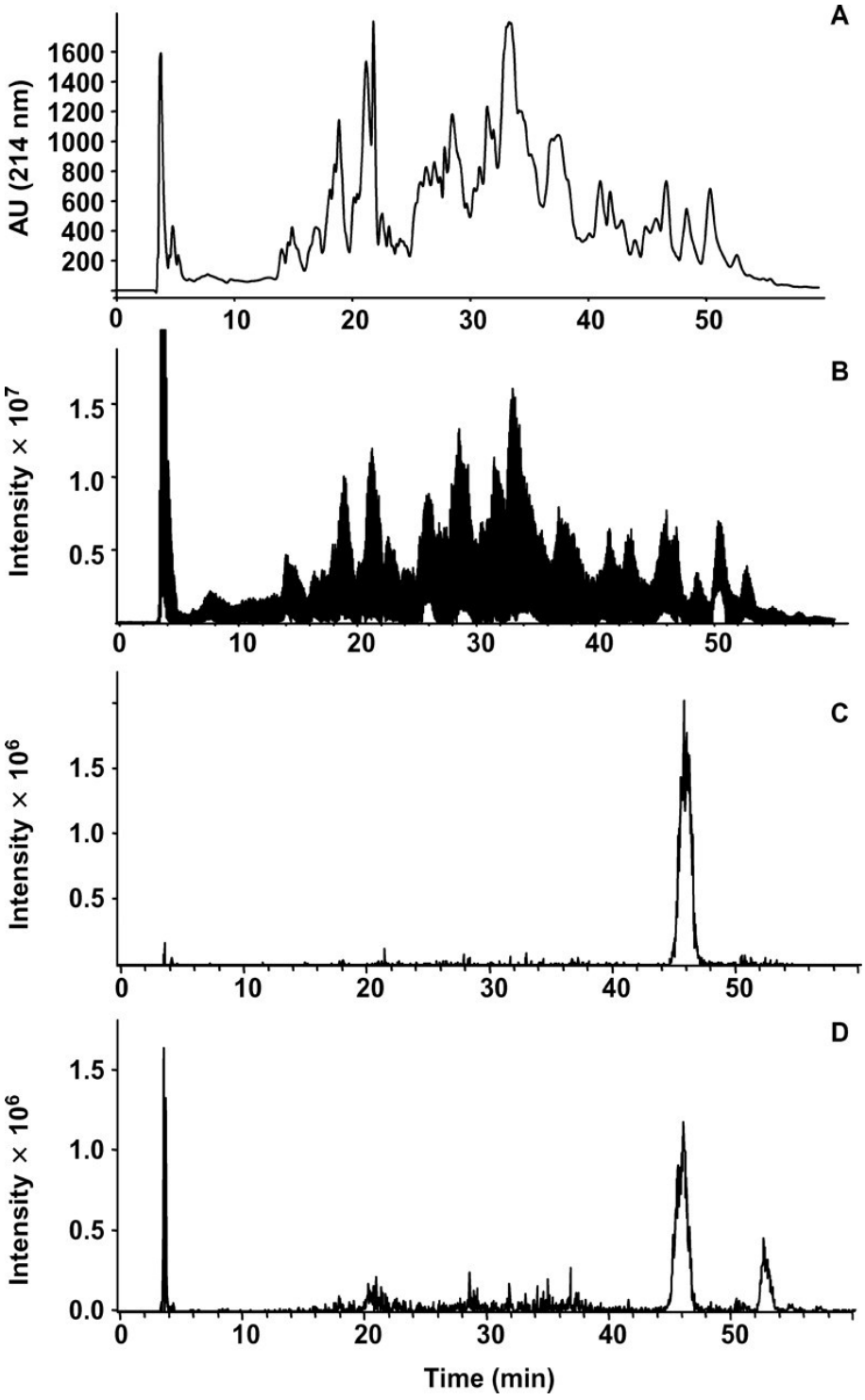
A
AU (214 nm)
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
B
Intensity × 10^7
1.5
1.0
0.5
C
Intensity × 10^6
D
0.0
Time (min)

Fig. 2

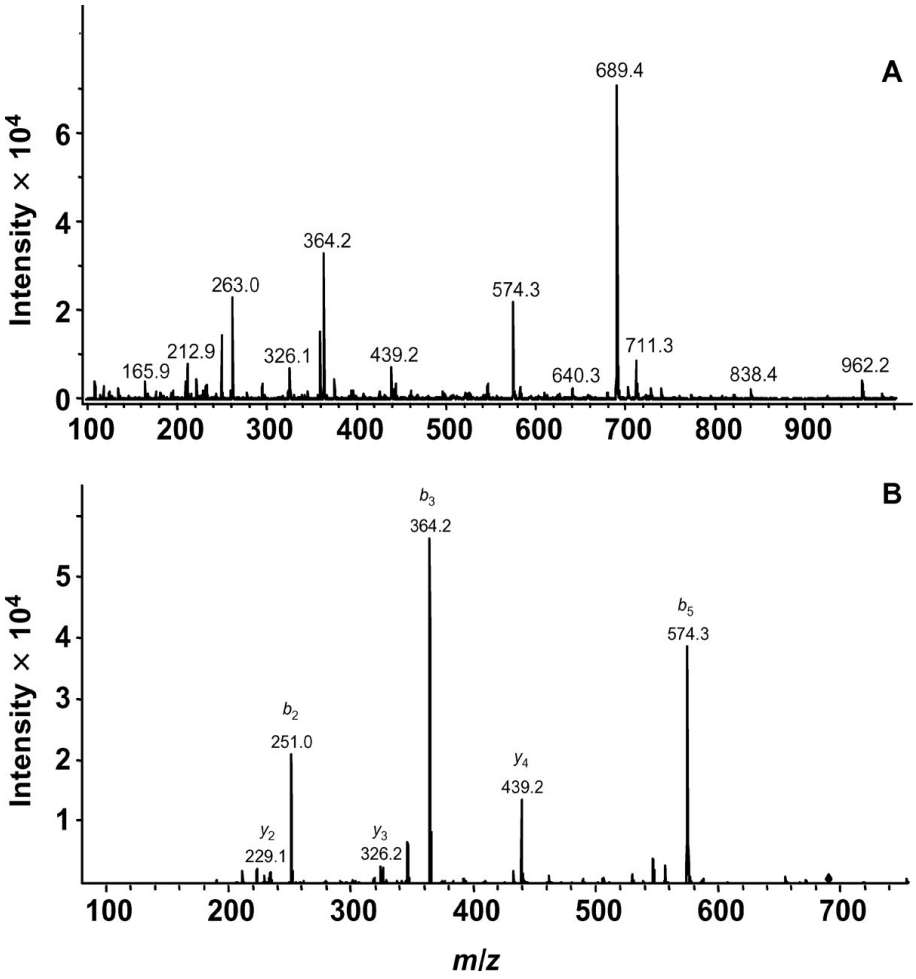
A
Intensity × 10^4
689.4
364.2
263.0
574.3
212.9
326.1
439.2
711.3
165.9
640.3
838.4
962.2
B
b3
364.2
b5
574.3
b2
251.0
y4
439.2
y2
229.1
y3
326.2
m/z

Fig. 3