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Review

Analysis of food proteins and peptides by chromatography and mass spectrometry

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Abstract

The research topics and the analytical strategies dealing with food proteins and peptides are summarized. Methods for the separation and purification of macromolecules of food concern by both high-performance liquid chromatography (HPLC) on conventional packings and perfusion HPLC are examined. Special attention is paid to novel methodologies such those based on multi-dimensional systems that comprise liquid-phase based protein separation, protein digestion and mass spectrometry (MS) analysis of food peptide and proteins. Recent applications of chromatography and MS-based techniques for the analysis of proteins and peptides in food are discussed.

Keywords: Reviews; Hydrophobic interaction chromatography; Perfusion chromatography; Mass spectrometry; Multi-dimensional liquid-phase-based separation techniques; Electrospray ionization; Matrix-assisted laser desorption ionization; Food analysis; Peptides; Proteins

Contents

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1. Introduction

In the last decade many research efforts have been done to develop techniques and methods for the separation, purification and characterization of food peptides and proteins. 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 [1,2]. Peptides are attributed with a number of different biological properties, as antioxidants, antimicrobials and surfactant agents [3]. Peptides have been also investigated for their sensorial properties, which are known to determine sweet and bitter taste in foodstuffs [4].

Nutritionally relevant proteins can be divided into animal (egg, milk, meat and fish proteins), plant (cereals, legumes and other proteins) and microbial proteins. Research on structural and physico–chemical properties of food proteins has been essential for elucidating their molecular structure responsible for their functionality in food. In addition, the development of methods for the purification of proteins has been of utmost interest in biotechnology research. In fact, the purity of a protein is a pre-requisite for its structure studies or its application, low degree of purity being requested for industrial application in food industry.

If high-performance liquid chromatography (HPLC) in different modes is a well established technique in food peptide and protein research, the new emergent technique capillary electrochromatography (CEC) is expected to have great potential in the separation of peptides and proteins as well [5]. Analytical approaches based on the use of mass spectrometry (MS) are also well established in protein food analysis. In CEC, which is also called ‘‘liquid chromatography on electrophoretic platform’’, the retention mechanism of charged species consists of a series of partitioning steps between the mobile and stationary phases and is controlled by the relative importance of three parameters: reduced mobility of a sample component with the electrosmotic mobility as the reference, the CEC retention factor and the ratio of the electrophoretic migration velocities of the migrant at the stationary phase surface and in the mobile phase. As depicted in Fig. 1, ionized sample components in CEC can migrate in the same direction as that of electroosmotic flow or in the opposite direction to that of electroosmotic flow depending on the charge. Recently, the research group of Horwáth successfully investigated silica-based tentacular weak cation-exchanger particles in CEC of peptides on open tubular and packed capillary columns [6]. Currently, various approaches to monolithic columns for CEC are under development, these materials being useful and versatile for the preparation of packed capillary columns [7,8]. In CEC peptides and proteins can also be separated with chemically modified etched fused-silica capillary, as demonstrated in a recent investigation aimed at studying selectivity differences in the separation of peptides due to the surface properties of the etched fused capillaries able to determine differences in solute-bonded phase interactions [9]. The development of new stationary phases for CEC and the

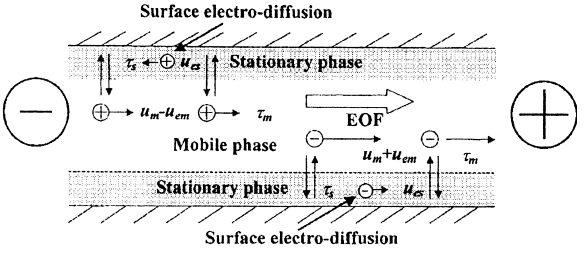

Fig. 1. Schematic illustration of a simple random walk by ionized sample components in CEC. Negatively charged sample components migrate codirectionally toward the anode while the positively charged sample components migrating counterdirectionally. Reprinted with permission from Ref. [5].

versatility of this technique could make this novel approach an attractive analytical tool for determination of peptides and proteins also in food analysis.

As for the use of mass spectrometry in the analysis of peptides and proteins, in particular, matrix assisted laser desorption ionization-mass spectrometry (MALDI-MS) with time-of-flight (TOF) analyzers and electrospray-mass spectrometry (ESI-MS) with quadrupole mass spectrometers became a major breakthrough in accurately measuring molecular masses of proteins up to 100 000 Da or more [10–12]. Further, because of the ability of ESI-MS to directly analyze compounds from aqueous/organic solutions providing valuable structural information, it is the most desiderable and powerful liquid chromatography (LC) detection system available.

A number of review papers on the application of chromatography and MS to food peptides and proteins have appeared in the recent literature, attesting a large increase in related publications and the increasing interest and efforts made in this direction [13–19]. Applications of liquid chromatography off-line with ESI, MALDI, fast atom bombardment (FAB) and on-line coupled with ESI and flow-FAB have been discussed in the research area concerning food proteins and derived peptides by Léonil et al. [13]. In this overview attention was also paid to the characterization of food protein hydrolyzates having functional, nutritional and biological interest by LC and MS.

The application of ESI-MS and MALDI-TOF-MS to the structural characterization of proteins in some common protein foods is discussed in the review article by Alomirah et al. with particular regard to their advantages in terms of sensitivity, mass accuracy and short analysis time [14]. The potential of MS for molecular mass determination, peptide sequencing, identification of post-translational modifications, study of protein–protein or protein–ligand interactions is also discussed.

The research performed in modern feed analytical laboratories is outlined by Rutherfurd et al. [15]. This review paper underlines the increasing use of techniques such as fast protein liquid chromatography (FPLC), MS and high-performance capillary electrophoresis (CE) for feed evaluation. The analysis of peptides is of great importance also for feed evaluation scientists, as the area of biologically active peptides starts to open up. In this context, it can be expected that techniques such as LC and MS will be applied more and more for the separation and characterization of peptides.

Recently, Bayard and Lottspeich overviewed the research carried out for characterization of post-translational modifications of allergenic proteins, underlying the role of MS-based methods for analysis of allergenic and non-allergenic proteins [16].

Selected applications of chromatographic and MS methods for the analysis of peptides and proteins are described in a review paper dealing with analytical methods for the characterization of these components in wine [17].

Very recently, advances in the application of MS to food protein research have been overviewed by Careri et al., emphasizing the role of MS and tandem MS as powerful tools in peptide and protein characterization [18], whereas a survey of new proteomics technologies with emphasis on their applications to nutritionally important proteins has been presented by Kvasnička [19].

This review addresses the contribution of the different techniques of chromatography to the separation and purification of food peptides and proteins with selected examples that have been published mainly during the past 2 years with emphasis on novel chromatographic systems. The most recent applications of the different techniques of ionization in mass spectrometry and the progress made in combining LC with MS for the analysis of food proteins and peptides will be also subject of discussion.

2. Chromatography

2.1. Purification and separation by high-performance liquid chromatography on conventional packings

Although nowadays other analytical techniques such as CE and micellar electrokinetic chromatography (MEKC) are gaining in application, the most widely used techniques for peptide and protein isolation and separation are reversed-phase (RP) LC,

ion-exchange chromatography (IEC) and size exclusion chromatography (SEC). Hydrophobic interaction chromatography (HIC) has also become dominant for this purpose. HIC results to be widely used in protein purification in the research laboratory as a complement to other techniques which separate according to other parameters such as charge (IEC), size (SEC) or biospecific recognition (affinity chromatography). The order in which the different techniques are combined is also of great importance.

Recently, polymeric materials have been designed for large-scale preparative and process-scale purification of synthetic peptides and recombinant proteins [20]. Rigid macroporous copolymers of styrene and divinylbenzene providing high chemical and mechanical stability were prepared. Attention was paid to the pore size and pore morphology in such a way to enable unrestricted solute diffusion while offering maximum available surface area for higher loading capacity. The chemical stability of the material makes it compatible with 1 *M* sodium hydroxide without particle dissolution or a decrease of selectivity.

The advantages of using an automated on-line two-dimensional 2D-LC system with integrated sample treatment has been described by Wagner and co-workers for the analysis of proteins and peptides with a molecular mass below 20 kDa [21]. High-speed separations combined with high resolution were claimed for this novel system that may be considered complementary to 2D gel electrophoresis in proteome research. After a size-selective sample fractionation step, in the first dimension new silica-based restricted access materials (RAM) in IEC mode were used, whereas hydrophobic interactions using short RP columns were exploited for separation in the second dimension. In addition, in this dimension a new column-switching technique consisting of four RP columns was used for on-line fractionation and separation. Selected peaks were collected and analyzed off-line by MALDI-TOF-MS. Even though this approach was proved for protein mapping of biological samples, it could be useful also in food research.

The problem of the quantitative determination of peptides and proteins separated by reversed-phase LC has been successfully addressed by Moffatt et al. [22]. The authors developed a protocol based on the calculation of a protein concentration directly from the peak area relative to an internal standard without the use of an analytically pure reference sample. Application of this approach to purified samples and potentially to crude biological extracts was claimed and sources of error and limitations of the method were thoroughly discussed.

Improved reversed-phase LC separation of peptides has been demonstrated in a recent investigation dealing with the development of surface-alkylated polystyrene monolithic columns suitable for capillary LC-ESI-MS [23]. Monolithic columns, which usually are composed of one piece of polymeric or silica-based monoliths having flow-through pores, offer advantages of eliminating the technical problems of frits to retain the packed bed, while providing a stable chromatographic bed, adjustable pore diameter, and low column back pressure under high eluent flows. Surface octadecylation provided better results in terms of peptide separation with respect to those achieved using an unmodified monolithic poly(styrene–divinylbenzene) column. By contrast, comparable resolution was proved for protein separation when using modified and unmodified columns. The usefulness of this approach was demonstrated by analysing tryptic protein digests and standard mixtures of peptides and proteins.

An ion-pair reversed-phase LC separation suitable for ESI-MS detection has been recently devised for analysis of underivatized small peptides [24]. Attention was focused on the separation of isomers and isobars dipeptides (e.g. Gly-Ile, Gly-Leu, Leu-Gly, Gly-Lys, etc.), which is needed because of the intrinsic MS problems with these compounds. Among the ion pairing reagents tested (i.e. trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), tridecafluoroheptanoic acid (TDFHA), nonafluoropentanoic acid (NFPA) and pentadecafluorooctanoic acid (PDFOA)) on a C_{18} stationary phase, good results were achieved using NFPA and TDFHA. As illustrated in Fig. 2, the best selectivities for the separation of six dipeptides were achieved when using 0.5 *M* TDFHA. Further, optimization of MS parameters indicated different collision energy values between glycyl peptides and lysyl peptides for optimum MS/MS experiments. In general, under

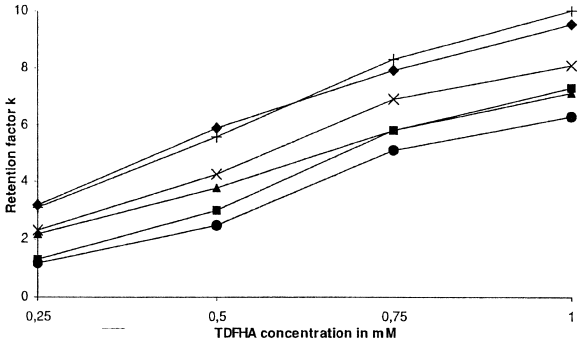

Fig. 2. Influence of the tridecafluoroheptanoic acid (TDFHA) concentration in an aqueous mobile phase on the retention factor *k* of six dipeptides (●) Asp-Asp; (▲) Gly-Asn; (■) Asp-Gly; (×) Gly-Asp; (♦) Gly-Gly; (+) Gly-Gln. Reprinted with permission from Ref. [24].

selected reaction monitoring (SRM) conditions higher sensitivity than that achieved in MS mode was evidenced.

2.1.1. Chromatography of peptides

Isolation of peptides in food is a difficult task because they are present in a complex mixture containing various substances such as acids, free amino acids, sugars, salts. For this purpose, classical preparative chromatographic methods and ultrafiltration are normally used. A further problem is a limitation in the sensitive and selective detection of peptides after a chromatographic separation, since particularly small peptides often lack a suitable chromophore, fluorophore or electrophore. In this context, derivatization procedures adequate for the different detection methods have been developed, as discussed in a review by Koller and Eckert [25]. In addition, the development of mass spectrometry has opened a new era in structure elucidation of peptides. On the other hand, when using MS as detection system, the chromatographic method has to be developed taking into account also the features of the mass spectrometer.

Analytical separations of peptides in various food products among which protein hydrolyzates, dairy and meat products have been attempted through LC techniques according to their hydrophobicity, molecular size and net charge [26–47].

A method based on the application of gel filtration chromatography on Sephadex LH20 followed by LC on a porous graphitic carbon (PGC) column Hypercarb column (100×4.6 I.D., 5 μm particle size, 250 Å pore diameter) has been reported for the isolation, separation and purity evaluation of small peptides from wine [26]. After a pre-treatment consisting in ultrafiltration of wine on hollow fibre, multi-step fractionation was needed because of the complexity of the matrix, which contains a number of non-peptidic components together with small peptides. The use of PGC stationary phase proved to be advantageous with respect to a traditional C_{18} phase, enabling good separation of peptides from phenolic compounds (Fig. 3).

Recently, a RPLC method has been devised to investigate the formation of gamma-glutamyl-beta-alanylhistidine and related peptide in the macromolecular fraction of beef soup stock [27]. For this purpose, a model solution made up of amide-containing amino acids and carnosine was heated and the products were analyzed by LC in RP mode using an octadecyl-silica column. A sample preparation stage prior to high resolution analysis including proteolytic digestion and fractionation by solid-phase extraction (SPE) was performed. After derivatization with phenylisothiocyanate, the qualitative and quantitative analysis of the fraction containing the peptides allowed to evidence an increase of the analyte in the heated beef soup stock solution, suggesting that the formation of the isopeptide occurs during the heating process of carnosine-containing foods.

RPLC using a FPLC system proved to be valuable for the fractionation of the soluble nitrogen fraction of fermented milks containing angiotensin-I-converting-enzyme (ACE) inhibitory peptides [28]. After a further purification of the fractions that showed the highest ACE-inhibitory activity, the related peptides were sequenced by tandem FAB-MS. Using a reversed-phase column off-line with FAB-MS allowed Suetsuna and Nakano to separate and identify ACE inhibitory peptides derived from wakame (*Undaria pinnatifida*), the most popular edible seaweed in Japan [29]. IEC and SEC in gel filtration (GFC) mode were firstly used to isolate the peptide fraction having activity against ACE from the peptic digest of protein prepared from wakame sample. Other authors purified peptides exhibiting ACE inhibitory activity

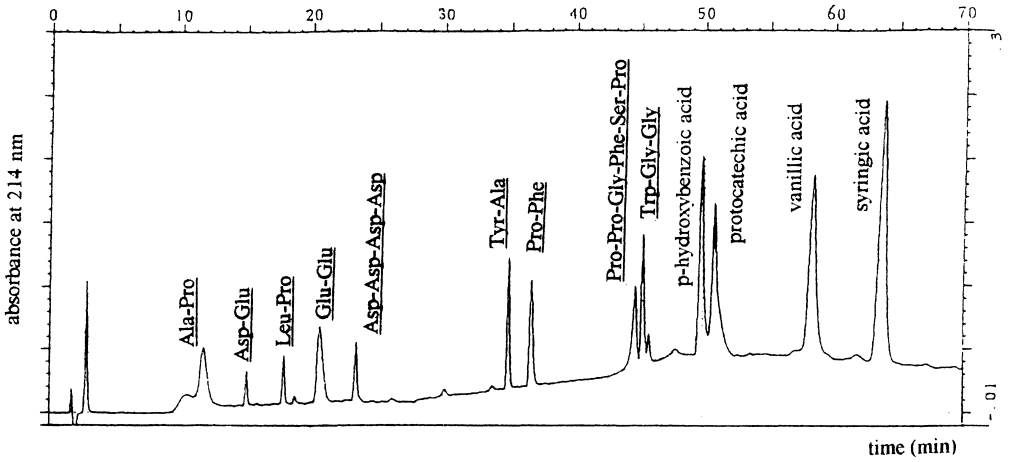

Fig. 3. HPLC profile at 214 nm of a standard solution of peptides and phenolic compounds Hypercarb PGC, 5 μm column (100×4.6 mm I.D.). Eluent A: 0.1% TFA in water. Eluent B: 0.1% TFA in acetonitrile. Flow-rate: 0.8 ml/min. Peptides are underlined. Reprinted with permission from Ref. [26].

isolated from bovine skin gelatin hydrolyzate by sequential LC methods consisting of GFC, IEC and RPLC [30]. The purified peptides comprised Gly-Pro-Leu and Gly-Pro-Val. Peptides exhibiting potent inhibitory activity to ACE have been also recently isolated from Gouda cheese samples by using RP chromatography and GFC [31].

A ubiquitin-like peptide has been recently studied for its potent ribonuclease activity toward yeast transfer RNA [32]. For this purpose, isolation from an extract of the edible mushroom *Pleurotus sajor-caju* was performed by affinity chromatography on Affi-gel Blue gel, IEC on DEAE-cellulose and GFC on Superdex 75. A 9.5 kDa bioactive peptide with an N-terminal sequence similar to ubiquitin was isolated with a yield of 0.25 mg/kg mushroom.

Hydrolyzed proteins are widely used in foodstuffs because of their nutritional or functional properties. In this context research has been focused on the purification and characterization of the peptide fraction deriving from enzymatic treatment [33–35]. Peptides generated by hydrolysis of goat whey in an ultrafiltration membrane enzymic reactor have been separated by RPLC and characterized by MALDI-TOF-MS [33]. A wide range of products deriving from α-lactalbumin were identified ranging from dipeptides with a molecular mass lower than 600 Da to very large peptides having a molecular mass higher than 2000 Da. Hydrophobic interaction chromatography has been recently applied for a semi-large purification of lactoferricin, a potent antimicrobial peptide contained in a pepsin hydrolyzate of lactoferrin used also in infant formula [34]. Gel permeation chromatography (GPC) and RPLC have been successfully applied for fractionation of an enzymatic hydrolyzate of deamidated wheat gluten, which is used as ingredient in food products because of its glutamate-like taste [35]. Results evidenced two fractions both exhibiting this sensorial feature attributable to the formation of pyroglutamyl peptides.

The analytical problem of the identification of substances involved in the development of bitter taste in cheese products has been recently addressed by Broadbent et al. [36]. By applying RPLC to aqueous extracts of reduced fat Cheddar cheese samples, the authors found that accumulation of peptides f 1-9, f 1-13, f 1-16, and f 1-17 derived from α_{s1}-casein was affected by *Lactococcus lactis* extracellular proteinase (lactocepin) specificity. The investigation was carried out in Cheddar cheese samples manufactured with a starter made up of isogenic strains of *Lactococcus lactis*; one of isogens did not produce lactocepin, whereas the others produced lactocepin specificity. In particular, results showed that propensity for development of bitterness was highest in cells which produced a lactocepin with group h specifity. Findings suggest a possible

way to control the formation of bitter flavour defect in cheese by proteinase gene exchange or gene replacement.

Reversed-phase LC has been applied in investigations aimed at clarifying the role of peptidases of thermophilic lactic acid bacteria in the proteolysis of Swiss cheeses during ripening [37,38]. Chromatographic profiles evidenced a more extensive proteolysis for the *Lactobacillus helveticus* strain ITGLH1 compared with ITGLH77 [37]. In a subsequent study the same group compared the peptidase activities toward a dairy substrate [38]. For this purpose, a tryptic/chymotryptic hydrolyzate of purified β-casein comprising 34 peptides from 3 to 35 amino acids was used. From the LC assessment it was possible to conclude that *Lactobacillus helveticus* was the most efficient in hydrolyzing peptides of β-casein despite strain.

Other studies regarding the chromatographic analysis of peptides in dairy products with different purposes have been recently reported [39–44]. Chromatographic methods have been developed for the characterization of peptides from milk and derived products [39–42]. Anion-exchange FPLC and RPLC were applied to study heterogeneity of ovine casein macropeptide (CMP) [39] and caprine CMP [40]. The degree of glycosylation and phosphorylation was determined by using off-line and on-line mass spectrometry following LC separation. Among the most abundant ovine CMP components a diphosphorylated carbohydrate-free form, one and two monophosphorylated and a non-phosphorylated asialo-aglyco species were detected [39]. In the case of caprine CMP, the authors reported the occurrence of two non-glycosylated and diphosphorylated species as well as two monophosphorylated forms, each corresponding to a genetic variant of caprine κ-casein [40]. Peptides deriving from hydrolysis of CMP by endoproteinase Glu-C have been tested for activity against the pathogens *Streptococcus mutans* and *Porphyromonas gingivalis* after fractionation with RPLC and GFC [42]. Fractions were characterized by N-terminal sequence analysis and MALDI-TOF-MS. The authors concluded that phosphorylation is essential for antibacterial activity, since only the phosphorylated forms exhibited growth-inibitory activity against *S. mutans*.

A subject of technological interest is the role played by natural fat content and commercial fat mimetics on the proteolysis, sensory and texture properties of cheese samples. In a study regarding changes in the microbiology and proteolysis of Cheddar cheese, chromatographic peptide profiles obtained by reversed-phase LC and GPC evidenced a role of the fat content of the product on the proteolysis pattern [43]. More recently, peptide RPLC profiles obtained by comparing low-fat white-brined cheeses (~60% fat reduction) made from bovine milk and containing two hydrocolloid-fat replacers with analogous cheese products without fat replacers and the full-fat cheese sample evidenced that proteolysis was not largely influenced by the hydrocolloids [44]. Instead, fat replacers proved useful in determining a great improvement in cheese texture.

Concerning technological processes occurring in meat products, peptides and other nitrogen compounds have been evaluated as biochemical markers of pork meat quality at 2 h post-mortem [45] and during ageing [46] in investigations dealing with the analysis of peptides and amino acids in different pork meat qualities. Cation-exchange and reversed-phase chromatography were applied for isolation of peptides paying attention to prevent the interference from other nitrogen compounds. When following the evolution of the four fractions separated by RPLC, major differences among qualities were evidenced for peptide fractions 3 and 4 [46]. Meat quality was found to influence the system involved in the production of the final proteolytic products, such as peptides and amino acids.

Proteolysis of fermented meat products is recognized to result in changes of texture and flavour development, which are of concern for the acceptability of the products. To predict the suitability of *Lactobacillus casei* CRL 705 as a starter culture for dry fermented sausages processing, a method based on the use of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and RPLC was successfully applied for the study of proteolysis of pork muscle sarcoplasmic and myofibrillar proteins [47]. Production of hydrophilic peptides and free amino acids resulted to be affected by addition of ascorbic acid and by the presence of curing salts. In a very recent work, proteolytic changes obtained inoculating a known proteolytic

starter culture in fermented sausages were compared with those occurring in control sausages [48]. RPLC with UV detection was applied to qualitatively analyze the proteolytic changes in the sarcoplasmic and myofibrillar proteins during sausage ripening. Preliminary trials were necessary to devise the chromatographic method in such a way to obtain adequate resolution of peptides even at the cost of long analysis times, i.e. about 110 min. Operating in gradient elution mode, RPLC profiles of both the control samples and the sausages inoculated with *S. carnosum* MC1 appeared very complex and similar, with the formation of a plethora of hydrophilic peptides, being eluted between 35 and 75 min, i.e. between ~8 and 21% acetonitrile (Fig. 4). The most evident difference was a lower concentration of some peaks in the sausages inoculated with the starter culture as ripening proceeded, indicating a further hydrolysis of the peptides by enzymes of *S. carnosum* MC1. After RPLC analysis, peptides were identified by N-terminal amino acid sequence analysis. From this investigation it was concluded that indigenous proteinases are responsible for the formation of hydrophilic peptides from both the protein fractions, since bacterial peptidases determined mostly the release of free amino acids. Further, most of the small hydrophilic peptides generated during sausage ripening result to originate from both sarcoplasmic and myofibrillar proteins.

2.1.2. Chromatography of proteins

As attested by the literature, in the last years researchers namely used types of chromatography such as SEC in both GFC and GPC modes, and IEC to separate and analyze proteins of nutritional interest. Most of the novel methods developed are also based on hydrophobic interaction chromatography of proteins both for preparative and analytical applications in the research laboratory.

2.1.2.1. Reversed-phase, ion-exchange and size exclusion chromatography

Wang et al. applied FPLC–GFC on a Superose 12HR 10/30 column to estimate the molecular mass of a protein capable of inhibiting cell-free translation including antiviral and anticancer activities from cultured mycelia of the edible mushroom *Tricholoma lobayense* [49]. IEC on different stationary phases (DEAE-cellulose in a first fractionation step and CM-cellulose in a further step) and affinity chromatography were demonstrated helpful in the purification of the investigated protein. The strategy followed by the authors was to focus on the peak with the strongest translation-inhibitory activity at each chromatographic step and to submit this fraction to a further purification. A molecular mass of 30 kDa was estimated by comparing the retention time of the peak of interest with those of standard molecular mass marker proteins.

The combination of multi-angle laser light scattering (MALLS) technique and GPC has been proved powerful in analyzing the time-dependent change of molecular size of α-lactalbumin polymers formed by mammalian and microbial transglutaminases (TGase) [50]. TGase is an enzyme of interest for applications in food systems being capable of catalyzing the acyl-transfer reaction between peptide-bound glutamine residues and primary amines [51]. Therefore, in food processing it could improve functional properties of proteins and give a desiderable texture to various foods. Further, since the physical states of many food systems are affected by the presence of Ca^{2+}, the effect of calcium on the ability to induce cross-linking of α-lactalbumin by TGase was studied by performing experiments in a Ca^{2+}-free system as well as in the presence of Ca^{2+}. Different behaviour of the two enzymes was demonstrated. Irrespective of the presence or absence of calcium, at the early stages of incubation the mammalian TGase-catalyzed polymerization exhibited better efficiency than the microbial-catalyzed one, as shown from the molecular mass distribution curves at 60 min (Fig. 5). By contrast, microbial TGase was found capable of forming larger polymers in the late stage for prolonged incubation.

Elgar and co-workers devised a precise, sensitive and reliable RPLC method for the simultaneous determination of proteins such as α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin G including caseinomacropeptide and proteose peptone in bovine whey samples [52]. The separation was achieved on the Resource RPC column (Pharmacia Biotech) in 30 min and resulted to be applicable to the analysis of soluble proteins in various commercial and laboratory whey products. Evaluation of protein heterogeneity and quality was

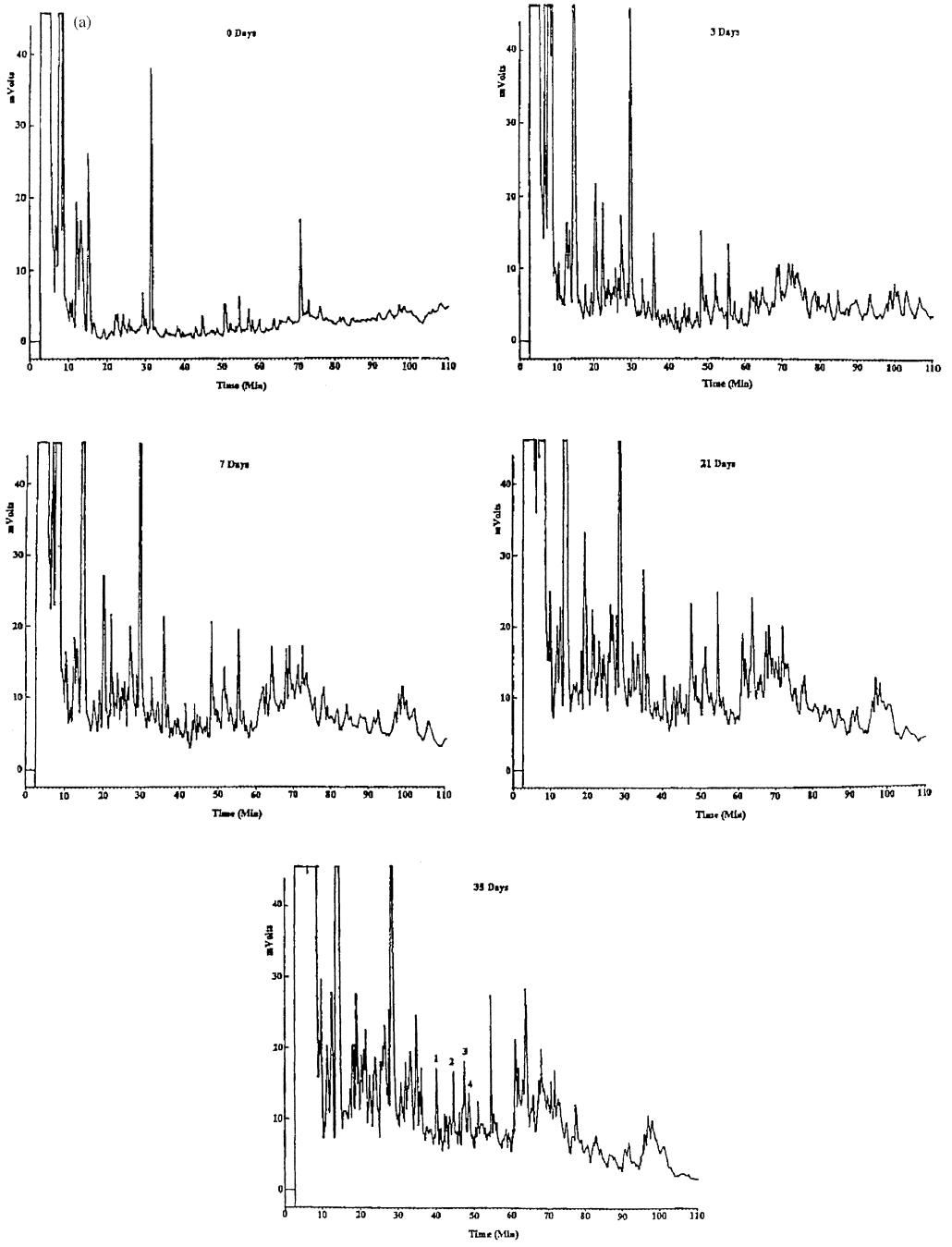

Fig. 4. (a) Reversed-phase RP-HPLC (C_{18}) elution profiles of 2% TCA-soluble peptides produced throughout the ripening of the control sausages. (b) Reversed-phase RP-HPLC (C_{18}) elution profiles of 2% TCA-soluble peptides produced throughout the ripening of sausages inoculated with *S. carnosus* MC1. Reprinted with permission from Ref. [48].

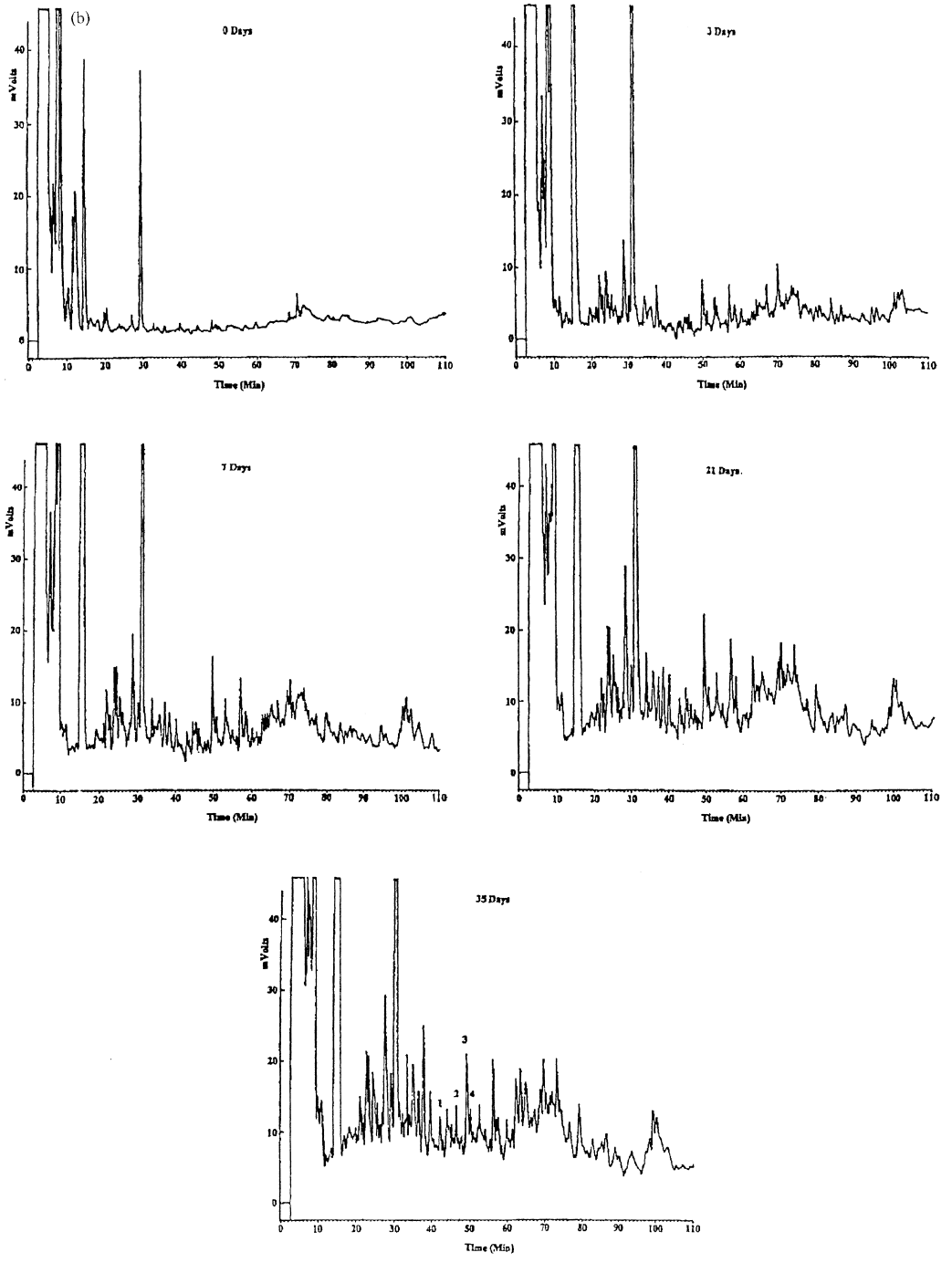

Fig. 4. (*continued*)

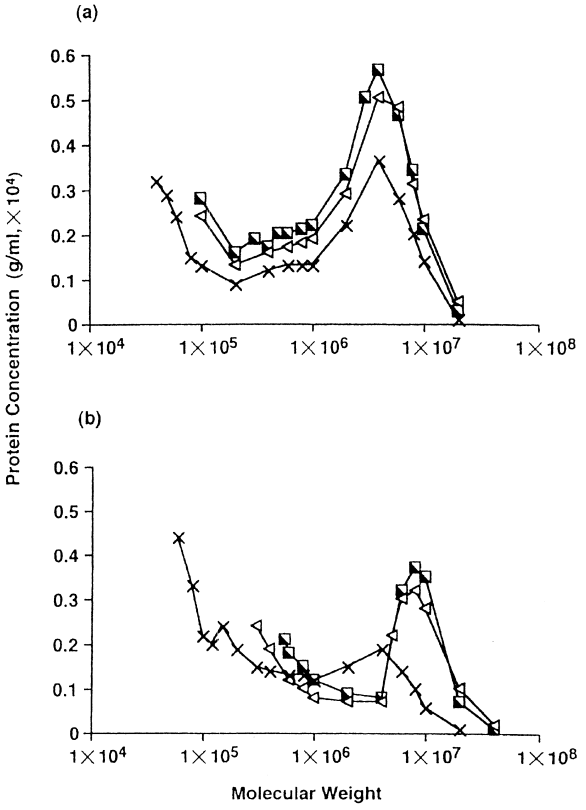

Fig. 5. Molecular weight distribution curves of α-LA polymers formed by the incubation with TGases from 60 to 240 min in the absence of Ca^{2+}. (a) and (b) indicates the results of GTGase and *Streptoverticillium* S-8112, respectively. Reaction time: (×), 60 min; (△), 120 min; (□), 240 min. Reprinted with permission from Ref. [50].

also feasible from the RPLC determinations with additional data obtained from on-line electrospray-mass spectrometry. Analyzing whey protein concentrate and whey protein isolate samples, limits of detection and quantitation of 0.6 and 2.0% were achieved for immunoglobulin G. These values were lower for the other proteins, since limits of detection and quantitation were lower than or equal to 0.3% and lower than or equal to 1.0% powder mass, respectively.

A novel method for large-scale isolation of native β-lactoglobulin from cheese whey has been developed by Konrad et al. [53]. The method was based on a peptic hydrolysis of whey protein and membrane filtration under mild conditions for isolating β-lactoglobulin as a pure compound. Since one of the scopes of the study was the evaluation of the applicability of the process for industrial practices, the method was applied to 10 000 l batches of processed whey and the preparation isolated according to the new method was compared with pilot-scale results of three other procedures: selective thermal precipitation, 3% (w/w) trichloroacetic acid precipitation and a salting-out procedure. Analysis of purity performed by both electrophoresis and FPLC-IEC in gradient mode revealed maximum purity and nativeness of the protein isolated by the new method, which proved to be reproducible and selective. In addition, any technical problems or variations in the purity of the isolated β-lactoglobulin attested method robustness when applied to processing three 10 000 l batches.

Another process of interest for food industry is the heat-induced aggregation of whey and pure β-lactoglobulin. In fact, aggregation can be undesiderable, e.g. in pasteurization and sterilization of milk, or it can be exploited for possible applications of protein aggregates and gels. Very recently, SEC experiments and LC measurements under RP conditions have been carried out with the aim to studying the influence of genetic variance on the structures of heat-induced aggregates of β-lactoglobulin [54]. SEC was performed at room temperature using two columns (TSK® PW 5000, 30 cm+PW 6000, 60 cm) in series and eluting with 0.1 *M* $NaNO_3$ at a flow-rate of 1 ml/min. RPLC separations were carried out using a C_{18} *Pluresil* (4.6×150 mm, particle size: 5 μm, pore size: 12 nm) column in gradient mode at 30 °C and pH 2.1. Aggregates formed by pure variants A and B of the investigated protein and by a mixture containing these variants in about equal amounts were compared. In particular, SEC was applied to measure the quantity of unaggregated proteins, whereas RPLC was used to determine the ratio of the two variants. In contrast with literature [55,56], results of the study evidenced that in a mixture containing β-lactoglobulin variants A and B in about equal amounts, the aggregation rate is the same for both variants over a range of pH (2.1–7), protein concentrations (36–115 g/l) and heating conditions (55–73 °C) (Table 1).

A purification procedures involving anion-exchange chromatography, GFC and chromatography on hydroxylapatite has been developed in order to isolate an esterase from tomato from cell cultures of *Lycopersicon esculentum* [57]. The native enzyme

Table 1
Total fraction of non aggregated proteins (F) and ratio of the two variants (R) at various conditions [54]

C (g/l)	pH	T (°C)	F	R
115.5	7	55	0.45	0.89
52.3	7	73	0.22	0.82
52.3	7	70	0.14	0.75
52.3	7	60	0.30	0.82
37.2	7	60	0.42	0.79
37.2	7	73	0.24	0.89
37.2	7	70	0.27	0.85
42.2	2.1	70	0.68	0.72
36.2	5.5	70	0.61	0.85

showed a molecular mass of 26 kDa as measured by GFC, which was similar to that determined by SDS–PAGE and MALDI-TOF-MS analysis (M_r of 28547 kDa).

A novel protease from *Penicillium chrysogenum* (PG222) isolated from dry-cured ham has been purified by a multi-step procedure including IEC in order to evaluate the hydrolytic properties of the purified enzyme on meat proteins [58]. SDS–PAGE analysis and GFC allowed to establish a molecular mass of about 35 kDa for the purified fraction. Based on the results of this investigation, the authors concluded that the activity of this novel proteolytic enzyme could be of interest in ripening process and in generating the flavour of dry-cured meat products.

In a recent work, column chromatography, i.e. anion-exchange chromatography (AEC) followed by GFC, together with other methodologies (cryo-precipitation and isoelectric precipitation) has been used to prepare amandin, the major storage protein in almond (*Prunus dulcis* L.) [59]. According to SDS–PAGE and RPLC analyses, the protein resulted to comprise two major types of polypeptides with estimated molecular masses of 42–46 and 20–22 kDa linked *via* disulfide bonds besides to other minor polypeptides.

2.1.2.2. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography, which was introduced by Hjertén in 1973 to describe salt-mediated separations of proteins in weakly hydrophobic carbohydrate gel matrices [60], has been proved to be a powerful and very promising technique for purification and separation of complex mixtures of proteins. HIC lies on hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the surface of proteins, thus exploiting the hydrophobicity of proteins to achieve their separation. As reported in a recent overview, the applicability of this type of chromatography has been successfully evaluated as powerful bioseparation technique in laboratory-scale, as well as in industrial-scale purifications of proteins [61].

Analytical separations of caseins by HIC have been discussed by the research group of Bramanti [62–64]. Caseins are milk proteins which have long been known to play an essential role in biological defense systems. In addition, a key role in dairy industry is assigned to caseins, being frequently used as additives in foods because of their emulsifying properties. Rapid and efficient chromatographic methods to separate and analyze casein fraction components in food systems are thus needed, since methods based on CE and MEKC are laborious and time-consuming. In a first study a novel method for the separation and quantitation of denatured α-, β- and κ-caseins in commercial raw samples by HIC has been described [62]. The method is based on an easy solubilization of real samples by 4.0 *M* guanidine thiocyanate (GdmSCN) and separation on a TSK-Gel® Phenyl-5PW column (TosoHaas) eluting in the presence of 8.0 *M* urea in the mobile phase. High levels of denaturants such as GdmSCN and urea were used to avoid aggregation of caseins. Good separation of caseins was achieved also in the presence of whey proteins without requiring preliminary separation or precipitation procedure of the casein fraction. The sensitivity showed was in agreement with the extinction coefficient values of the proteins investigated and detection limits in the 0.33–0.65 μ*M* range were claimed (Table 2). The developed method, applied for quantitative determination of α-, β- and κ-caseins in whole milk powder and fat-free yoghurt samples, requires common LC instrumentation and is not expensive. The same authors reported validation of the method by the analysis of reference skim milk powder (BCR-063R) certified for total nitrogen content [63]. The mean casein content found in 10 aliquots of reference material calculated with respect to the total protein

Table 2
Results of linear fitting of calibration data of α-, β- and κ-caseins [62]

Data	α-casein (t_R = 45.3 min)	β-casein (t_R = 41.5 min)	κ-casein (t_R = 44.0 min)
Injected concentration range (μM)	0.5–65	0.5–63	0.5–46
R^2 (number of points = 8)	0.9990	0.9997	0.9956
Slope (μM^{-1})	16044	8100	13701
RDS (%)[a]	2.5	2.2	2.7
LOD (μM)[b]	0.33	0.65	0.40

[a] Average value of five replicate determinations for standard solution whose protein concentration injected was 20 μM.
[b] LOD = 3σ/slope, where σ has been estimated on the basis of baseline noise.

content was 79±2.7%. A comparison of the results of linear fitting of casein calibration data with results of linear fitting of standard addition data of α-, β- and κ-caseins to BCR-063R was also performed. Quantitation was carried out on 31 real raw dairy samples (processed cow's milk, milk powders, cream, cheeses, casein-free infant formulae, cookies for babies containing milk proteins), concluding that the approach used has to be considered of great worth in the quality control of milk products. Very recently, an improvement of the HIC chromatographic method has been reported by the same group [64]. The use of TSK-Gel Ether-5PW column (Tosoh Biosep) was proposed for the separation of caseins, allowing separation of α-, β- and κ-caseins in less than 22 min, since TSK-Gel Ether-5PW column contains a HIC phase less hydrophobic than the phenyl one. In addition, separation of α-casein in α_{s1}- and α_{s2}-casein fractions was achieved. The method was validated in terms of linearity, detection limits, precision and accuracy by the analysis of reference skim milk powder (BCR-063R) certified for total nitrogen content. The method was applied to commercial caseins and to 30 real raw samples. Fig. 6 illustrates HIC chromatograms of four dairy product samples injected after a fast preparation consisting of sample centrifugation and diluting in the LC gradient starting buffer. A statistical comparison was performed between results on quantitation of α-, β- and κ-caseins obtained by TSK-Gel Ether-5PW and TSK-Gel Phenyl-5PW HIC columns, showing more accurate results for chromatographic analysis performed by the ether column.

HIC has been used to isolate protein fractions from two Acacia gums [*Acacia senegal* (L.) Willd. and *Acacia seyal* Del.] with the aim of studying their action mode in terms of interfacial properties [65]. Analytical data of the three HIC fractions of two Acacia gums showed a higher protein content in *Acacia senegal* than *Acacia seyal* gums. The behaviour of the gums resulted to be strongly affected by their arabinogalactan–protein complex.

HIC of microbial proteins has also been described [66]. Tyrosine decarboxylase (EC 4.1.1.25) (TDC) from the wine *Lactobacillus brevis* IOEB 9809 was purified by a fast method involving anion-exchange chromatography, ultrafiltration and HIC. The protein was composed of two subunits of identical molecular mass (approximately 70 000 Da). The method allows the purification of the enzyme to near homogeneity in 1 working day.

Debittering of protein hydrolyzates is a good example of current applications of HIC in producing biotechnological products [67]. The enzymatic treatment of various food proteins frequently results in a bitter taste due to the formation of low-molecular mass peptides composed of mainly hydrophobic amino acids. The formation of bitter peptides thus represents a serious problem in the use of protein hydrolyzates, limiting the sensory acceptability of food proteins. A recent review paper describes the various attempts made to debitter protein hydrolyzates including selective separation such as treatment with activated carbon, extraction with alcohol, isoelectric precipitation, chromatography on silica gel, HIC and masking of bitter taste. The authors discuss applications of HIC for reduction in bitterness of protein hydrolyzates. This type of chromatography exploits hydrophobicity of bitter peptides, which can be completely removed on hydrophobic

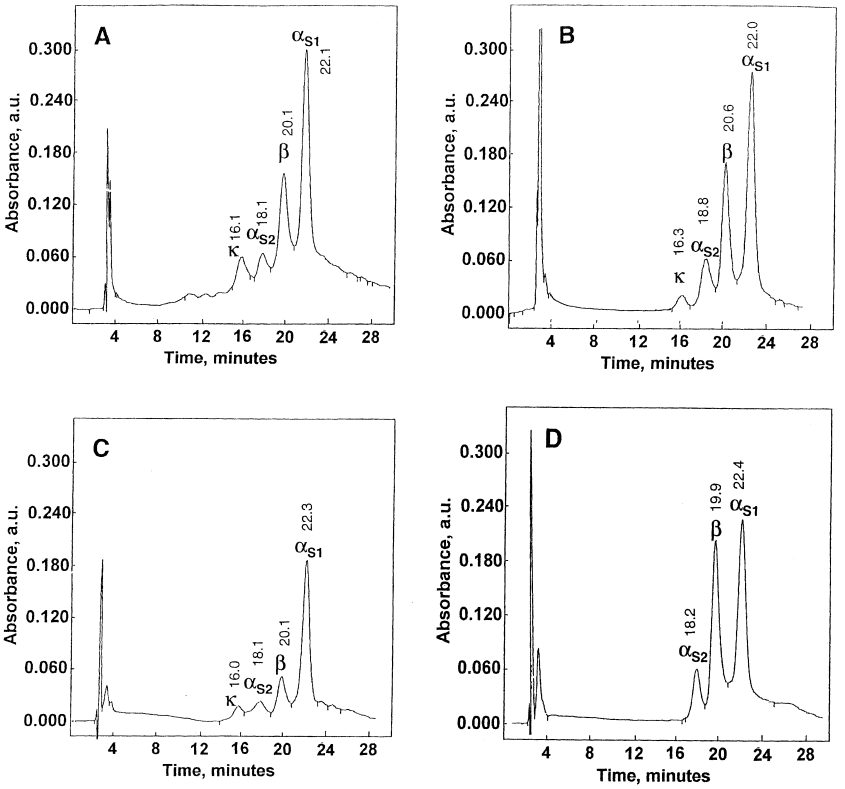

Fig. 6. HIC chromatograms of cheese samples dissolved in 4.0 *M* GdmSCN and centrifuged. (a) Robiola cheese (retention times: $t_{\alpha s1}=22.1$; $t_{\alpha s2}=18.1$; $t_{\beta}=20.1$; $t_{\kappa}=16.2$); (b) ewe's milk ricotta cheese (retention times: $t_{\alpha s1}=22.0$; $t_{\alpha s2}=18.8$; $t_{\beta}=20.6$; $t_{\kappa}=16.3$); (c) cow's milk mozzarella cheese (retention times: $t_{\alpha s1}=22.3$; $t_{\alpha s2}=18.1$; $t_{\beta}=20.1$; $t_{\kappa}=16.0$); (d) buffalo's milk mozzarella cheese (retention times $t_{\alpha s1}=22.4$; $t_{\alpha s2}=18.2$; $t_{\beta}=19.9$). Reprinted with permission from Ref. [64].

stationary phases like hexylepoxy Sepharose or phenolic formaldehyde resins.

Recent applications of HIC concern the isolation and analysis of κ-casein glycomacropeptide (GMP) from goat sweet whey [68] and the chacterization of the haze-active protein in apple juice [69]. Analyzing sweet whey, a by-product of cheese manufacturing, unreported data on the composition of goat GMP were obtained [68]. The goat glycomacropeptide prepared using a two-step procedure with AEC and HIC on phenyl-agarose resulted to be of high purity. In addition, valuable data on the concentration of sugars, such as sialic acid and galactose, in the GMP fraction were discussed. Similarly, a procedure involving HIC on phenyl-Sepharose and SPE proved useful in fractionating a potential haze-active protein in apple juice [69].

2.2. Separation and purification by perfusion high-performance liquid chromatography

In the last 10 years new types of support materials with improved mass transfer properties have been developed for the separation of proteins [70,71]. In particular, the group of Afeyan introduced perfusion chromatography as a powerful technique for the fast separation of biopolymers [70]. In perfusion chromatography macroporous polymers based on synthetic polymers are used in order to increase particle permeability by providing large pores for transport

connected to short diffusive pores. Typical perfusive packings have ''large-pores'' with a diameter of 6000–8000 Å and small diffusive pores of diameter of 800–1500 Å providing a large adsorption area and allowing proteins to enter more readily into the diffusive pores. Thanks to increased diffusivity by intraparticle convection, perfusion chromatography allows better column efficiency and higher separation speed than that achieved with conventional packings [72,73]. Very recently, the group of Rodrigues have characterized mass transfer mechanisms inside permeable media such as POROS HQ/M (PerSeptive Biosystems) particles [74]. For this purpose, elution chromatography experiments under non-retained conditions to avoid extra effects due to protein adsorption and frontal chromatography experiments in retained conditions of two proteins myoglobin and bovine serum albumin were performed. Elution chromatography studies show that the mass transfer resistance in POROS HQ/M is reduced by intraparticle convection. Three mathematical models, accounting for adsorption of proteins on the surface as well as inside microparticles of POROS pellets and intraparticle convection in the network of through-pores connecting the particles, were developed in this investigation.

2.2.1. Chromatography of proteins

Recent applications of perfusion chromatography for the separation of food proteins have been described by Garcia and co-workers [75,76] and by the group of Savary [77–79]. With the aim to determining soybean proteins in commercial soybean products obtained directly from whole soybeans, perfusion RPLC was applied [75]. Separation was achieved on a Poros R2/H (PerSeptive Biosystems) perfusion column packed with crosslinked polystyrene–divinylbenzene beads using a linear binary gradient acetonitrile–water (both with 0.1% trifluoroacetic acid) in 3 min at a flow-rate of 3 ml/min, operating at a temperature of 60 °C. Chromatographic method was able to separate up to eight proteins in less than 3 min, as illustrated in Fig. 7. Using soybean flour as external standard for quantitation purposes, the method was validated in terms of detection limits, precision and accuracy, and applied to the analysis of soybean liquid and powdered milks and textured soybean. Detection up to 5–7 mg of soybean proteins/ml of soybean liquid milk, 25–50 mg of soybean proteins/g of powdered milk, and 50 mg of soybean proteins/g of textured soybean was proved. Further, good precision and accuracy was demonstrated, making the method developed a suitable alternative to the laborious Kjeldhal method usually applied for this purpose.

Previously optimized methods of conventional chromatography [80] and perfusion chromatography [81] were applied and compared by the same group for the characterization of a number of commercial soybean products (soybean protein isolate, soybean flour, textured soybean, liquid and powdered soybean milks, and soybean infant formulas) [76]. Data obtained analyzing protein profiles by both chromatographic methods were processed using multivariate methods such as principal components and discriminant analysis. They concluded that a more rapid separation of high efficiency can be obtained by using perfusive materials than conventional ones.

Perfusion chromatography has been applied for purification purposes [77,78] and more recently for characterization of proteins [79]. A Poros HS (a strong cation-exchanger) column was used to separate the fruit-specific pectin methylesterase isoform from other enzymes present in commercial tomato enzyme preparation [77]. The method was found simple and fast, being capable of providing monocomponent enzymes useful for modifying pectin structure and functional properties. The same group devised an improved method for purification of hexose oxidase (HOx), an enzyme with interesting food and biotechnological potential, from the marine red alga *Chondrus crispus* [78]. After preliminary treatments, perfusion chromatography using different Poros packings (Poros DEAE-50, Poros HP2 and Poros HQ) for sequential purification steps allowed to achieve fast separations of high resolution. Characterization of the purified enzyme consisted of determination of enzyme activity and of the native size by SEC. The final goal of the development of novel enzyme technologies was their use in bioconversion of low-value sugars, obtained as by-products from food processing, into value-added substances. In the case of Hox, the enzyme could be utilized for converting lactose into lactobionic acid. Hydrophobic-interaction perfusion chromatography has been

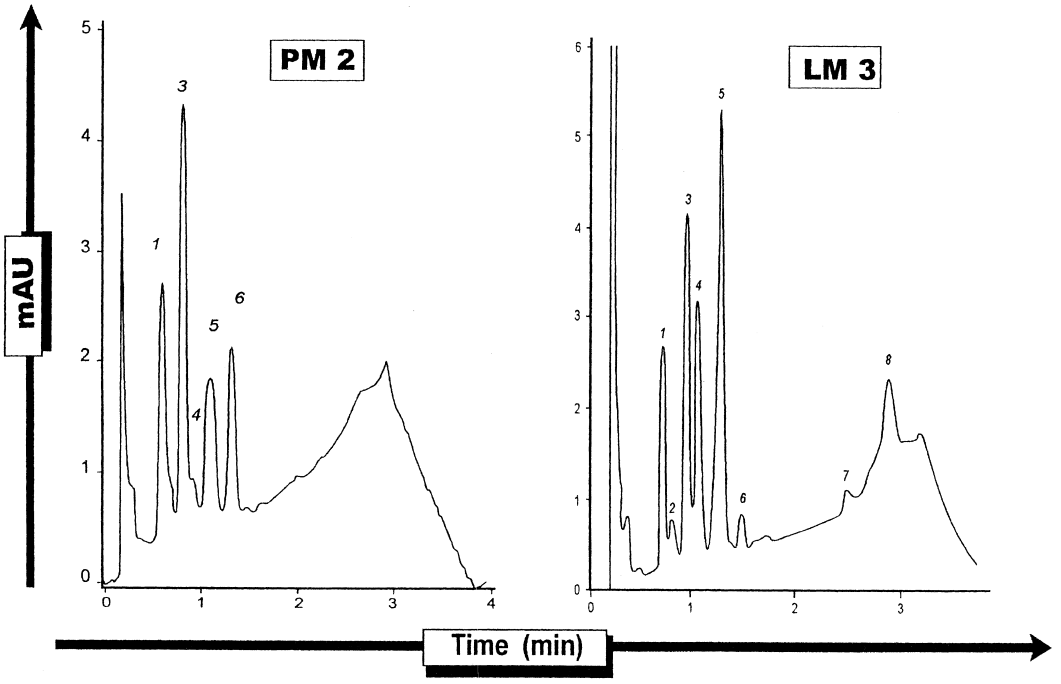

Fig. 7. Perfusion RP-HPLC separation of aqueous solutions (as dry basis) of a powdered milk (PM 2) (1.20 mg/ml) and a liquid milk (LM 3) (9.97 mg/ml). Reprinted with permission from Ref. [75].

also applied by Savary et al. to characterize the pectin methylesterase (PME) purified from Valencia orange peel in order to establish its identity among the other Valencia orange PME isozymes [79].

3. Mass spectrometry

3.1. Advances in mass spectrometry of peptides and proteins

MS techniques now play a pivotal role in the analysis of food peptides and proteins, as illustrated in recent overviews [13–19]. The revolution realized by electrospray ionization mass spectrometry and MALDI-MS in the analysis of biopolymeric materials is also highlighted in the recent book of Mellon et al. [82], since ESI-MS has been successfully used to examine macromolecular polar compounds such as proteins and MALDI-MS techniques have been demonstrated highly effective for analyzing complex protein mixtures in various food samples as well.

3.1.1. Matrix-assisted laser desorption ionization mass spectrometry

In the last years the literature has reported many applications of MS-based techniques in food peptide and protein analysis, selected examples of which will be presented and discussed in this overview. Additional information on this subject can be obtained from Ref. [18].

An important fundamental study aimed at evaluating the effect of various buffers and surfactants commonly used in MEKC on MALDI-TOF-MS detection of peptides has been performed by the group of Regnier [83]. The type and the concentration of buffers resulted to affect signal-to-noise ratio without influencing mass accuracy. In the case of organic additives, such as anionic surfactants, non-ionic surfactants and cyclodextrins, their ionization was found buffer dependent and capable to hinder MALDI-MS of peptides when the mass of the additive was in the range of the peptide mass. Among buffer tested, the ammonium ion resulted a poor cationizating agent in contrast with sodium and potassium ions. Consequently, MALDI spectra of

peptides were effected by ionization of neutral surfactants in sodium and potassium containing buffers. As shown in Table 3, the effect of buffer and additive concentration on the production of mass spectra was different depending on the salt. Ammonium acetate, glycine and guanidine could be used at concentrations ranging up to 500 m*M* without affecting ionization of a peptide having a molecular mass of 1059, whereas sodium phosphate, sodium borate and 2-(*N*-morpholine)ethane sulphonic acid (MES) had to be used at less than 100 m*M*.

Recent applications of MALDI-TOF-MS concern characterization of the nonenzymatic reactions of food proteins with selected phenolic compounds as secondary plant metabolites [84], identification of sites of phosphorylation in the caseinomacropeptide sequence [85], phosphopeptide sequencing by tandem MS [86], evaluation of allergenicity of novel food proteins [87], determination and localization of enzymatically modified glutamine residues in a glutamine-rich protein [88], study of high-pressure effects on protein expression in microorganisms of food concern [89]. Other applications have been previously discussed [33,42].

The capability of MALDI-TOF tandem mass spectrometer to sequence a series of phosphopeptides which resulted from gel in situ digestion of phosphoproteins has been successfully evaluated by Bennet et al. [86]. The technique proved helpful in localizing up to four phosphorylated amino acid residues in phosphopeptides up to 3.1 kDa. Further, the abundant neutral loss of phosphoric acid (−98 Da) resulted to be advantageous for detection of phosphoserine-containing peptides at femtomole level. High mass resolution and mass accuracy were the main features of the method proposed.

A combined MALDI-TOF-MS and nanoESI-MS/MS approach has been proposed for determination of the extent of modification of a glutamine-rich protein (dB1) and for localization of modified glutamine residues at the amino acid level [88]. Usefulness of this method was the feasibility to monitor the modification reaction time at the amino-acid sequence level, as illustrated in Fig. 8.

The knowledge of the effect of stress to bacteria due to environmental changes such as temperature, high hydrostatic pressure or pH shift is of interest for food industry because stable starter cultures are

Table 3
Effect of the salt concentration on the mass determination by MALDI-TOF-MS[a,b] [83]

Buffer (salt)	Concentration (m*M*)									
	20	50	100	150	200	250	300	350	400	500
Ammonium acetate	+	+	+	+	+	+	+	+	+	+
Guanidine	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+
TRIS	+	+	+	+	+	+	+	+	−	−
Ammonium bicarbonate	+	+	+	+	+	+	−	−	−	−
Imidazole	+	+	+	+	+	+	−	−	−	−
EPPS	+	+	+	+	+	+	−	−	−	−
Sodium acetate	+	+	+	+	+	−	−	−	−	−
CAPS	+	+	+	+	+	−	−	−	−	−
Sodium carbonate	+	+	+	+	+	−	−	−	−	−
Sodium citrate	+	+	+	+	−	−	−	−	−	−
HEPES	+	+	+	−	−	−	−	−	−	−
Sodium phosphate	+	+	+	−	−	−	−	−	−	−
Sodium borate	+	+	+	−	−	−	−	−	−	−
MES	+	+	+	−	−	−	−	−	−	−
ADA	+	+	−	−	−	−	−	−	−	−

[a] Operating conditions: the salts were dissolved in deionized water at different concentrations. All spectra were obtained using the peptide H-TRNLADQED-NH_2 ($M_w = 1059$).

[b] The mass of the peptide could be determined. Percent deviation from the real mass [(theoretical mass − observed mass)/theoretical mass] was found to be 0.01–0.06%.

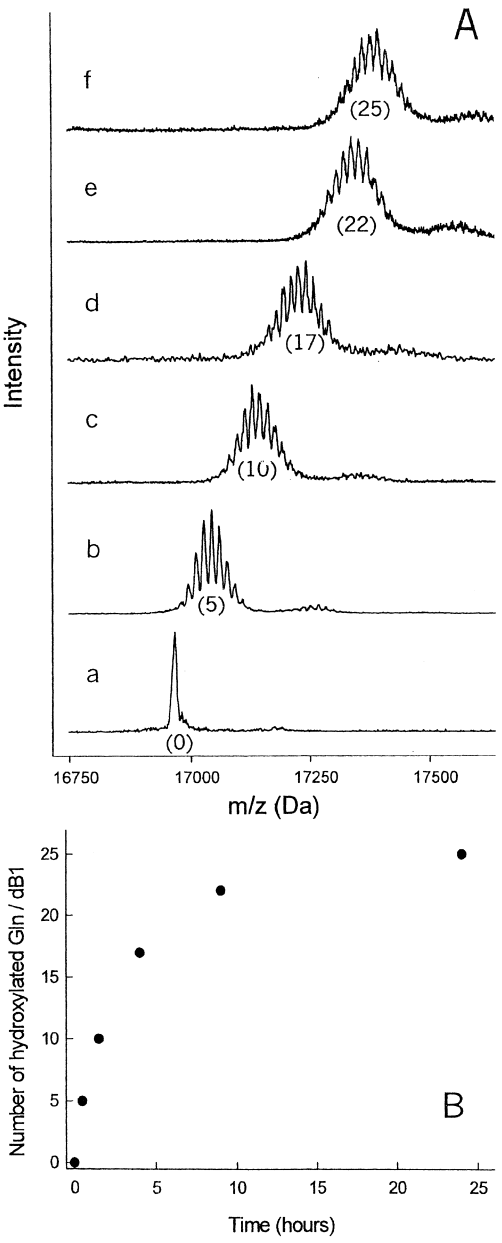

Fig. 8. MALDI-MS spectra of dB1 as a function of incubation time. (A) (a) $T=0$ min, (b) $T=30$ min, (c) $T=90$ min, (d) $T=4$ h, (e) $T=9$ h and (f) $T=24$ h. The number of hydroxylated Gln residues giving the highest intensity mass peak (weighed average number of hydroxylations) is noted within parentheses. (B) the average number of hydroxylated Gln per dB1 molecule, as a function of incubation time, is shown. Reprinted with permission from Ref. [88].

demanding for food preservation. A recent investigation focused on protein expression when *Lactobacillus sanfranciscensis* microorganisms were submitted to high pressure steps [89]. For this purpose, an approach based on 2D-electrophoresis, peptide mass fingerprinting by MALDI-TOF-MS and peptide fragmentation by LC-Q-TOF–MS/MS was successfully applied. Five pressure-affected proteins were identified by LC–MS/MS as cold shock proteins of *Lactococcus lactis*, which are known to participate in carbohydrate and purine metabolism. This study demonstrated the presence of some proteins which are maximally induced or repressed at different pressures or modified by pressure stimulation.

3.1.2. Electrospray ionization mass spectrometry

Several researchers have used ESI-MS systems coupled with a high resolution separation technique such as LC [90–97]. Coupled techniques are known to extend the automation of various procedures providing additional information for the structural elucidation of peptides and proteins in complex matrices as foods. Further, the use of ESI-MS with quadrupole or time-of-flight analyzers or with tandem MS systems proved to provide valuable information for the analysis of food peptides and proteins in many recent studies [18,52,99,100].

ESI-MS techniques are recognized as having high potential for mass determination of food proteins, detection of modifications in proteins caused by insertion, deletion or modification of amino acids, identification of genetic variants and post-translational modifications like deamidation, oxidation, glycosylation, phosphorylation, sulfation [13,14,18]. In this context, a comprehensive rapid and sensitive LC-ESI-MS method has been devised by the group of Ferranti to evaluate the gliadin and glutenin components in different wheat varieties [90]. Accurate mass measurements of the two gluten protein components and detection of more than 40 species for each fraction revealed high gluten heterogeneity in different wheat varieties. As illustrated in Table 4, the difference in the molecular masses of two components of the gliadin fraction from the wheat varieties examined ranged from a few mass units indicative of amino acid substitutions up to about 1000 mass units corresponding to peptide insertion

Table 4
Measured masses of components A and B of the gliadin fraction from the seven wheat varieties examined [90]

Wheat variety	Component A measured mass[a] (Da: mean±SD)[b]	Component B measured mass[a] (Da: mean±SD)[b]
Adamello	38822.9±1.2	34633.0±1.4
Ofamo	38822.4±1.3	34632.6±1.1
Creso	38826.0±1.7	34634.6±1.2
Bronte	38827.4±1.9	34656.8±3.2
Trinakria	38830.7±1.9	35547.5±1.4
Nodura	38827.3±2.4	34633.7±2.8
Messapia	38826.1±2.3	33539.7±2.2

[a] Average molecular mass calculated by integrating the multiple peaks corresponding to each molecular species and differing only in the total number of charges measured by ESI-MS.
[b] SD = standard deviation.

or deletion. Findings suggested the use of these gliadin components as markers to detect traces of wheat in gluten-free foods for celiac patients.

LC-ESI-MS in reversed-phase mode and flow-injection (FIA)-ESI-MS have been applied for the characterization of ovine [91] and caprine milk proteins [92]. Usefulness of these ESI-MS-based approaches relies particularly in evidencing the great heterogeneity of ovine and caprine milk proteins due to post-translational modifications, and to the presence of genetic variants and of multiple forms of proteins. Similar results were also reported by other research group, which developed an analytical method based on the separation by RPLC and ESI-MS detection to study the protein composition of commercial whey samples [93]. Valuable information on the modifications contained in industrial whey proteins allowed to better understand the influence of industrial processing on protein modifications.

The growing interest for the identification of genetic variants and post-translational modifications using ESI-MS analysis is attested by a recent fundamental study [94]. In this work the authors devised and validated a protocol based on the use of LC with ESI-MS detection for determination of specific phosphorylation sites in proteins. As illustrated in the scheme (Fig. 9), the degree of phosphorylation was measured from the ratio of the phosphopeptide peak area to the peak area of the unmodified peptide used as reference. Even though the usefulness of the method was demonstrated on proteins of biological concern, it was stated that it would be applicable to study a variety of posttranslational modifications as well as to directly quantify site-specific phosphorylation in proteins in different physiological state.

LC-ESI-MS and MS/MS techniques have been successfully used for peptide mass fingerprinting purposes by Kochhar and coworkers [95,96]. LC–MS, SDS–PAGE and NH(2)-terminal sequence analysis of purified albumin from Theobroma cacao seeds indicated apparent homogeneity for the protein [95]. LC-ESI-MS showed that the tryptic hydrolyzate of the purified protein contains sixteen peptides (Fig. 10) having masses with close agreement with the expected tryptic peptides calculated from the amino acid sequence available from the cDNA of the 21 kDa cocoa albumin. MS/MS analysis of the C-terminal peptide isolated from the cyanogen bromide cleavage products provided unambiguous evidence that the mature cocoa albumin protein is nine amino acid residues shorter than expected from the reported cDNA of its corresponding gene due to post-translational modifications. The analysis by LC–MS of fermented cocoa extracts has been the subject of another study of the same Research Center in cooperation with other scientists [96]. Combining the information by SDS–PAGE and GPC-ESI-MS performed on an ion trap mass spectrometer it was found that changes in the amount and composition of the major proteins occurred during fermentation. In addition, production of a large number of peptides was evidenced. The MS/MS analysis revealed the presence of two related peptides, a hexapeptide and a nonapeptide, formed from vicilin, one of the cocoa storage protein.

There has been increased interest in understanding

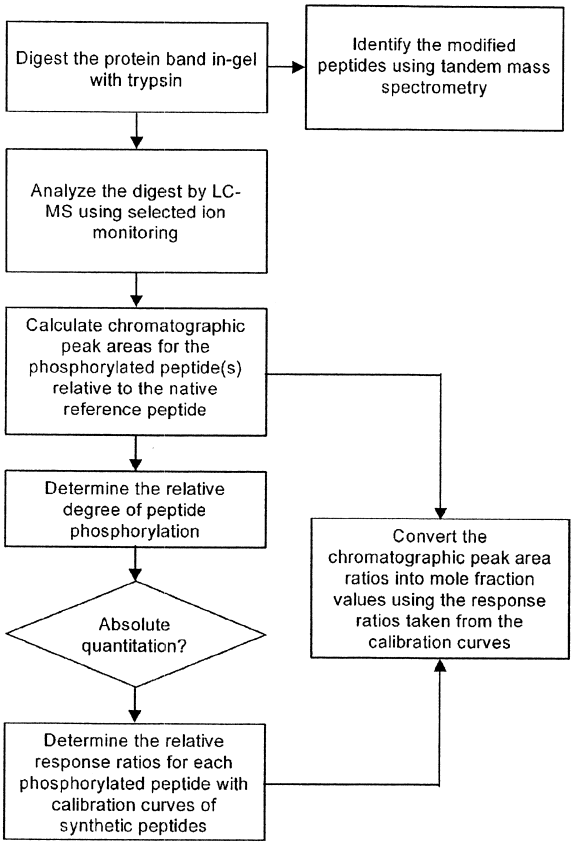

Fig. 9. Schematic diagram of the native reference peptide method. Initially, the protein of interest is digested by trypsin and the digest characterized by tandem mass spectrometry to determine the identity of the peptides in the digest, including modified and unmodified peptides. These data are used to develop a list of analyte peptides and a native reference peptide to be used for quantitation. In subsequent experiments, LC/MS analysis with selective ion monitoring is used to record the chromatographic peak area of the peptides of interest. In general, a minimum of three peptides would be monitored-the phosphopeptide, a control peptide, and the native reference peptide. The peak area ratios of the analyte peptides, relative to native reference peptide, provide direct albeit unitless, quantitation of the amount of the analytes in a given sample. Calibration, using parallel analyses of synthetic peptide standards, is used to convert the peak area ratios into mole percent ratios. Reprinted with permission from Ref. [94].

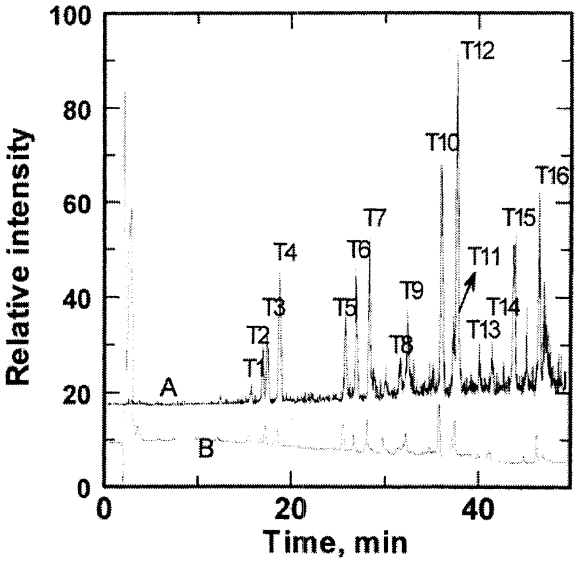

Fig. 10. Tryptic peptide mass fingerprint of the cocoa albumin trypsin inhibitor CoATi. RP-HPLC/ESI-MS measurements were performed using a FinniganMat LCQ mass spectrometer interfaced with a Spectra HPLC system (FinniganMat). Conditions: RP C_{18} column [Nucleosil 100-3 C_{18} HD (3 μm, 2×150 mm), Macherey–Nagel] with a linear increase of solvent B [0.05% TFA/80% ACN (v/v) in water] in solvent A [0.045% TFA (v/v) in water] as follows: 0–60% B in 60 min, 60–100% B in 20 min, followed by an isocratic elution at 100% B for 5 min. The flow-rate was 0.2 ml/min and UV detection was at 215 nm. A, TIC trace; B, UV trace. Modified from Ref. [95].

the structure–function relationships of a protein to explain certain characteristics of a food product; this requires elucidation of the molecular characteristics of the proteins. To date the use of MS for characterizing complexes between proteins from milk has been somewhat limited. The applicability of on-line LC-ESI-MS to the study of covalent interactions between casein micelles and β-lactoglobulin from goat milk has been recently explored by Henry et al. [97]. For this purpose the authors developed a multi-step approach comprising heat treatment of a mixture of purified β-lactoglobulin and casein micelles, ultracentrifugation, chromatographic analysis and fractionation, sequential enzyme digestion of disulphide-linked oligomers, and identification of heat-induced disulfide bond(s) by LC-ESI-MS and MS/MS. Three types of disuphide bonds involving one molecule of β-lactoglobulin and one molecule of κ-casein, two molecules of κ-casein, and two molecules of β-lactoglobulin were identified. The occurrence of a covalent linkage between β-lactoglobulin and κ-casein could account for the low heat stability of goat milk.

The allergenicity of food proteins is well docu-

mented and is a hazard that must be guarded against when introducing any novel protein into the food chain. The analytical problem of food allergen isolation has been well documented in a recent review paper of Pastorello and Trambaioli, in which the authors underline the importance of allergen purification from natural sources as foods for subsequent molecular characterization and allergological studies [98]. In a study aimed at characterizing maize major allergens and cross-reactivity with other cereals, using ESI-MS Pastorello et al. identified the major allergen as a protein with a molecular mass of 9047.0 Da [99]. The 9 kDa allergen was recognized to be a lipid transfer protein (LTP) having high homology with the peach LTP, the major allergen of the Prunoideae subfamily. In a subsequent work, the same research group recently determined the primary structure of apricot LTP by micro-sequencing and ESI-MS analyses, demonstrating that the major allergen of apricot is a protein of molecular mass (M_r) 9000 [100]. This allergen resulted in a 91 amino acid protein with a calculated M_r of 9170.6 having 91 and 94% sequence omology with peach and almond LTPs. As shown in Fig. 11, ESI-MS mass spectrum of purified apricot sample evidenced also the presence of a minor allergen having M_r of 7238.0. Amino acid sequencing of the M_r 7000 apricot LTP resulted in a protein of 68 amino acids with a molecular mass of 7237.5, which is in good agreement with that calculated by the ESI-MS technique.

Evaluation of allergenicity of a novel food protein and its glycoconjugates has been addressed through the assessment of sequence similarity to known

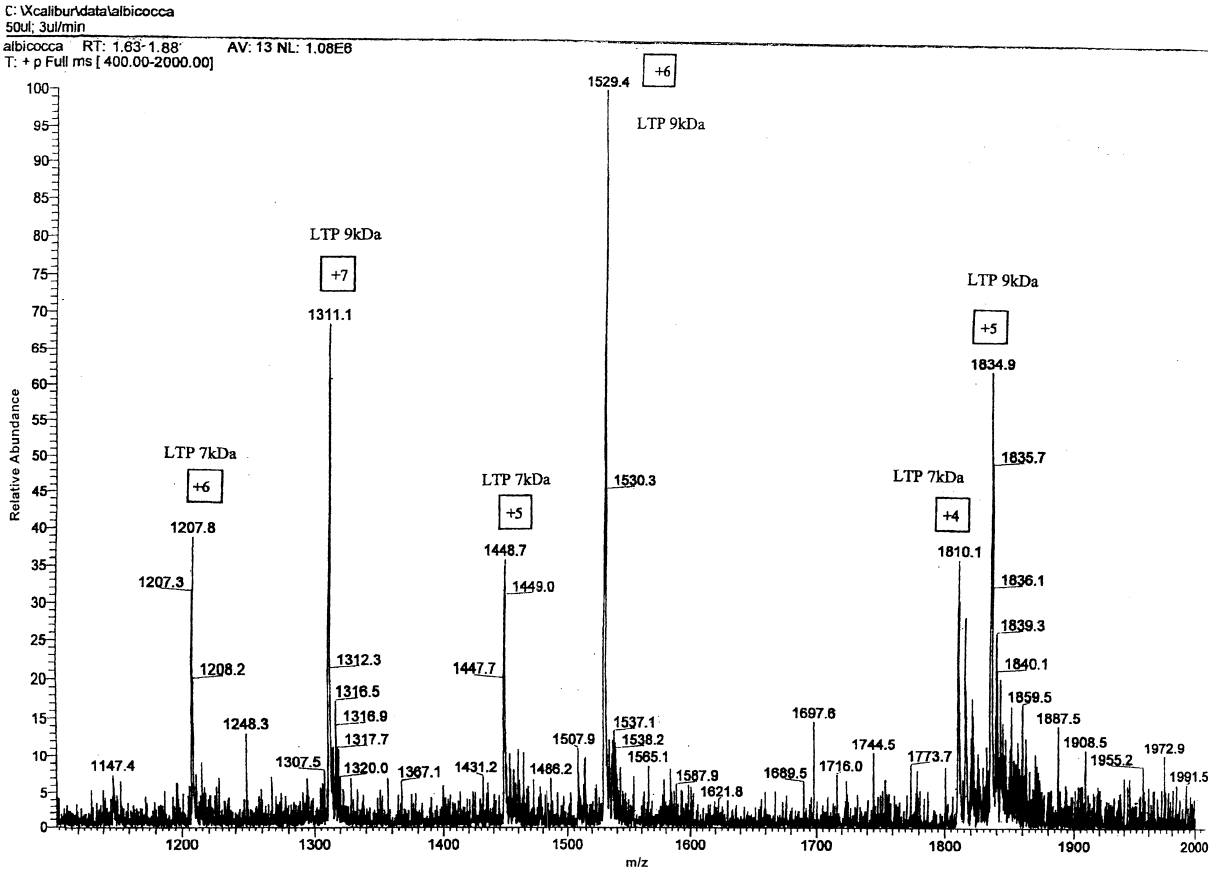

Fig. 11. Electrospray mass spectroscopic analysis of purified apricot sample. Molecular masses of the two components were deduced from the m/z ratio of multiply charged species of each protein. Reprinted with permission from Ref. [100].

allergens and the assessment of the pepsin resistance [87]. Protein under investigation was the ice structuring protein type III HPLC 12 (ISP type III) belonging to the anti-freeze proteins, which have been found in a wide variety of organisms in nature that need to protect themselves against freezing damage [101]. Intake by human populations of fish containing these proteins demands assessment of their safety in foods. Results of the analysis of ISP type III and ISP type III glycoconjugates by sequence analysis together with lack of resistance to pepsin hydrolysis allowed to conclude that both are unlikely to present a potential risk. MALDI-TOF-MS was effective in confirming the identity of the proteins contained within the primary test material separated by gel filtration chromatography. This material was a freeze-dried fermentation product containing 33.3% (w/w) ISP type III protein, 24% (w/w) ISP type III glycoconjugates and 43 (w/w) other proteins. MALDI-TOF-MS and LC–MS were also applied to qualitatively and quantitatively analyze glycoconjugates, since the degree of glycosylation is another structural information of relevance for assessment of protein allergenicity.

Other recent investigations in which MS-based techniques were successfully applied concern identification of allergens in raw and roasted hazelnuts [102], identification of the major allergens in the edible nests of Collocalia spp (''Bird's nests'') [103], and the characterization of one of the most frequent IgE defined food allergens, fish parvalbumin [104].

The above discussed studies prove the potential of MALDI-TOF-MS, ESI-MS and ESI-MS/MS techniques in the research of peptides and proteins of food concern as a powerful tools for mass determination purposes and for detection of protein modifications induced by processing.

3.1.3. Emergent MS-based techniques for proteomics

Emergent approaches nowadays are aimed at developing automated multi-dimensional systems that comprise liquid-phase based protein separation, protein digestion and MS identification system [105–107]. A microanalytical platform for automated sample treatment, reduced sample loss and minimized sample consumption has been recently presented by Jiang and Lee [105]. High-performance membrane chromatography was miniaturized by sandwiching a poly(vinylidene fluoride) membrane between two poly(dimethylsiloxane) substrates and then on-line coupled with a micro-enzyme reactor to perform fast protein digestion and with ESI-MS for protein identification. High throughput, improved detection sensitivity and dynamic range for the analysis of proteins in complex mixtures were highlighted. Similar approaches were addressed by other research groups [106,107]. Strategy proposed by Riggs and coworkers involves the automation of multidimensional chromatographic techniques using an immobilized enzyme column, transfer of the digest to an affinity chromatography column and to a RPLC column for further fractionation [106]. Effluent from the RPLC column is directed to an ESI-TOF mass spectrometer with an analysis time lower than 2 h for the entire process. Coupling of LC with high-resolution ion mobility/time-of-flight mass spectrometry (IMS) has been reported for the analysis of complex protein mixtures [107]. In this approach, peptides separated by RPLC are electrosprayed into an ion mobility/TOF mass spectrometer in which ions are separated on the basis of differences in mobility through a buffer gas and further separated according to their mass-to-charge (m/z) ratios in the mass spectrometer. Feasibility of this strategy relies in the large differences in timescales of the chromatographic separation (min), ion mobility (ms) and time-of-flight (μs) techniques. Bovine albumin was one of the five proteins tested to illustrate this combined approach.

In the context of multidimensional liquid phase-based separation techniques other promising approaches describe micro- and nano- LC in conjunction with ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) [108–110]. Using microcapillary LC columns on-line coupled with nano-ESIFTICR-MS, peptide sequence analysis at subfemtomole level was achieved [108]. Further, MS/MS analysis provided accurate precursor and product ion mass information which allows to identify primary amino acid sequences differing by Δm as low as 0.036 Da. High-throughput proteome analysis was the goal of a study dealing with the design and application of multiple-capillary LC system integrated with ESI-FTICR-MS [109,110]. System comprises a number of serially connected

dual-capillary columns and ESI sources which were operated independently. In a first study the separation efficiency of the dual-capillary column approach, optimal chromatographic parameters (capillary dimensions, eluent compositions) and their compatibility with ESI-MS were evaluated [109]. They found that this high-throughput technique was capable to detecting more than 100 000 polypeptides in yeast cytosolic tryptic digests. On the other hand, the high mass accuracy of FTICR spectrometer allowed to characterize approximately 1000 proteins from a single capillary LC-FTICR analysis. The same research group proposed a multiplexed-MS/MS approach on-line coupled with capillary LC for the characterization of peptides in complex mixtures such as those in whole cell lysate digests [110]. This technique was successfully demonstrated using a bovine serum albumin tryptic digest as a model sample in which several peptides were separated by capillary LC and identified in single tandem FTICR-MS acquisitions. The major advantage of these novel strategies is its high throughput proteomics feature.

Palmblad et al. [111] introduced a new approach to the characterization of peptides in enzymatic digests of proteins to identify post-translational modifications, based on the coupling of LC with FTICR-MS using the recently introduced fragmentation method electron capture dissociation (ECD) [112]. Method developed was applied to the analysis of a standard peptide mixture and to a bovine serum albumin tryptic digest. This application proved an interesting alternative approach to the LC-ESI-FTICR-MS technique in proteomics research.

Zhou et al. examined the usefulness of a LC/dual-ESI-MS system in studies of protein identification [113]. High accuracy of peptide mass measurement was claimed, since the dual-ESI-sprayer system consisted of one sprayer for sample introduction and a second sprayer for reference introduction. Internal calibration ensured improved accuracy with a range of mass errors between 0.16 and 5.37 ppm, thus making the method very reliable for protein identification.

Promising approaches in proteome research have been recently reported by different groups [114–116]. Vissers et al. developed a new variable flow interface for nanoscale LC coupling to ESI-MS with the aim to identifying proteins by MS/MS [114]. A flow reduction and an interruption in the LC gradient during tandem MS analysis preserved the peptide separation on the analytical nanoscale LC column. The usefulness of this strategy was proved by the automated analysis of standard peptide mixtures and protein digests using variable flow and automated database searching. Another approach has included automation of nanoscale microcapillary LC–MS/MS system with a vented column, which allowed on-column enrichment and desalting [115]. Complex peptide mixture were separated with high efficiency both from a single RPLC–MS/MS experiment and in a multidimensional LC–MS/MS analysis where several strong cation-exchange chromatography fractions from a lysate were desalted, enriched and automately analyzed. A new fused-droplet electrospray (FD-ESI) source has been developed with the goal of generating peptide and protein ions from high-salt solutions [116]. FD-ESI-MS mass spectra were obtained by fusing sample aerosol with charged methanol droplets in a reaction chamber. Spectral patterns similar to those of conventional ESI-MS experiments were obtained with the advantage of extremely high salt tolerance respect to the traditional technique.

Very recently, the group of Russell has proposed a MALDI-IM-TOF-MS-based method as a promising alternative to LC–MS and CE–MS for the analysis of complex protein mixtures [117]. Low picomole amounts of single proteins and enzymatically digested protein mixtures were successfully analyzed without the use of laborious clean-up steps. Rapidity and sensitivity were major features of this approach.

The determination of post-translational modifications of proteins is a problem of analytical concern which has been recently addressed following different strategies [118–120]. The advantages of on-line coupling of capillary LC with inductively coupled plasma mass spectrometry (ICP-MS) for identification of post-translational modifications in proteins have been demonstrated by Wind and coworkers [118]. The developed method proved useful in determining both the phosphorylation degree in phosphoproteins and phosphopeptides containing cysteine and methionine residues. Simultaneous detection of phosphorous and sulphur was feasible with this approach. The sulphur signal was used to quantify both the phospho and the dephospho form,

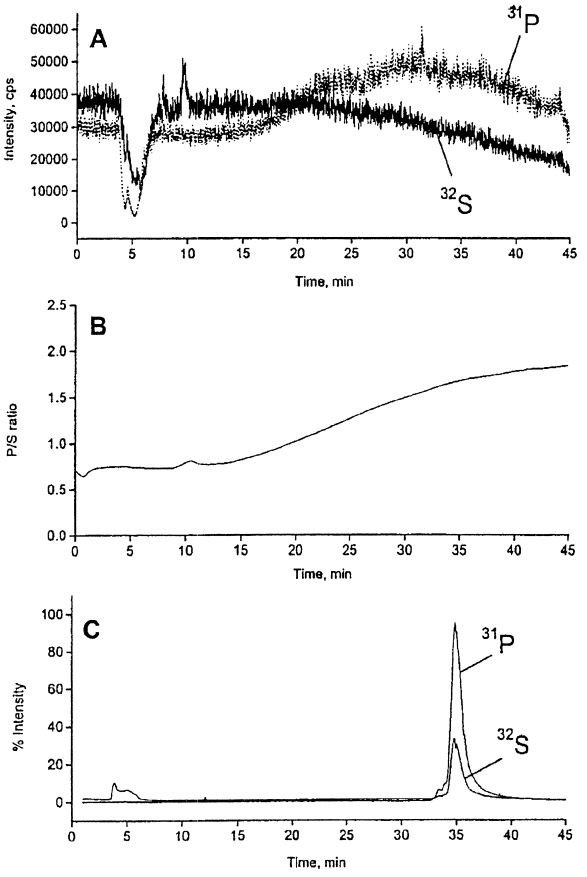

Fig. 12. Determination of the stoichiometric P/S ratio from μLC-ICPMS data and measurement of the P/S ratio in an intact phosphoprotein. (A) Gradient μLC-ICPMS run with spiked mobile phases, containing 10 μ*M* phosphate and 10 μ*M* cysteine each, pure water was injected; (B) uncorrected P/S signal intensity ratio of the experiment displayed in (A), smoothed; (C) α-casein analysis by a gradient μLC-ICPMS run. Reprinted with permission from Ref. [118].

whereas the phosphorous signal serves to determine selectively the phospho form. This investigation was successfully carried out on α-casein (eight phosphorylated sites), β-casein (five phosphorylated sites), three fractions of recombinant protein kinase A catalytic subunit containing two, three and four phosphorylated sites, and on synthetic phosphopeptides. As representative example, Fig. 12 illustrates α-casein analysis by μLC-ICP-MS in gradient mode.

The capability of a tandem quadrupole time-of-flight (Q-TOF) mass spectrometer to perform fast acquisition of mass spectra and product ion spectra has been exploited in a new precursor ion discovery approach aimed at identifying the site of phosphorylation in proteins [119]. This method was claimed compatible with on-line chromatographic separations of complex mixtures of proteins.

A recent promising technique for phosphoproteomics involves the use of a MALDI source coupled to an orthogonal injection quadrupole time-of-flight (QqTOF) mass spectrometer for the analysis of immobilized metal ion affinity chromatography (IMAC)-enriched peptides [120]. Direct affinity-bound peptide sequencing on IMAC beads applied to the MALDI target was feasible and univocal identification of the phosphorylation site was proved in a protein with unknown residues, even with peptides containing four potentially phosphorylated sites. This coupled method was claimed to overcome limitation of the ESI-MS/MS approach, which requires desalting prior to MS analysis, thus causing sample loss.

3.1.4. Pyrolysis-mass spectrometry

Pyrolysis-mass spectrometry (Py-MS) has been described as a novel, rapid and economical method for the screening and the quantitative analysis of complex bioprocess over producing recombinant proteins [121]. It consists in a quick decomposition of the sample at several hundred degrees Celsius followed by transfer of the produced gas to a mass spectrometer and analysis of the resulting fragments by MS. Py-MS analysis followed by multivariate analysis of the resulting mass spectra has great potential for characterization of food products and food ingredients. A recent overview describes the developments and applications of modifications involving high-temperature chemical reactions of macromolecules in Py-MS experiments [122]. However, it can be noticed that in spite of the usefulness of this technique in characterizing proteins, there are still very few recent applications of Py-MS in food analysis [123–125].

An approach based on the use of this tecnique for discrimination of pure milk samples from milk samples with added whey proteins [123] and for discrimination of ewe's cheese [124] has been taken up by the group of Anklam. Rapidity and versatility of Py-MS-based method was claimed for detection of whey proteins in milk [123]. On the other hand, in another study limitation of Py-MS in the differentia-

tion of two types of ewe's cheese was evidenced as a consequence of the high influence of fat and proteins on the fingerprinting of mass spectra [124].

Coupling of Py-MS with gas chromatographic separation (Py-GC–MS) has been demonstrated useful for the characterization of meal samples conventionally sold as feed supplements in the US [125]. Useful information on the mechanisms involved in the release of nitrogen species was obtained, Py-GC–MS data being dominated by compounds resulting from thermal peptide cleavage. Thermochemolysis of the same animal byproducts samples supported this finding.

4. Conclusions

It is expected that application of LC techniques for fast separation of peptides and proteins will facilitate their isolation both in analytical and preparative scales. In spite of its high analytical performance, progress in applying CEC for the separation of macromolecules is still slow.

The integrated approach based on multi-dimensional systems that comprise liquid-phase based protein separation, protein digestion and MS analysis of food peptide and protein will allow for accelerated implementation of knowledge in food design. In food technology it will reduce research and development time, thereby abbreviating time and reducing costs.

5. Nomenclature

AEC	anion-exchange chromatography
CE	capillary electrophoresis
CEC	capillary electrochromatography
ECD	electron capture dissociation
ESI	electrospray
FAB	fast atom bombardment
FD	fused droplet
FPLC	fast protein liquid chromatography
FIA	flow injection analysis
GFC	gel filtration chromatography
GPC	gel permeation chromatography
HIC	hydrophobic interaction chromatography
ICP	inductively coupled plasma
IEC	ion-exchange chromatography
IMAC	immobilized metal ion affinity chromatography
IMS	ion mobility mass spectrometry
MALDI	matrix assisted laser desorption ionization
MALLS	multi-angle laser light scattering
MEKC	micellar electrokinetic chromatography
MS/MS	tandem mass spectrometry
PyMS	pyrolysis-mass spectrometry
RAM	restricted access material
RP	reversed-phase
SDS–PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SPE	solid-phase extraction
SRM	selected reaction monitoring
TOF	time-of-flight

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