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Review article

Update on HPLC and FPLC analysis of nitrogen compounds in dairy products

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Summary — HPLC and FPLC methods for the analysis of proteins, peptides, amino acids and amines in dairy products, as well as their ability to resolve practical difficulties, are reviewed. The most pertinent literature is summarized in tables giving the chromatographic conditions used in the analysis and the main objectives of each reference.

HPLC / FPLC / dairy product / casein / whey protein / peptide / amino acid / nitrogen compound

Résumé — L'analyse HPLC et FPLC des composés azotés dans les produits laitiers (synthèse). *Cet article est une revue bibliographique concernant les méthodes HPLC et FPLC utilisées pour l'analyse des protéines, des peptides, des acides aminés et des amines dans les produits laitiers. Une analyse critique de l'intérêt de ces méthodes pour la résolution de problèmes pratiques a également été abordée. Les références citées dans l'article ont été résumées sous forme de tableaux donnant les conditions chromatographiques utilisées et les objectifs de chaque étude.*

HPLC / FPLC / produit laitier / caséine / lactosérum / peptide / acide aminé / matière azotée

INTRODUCTION

The nitrogen fraction in dairy products is of great interest in relation to nutritional, biological and technical aspects.

Fast, specific methods of analysis have been developed in response to high dairy production rates and the need for stringent analytical quality controls. Versatility, short analysis time, effective separation, high resolution, and adaptability to automatic procedures have made high performance liquid chromatography (HPLC) one of the basic techniques employed in the dairy sector.

Fast protein liquid chromatography (FPLC), a technique developed for the purification of proteins in their native state, has become an increasingly useful tool in the dairy sector. Since, FPLC only needs a relatively low backpressure to drive the high rates of flow at which the separations are performed, the risk of denaturation caused by shearing forces diminishes. Moreover, the mechanical components are resistent to corrosive buffer and there is no contamination or inactivation of the compounds of interest.

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The present paper reviews HPLC applications in the study of proteins, peptides, amino acids and amines, and FPLC applications in the study of proteins in dairy products. Their practical repercussions (detection of mixtures, study of proteolysis, and the like) are also considered.

Analysis of proteins

A number of techniques have been used to separate and analyze milk proteins: salt and organic solvent fractionation (McKenzie, 1971), starch gel electrophoresis (Aschaffenburg and Michalak, 1968), polyacrylamide gel electrophoresis (Andrews, 1981) isoelectric focussing (Trieu-Cuot and Gripon, 1981), as well as combinations thereof. Ion-exchange chromatography on DEAE cellulose and open-column gel permeation have been widely used, but the most significant advances have been achieved by applying high performance liquid chromatographic and fast protein liquid chromatographic techniques.

These techniques reduce analysis times from days or even weeks to just hours. HPLC and FPLC methods are also useful in isolating compounds present in small concentrations, and the separations carried out on a laboratory scale can be transferred to an industrial scale.

Practically all known mechanisms have been employed in the separation of proteins by chromatography, *ie*, separations based on molecule size (gel permeation chromatography), on charge (ion-exchange chromatography), on hydrophobicity (reversed phase chromatography and hydrophobic interaction chromatography), and even on combinations of these various mechanisms.

Table I summarizes the main papers in which HPLC and FPLC were applied to the study of proteins in milk products.

Caseins

Whole casein is an extremely complex mixture containing a number of fractions. Schlossberger (1846) separated casein into 2 fractions. The development of electrophoresis enabled Mellander (1939) to identify 3 components (α, β, and γ). Waugh and Von Hippel (1956) showed that the α fraction could be further separated into K-casein and the α_s fraction. Starch gel electrophoresis with urea was used by Wake and Baldwin (1961) to detect over 20 components in whole casein.

Various methods have been applied to casein analysis: polyacrylamide gel electrophoresis (PAGE) is undoubtedly the most common, but, in view of recent advances in HPLC and FPLC technology, these latter methods may be an alternative to PAGE in the study of caseins.

The molecular weights of the different casein fractions being quite similar, casein separation by gel permeation chromatography is unsatisfactory. Dimenna and Segall (1981) used 2 columns in series (a TSK-125 Toyo Soda 2 000 SW column and a MicroPak TSK 3 000 SW column) with molecular weight limits of 100 000 and larger than 350 000 Da, respectively. To separate the caseins from skim milk, elution was made with 0.05 mol•l^{-1} phosphate buffer (pH 6.8) containing 0.1 mol•l^{-1} sodium sulphate. α_{s1} and β caseins were eluted in a first peak, and the rest of the caseins along with BSA and I_g G were eluted in a second peak, while a third peak consisted mainly of β lactoglobulins along with contamination of α_{s1} and β caseins. Peak 4 contains only α-lactalbumin. According to the authors, separation of bovine skim milk proteins is highly dependent on sample size, a 10 µl *vs* 30 µl injection volume resulted in a better separation of casein from whey proteins and IgG from BSA. The results reported by Haasnoot *et al* (1987) on

caseins were not much better. They also employed 2 columns (a TSK 3 000 SW column and a TSK 2 000 SW column) in series with a 0.1 mol•l^{-1} Na_2SO_4 + 0.02 mol.l^{-1} NaH_2PO_4, pH 6.8 buffer and used 6 mol.l^{-1} urea to dissolve the caseins. Alpha$_{s1}$ and β-caseins and their breakdown products, *ie* α_{s1}-I and γ-caseins were eluted in the first peak; the second peak contains several components among which 2 could be γ_2 and γ_3 casein, which are breakdown products of β-casein. Both Haasnoot *et al* (1987) and Dimenna and Segall (1981) used PAGE for peak identification.

Better results can be achieved using ion-exchange chromatography. Since, the isoelectric points for the main milk proteins lie between 4.5 and 5.5, except for *para*-K-casein (pI = 9.4), they can be fractionated on anionic columns at a pH above 5.5 or on cationic columns at lower pH levels. Andrews *et al* (1985), using a Mono S CHR/5/5 cationic FPLC column with pH 3.2-3.8 buffers, separated caseins, but the results were somewhat less satisfactory than those obtained on anionic columns.

Both the TSK-DEAE-5PW and Mono Q HR 5/5 anionic columns (Humphrey and Newsome, 1984) yield better separations than those obtained on DEAE-cellulose (Mercier *et al,* 1968) with much shorter analysis time (1 h for TSK columns and 25 min for Mono Q HR 5/5 columns, compared to about 20 h for conventional DEAE-cellulose columns). In all cases the buffer was Tris HCl or imidazol at a pH of 7-8 and a concentration between 4.5 mol.l^{-1} and 8 mol.l^{-1} urea. The elution was obtained with a linear gradient of NaCl. Casein samples are normally dissolved in the initial solvent, after addition of dithioerythritol (DTT) or 2-mercaptoethanol (MCE) to achieve dissociation and to prevent casein aggregation.

Using these conditions, Andrews *et al* (1985) and Guillou *et al* (1987) separated 5 or 6 K-casein components in addition to the α_{s1}, α_{s2}, α_{s0} and β-caseins.

Using a Mono Q HR 5/5 column, Guillou *et al* (1987) succeeded in separating the β-C variant from the other variants of β-casein (A_1, A_2 and B) and K-casein variants A and B (fig 1). The method proposed by these authors is linear, repeatable and allows the quantification of bovine caseins.

Satisfactory casein separations have also been performed using reversed-phase chromatography (Barrefors *et al,* 1985; Visser *et al,* 1986; Mikkelsen *et al,* 1987).

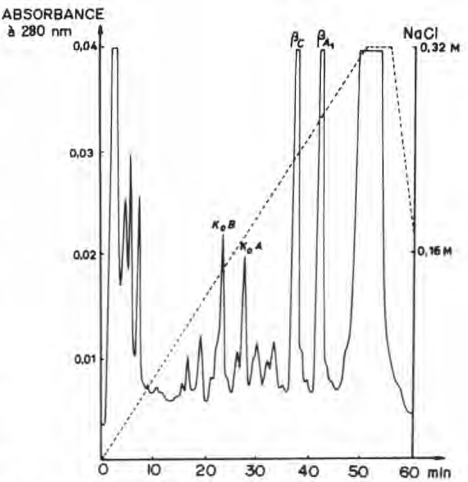

Fig 1. FPLC separation of caseins genetic variants on the Mono Q column. The sample of whole casein (k-A-k-B; β-C-β-A1; α_{s2}-A; α_{s1}-A) dissolved in a 5.10^{-3} mol•l^{-1} Tris-HCl; 4.5 mol•l^{-1} urea buffer pH 8.0 + dithiothreitol (8.10^{-4} mol•l^{-1}) was applied on the column and eluted at a flow rate of 1 ml/min using a 0–0.32 mol•l^{-1} NaCl gradient. Temperature: 40°C. [From Guillou *et al* (1986), *Lait* 67, 135 with permission].

Séparation par FPLC sur colonne Mono Q de la caséine entière d'une vache hétérozygote: k-A-k-B; β-C-β-A1; α_{s2}-A; α_{s1}-A. Débit 1 ml/min; température 40 °C. Tampon Tris-HCl 5.10^{-3} mol•l^{-1}; urée 4,5 mol•l^{-1}; dithiothréitol 6,4•10^{-5} mol•l^{-1}; pH 8.0. Elution par un gradient de NaCl de 0 à 0,32 mol•l^{-1}. Echantillon dans le tampon avec dithiothréitol 8•10^{-4} mol•l^{-1}.

Table I. HPLC application to the analysis of proteins in dairy products.
Application de la CLHP au dosage des protéines dans les produits laitiers.

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
Synchropac GPC-100 HP-RP-8	0.05 M-Tris pH 6.0, 0.2 M-NaCl a) 0.5 M-KH_2PO_4: 2-Methoxyethanol (98:2), pH 2 b) Isopropanol: 2-Methoxyethanol (98:2)	280 nm	Size exclusion reversed phase	Whey proteins BSA, α-la, β-lg	Separation of whey proteins	Diosady *et al* (1980)
TSK-125 + Micropak TSK 3000 SW	0.05 M-Phosphate buffer pH 6.8, 0.1 M-Na_2SO_4	280 nm	Size exclusion	Caseins and Whey proteins	Separation of milk proteins	Dimenna and Segall (1981)
Micropak TSK 3000 SW	0.04 M-TRIS HCl pH 7.6, 0.05 M-NaCl	220 nm	Size exclusion	Milk proteins	Separation of milk proteins	Bican and Blanc (1982)
Spherisorb S5 C6	a) 0.15 M-NaCl-HCl pH 2.1 b) CH_3CN	210 nm	Reversed phase	Whey proteins	Separation of whey proteins	Pearce (1983)
Toyo-Soda TSK 3000 SW	0.1 M-Phosphate buffer pH 6.8, 0.05 M-NaCl, 0.02% NaN_3 0.1 M-Phosphate buffer pH 6.8, 0.1% SDS	280 nm	Size exclusion	Proteins	Separation of native proteins Separation of denatured proteins	Gupta (1983)
TSK-DEAE-5 PW Mono Q HR 5/5	a) 0.02 M-TRIS-HCl, 4.5 M-urea, pH 8 b) Idem a) 0.5 M-NaCl a) Several buffer systems b) Idem a) + 0.5 M-NaCl	280 nm	Anion exchange	Caseins Whey proteins	Fractionation of caseins and whey proteins	Humphrey and Newsome (1984)

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
Micropak TSK 3000 SW	0.04 M-TRIS-HCl, 0.05 M-NaCl, pH 7.4	220 nm	Size exclusion	Whey proteins	Evaluation of heat treatment	Kneifel and Ulberth (1985)
Mono Q HR 5/5	a) H_2O b) 0.7 M-Sodium acetate, pH 6.3	280 nm	Anion exchange	Whey proteins	Evaluation of heat treatment	Manji and Kakuda (1986)
Mono S HR 5/5	0.05 M-Sodium formate pH 3.8, 8 M-Urea, 0.01 M-2-Mercapotethanol (0-0.03 M-NaCl gradient)	280 nm	Cation exchange	Caseins	Separation of milk proteins	Andrews *et al* (1985)
Mono Q HR 5/5	0.02 M-TRIS-HCl pH 7.0, 4.5 M-Urea 0.01 M-2-Mercaptoethanol (0-0.35 M-NaCl gradient)		Anion exchange	Caseins		
	0.02 M-TRIS-HCl pH 7.0 (0-0.35 M-NaCl gradient)			Whey proteins		
Superose 12 Hr 10/30	0.1 M-TRIS-HCl pH 7.0 0.5 M-NaCl, 0.01 M-NaN_3		Size exclusion	Whey proteins		
Mono Q HR 5/5	3.3 M-Urea, 0.01 M-Imidazole pH 7.0 (0-0.3 M-NaCl gradient)	280 nm	Anion exchange	Caseins	Analysis of different forms of K-Casein	Dalgleish (1985)
C8 HR 5/10	a) 0.1% TFA in 30% CH_3CN b) 0.1% TFA in 48% CH_3CN	280 nm	Reversed phase	Caseins	Separation of caseins	Barrefors *et al* (1985)

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
Mono Q Hr 5/5	0.01 M-Imidazole-HCl pH 7.0, 3.3 M-Urea 0.01 M-2-Mercaptoethanol (0-0.5 M-NaCl gradient)		Anion exchange	Caseins	Separation of caseins	Barrefors *et al* (1985)
Protein PAK DEAE-5 PW	0.02 M-TRIS-HCl, pH 7.2 (0-0.5 M-NaCl gradient)	280 nm	Anion exchange	Caseins and whey proteins	Separation of milk proteins	Bican (1985)
Bio-Gel HP HT	a) 0.005 M-KH_2PO_4, 6 M-Urea, 600 μM-$CaCl_2$ pH 6.8 b) 0.4 M-KH_2PO_4, 6 M-Urea, 7 μm-$CaCl_2$, pH 6.8	280 nm	Mixed mechanism	Caseins	Separation of milk proteins	Visser *et al* (1986)
Bio-Gel TSK-DEAE 5 PW	0.005 M-TRIS-HCl pH 8,2, 4 M-Urea, 0.03 M-NaCl (0.028-0.28 M-NaCl gradient)		Anion exchange			
Hi-Pore RP-318	a) 0.1% TFA in 10% CH_3CN b) 0.1% TFA in 90% CH_3CN		Reversed phase			
Bio-Gel TSK Phenyl RP	a) 0.1% TFA in 10% CH_3CN b) 0.1% TFA in 90% CH_3CN		Reversed phase			
Mono Q Hr 5/5	0.02 M-TRIS-HCl pH 8.5, 6 M-Urea (0-1 M-NaCl gradient)	280 nm	Anion exchange	Caseins	To detect mixtures of milk	Haasnoot *et al* (1986 a)

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
TSK 3000 SW + TSK 2000 SW	0.1 M-Na_2SO_4, 0.02 M-NaH_2PO_4, pH 6.8	280 nm	Size exclusion	Immunoglobulin G	Detection of colostrum in milk	Haasnoot *et al* (1986 b)
Mono S Hr 5/5	0.01 M-Imidazole-HCl pH 7.0, (0-1 M-NaCl gradient)	280 nm	Cation exchange	Lysozyme, lactoperoxidase, lactoferrin	Analysis of bactericides, proteins	Ekstrand and Björck (1986)
Phenil-superose Hr 5/5	a) 0.8 M-Sodium phosphate, 3.75 M-urea, pH 6.0 b) 0.05 M-sodium phosphte, 3.75 M-urea, pH 6.0	280 nm	Hydrophobic interaction	Caseins	Study of hydrophobic interaction	Chaplin (1986)
	a) 1.5 M-$(NH_4)_2SO_4$, 0.05 M-sodium phosphate, pH 7 b) 0.05 M-sodium phosphate, pH 7.0			Whey proteins		
Mono Q Hr 5/5	a) H_2O B) 0.7 M-sodium acetate, pH 6.3	280 nm	Anion exchange	Whey proteins	Separation of whey proteins	Hill *et al* (1987)
Superose 12	0.15 M-NaCl, 0.05 M-Phosphate, pH 7.0		Size exclusion			
PRO-RPC	a) 0.1% TFA in H_2O b) 0.1% TFA in 30% CH_3CN	215 nm	Reversed phase	Proteins, peptides	To determine the extent of proteolysis	Haasnoot *et al* (1987)
TSK 3000 SW + 2000 SW	0.1 M-Na_2SO_4, 0.02 M-NaH_2PO_4, pH 6.8, 6 M-urea	280 nm	Size exclusion			

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
Mono Q Hr 5/5	TRIS-HCl pH 8.5, 6 M-Urea (0-1 M-NaCl gradient)		Anion exchange	Proteins, peptides	To determine the extent of proteolysis	Haasnoot *et al* (1987)
Mono S Hr 5/5	Citric acid pH 3.2, 6 M-Urea (0-0.5 M-NaCl gradient)		Cation exchange			
Lichrosorb RP-8	a) 0.1% TFA in H_2O, 0.001 M-DTT b) 0.1% TFA in propan-2-ol	254 nm	Reversed phase	Caseins	Purification of caseins identification of α_{s1}-caseina	Mikkelsen *et al* (1987)
Mono Q HR 5/5	0.005 M-BIS-TRIS-Propane pH 7,3, 3 M-Urea (0-0.43 M-NaCl gradient)	280 nm	Anion exchange	Caseins	Quantitative fractionation of casein mixtures	Davies and Law (1987)
Vyadec 214 TP	a) 0.1% TFA in 30% CH_3CN b) 0.1% TFA in 45% CH_3CN	280, 210 230 250 nm	Reversed phase	Whey proteins	Determination of whey proteins and their functionality	Kim *et al* (1987)
Mono Q Hr 5/5	0.005 M-TRIS-HCl pH 8.0, 4.5 M-Urea 6.4×10^{-5} M-DTT (0.12–0.32 M-NaCl gradient)	280 nm	Anion exchange	Caseins	Quantitative analysis caseins	Guillou *et al* (1987)

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
TSK-G 2000 SW	0.1 M-KH_2PO_4, 0.01 M-K_2HPO_4, 0.15 M-Na_2SO_4	280 nm	Size exclusion	Whey proteins	To recover whey proteins	Murphy and Mulvihill (1988)
Zorbax GF-250	0.1 M-KH_2PO_4, 0.01 M-K_2HPO_4, 0.15 M-Na_2SO_4	280 nm	Size exclusion	Whey proteins	Evaluation of heat treatment	Van Den Bedem and Leenheer (1988)
Phenil-superose Hr 5/5	a) 0.05 M-TRIS-HCl pH 7.5, 1.27 M-$(NH_4)_2SO_4$ b) 0.05 M-TRIS-HCl pH 7.5 in 35% CH_3CN	280 nm	Hydrophobic interaction	Whey proteins	Evaluation of heat treatment	Dumay and Cheftel (1989)
Mono Q Hr 5/5	0.02 M-Piperazine, pH 6.0 (0.02-1 M-NaCl gradient)		Anion exchange			
Superose 12	0.6 M-Potasium phosphate pH 6.0, 0.15 M-Na_2SO_4		Size exclusion			

In acid conditions, it is necessary to dilute the sample with urea to avoid precipitation of caseins on the column. It is interesting to observe that with reversed phase (Visser *et al,* 1986) these authors obtain a better separation between α_{s2} and α_{s1}-casein than with a Mono Q column. This is also true with hydrophobic interaction column (Chaplin, 1986). So the latter could be used as a second step for rapid purification of α_{s2}-casein after a first step on an ion exchange column. Genetic variants can also be separated using longer analysis times (approximately 1 h) than those used for Mono Q columns. The advantage of this technique, compared to the other methods discussed above, is that volatile buffers are used, which enables recovery of proteins merely by freeze-drying the eluate.

Applications

One practical application of the HPLC analysis of caseins is to detect admixtures of milks of different species. Haasnoot *et al* (1986b) described a technique for detecting cow's milk in ewe's milk, goat's milk, and Gouda-type cheese made from these milks using ion-exchange chromatography on Mono-Q column. Bovine α_s-casein was used as an indicator to detect the presence of cow's milk in ewe's and goat's milk, curd, and non-proteolyzed cheese, with a detection limit of 2-4% and α_{s1}-I-peptide was used for old cheese, with a detection limit of 10%. These results are comparable to those obtained using electrophoretic and immunological methods.

HPLC analysis of caseins can also be used to determine the degree of ripening of cheese, a topic of great interest in the dairy sector. Chemical, physical, and sensory indices have been applied; among these, the chemical parameters, and in particular those related to proteolysis, appear to be most promising. The nitrogen index actually used furnished no information on individual components, and evaluation procedures are slow. HPLC separation and quantification of constituent compounds in caseins may provide a better indication of ripening time.

Haasnoot *et al* (1987) prepared cheese extracts at various stages of ripening in water, trichloroacetic acid, and urea and performed separations using reversed-phase, ion-exchange, and gel permeation chromatography. Separation by means of anionic exchange of urea extracts seemed to be the most appropriate method for determining the extent of proteolysis. According to the authors, the peak areas of γ and β-caseins are useful as a ripening index for old cheeses, those of α_{s1} and α_{s2}-caseins for young cheeses.

Whey proteins

The first HPLC separations of whey proteins were performed on gel permeation columns (Diosady *et al,* 1980; Dimenna and Segall, 1981), but the resolution of the major proteins (β-lg, α-la, and BSA) was incomplete, even though 2 columns were used in series. Today, the development of more efficient columns (Toya Soda 2 000 SW and 3 000 SW columns), combined with the use of phosphate buffer with sulphate or NaCl at a pH of 6.8, have made it possible to separate the Ig G, BSA, β lg, and the dimeric and monomeric forms of α-la in about 30 min (Gupta, 1983; Murphy and Mulvihill, 1988). When elution takes place in the presence of SDS (Gupta, 1983), there is only 1 peak of α-la.

Diosady *et al* (1980) described a nearly complete separation by reversed-phase

chromatography on a C_s column. Pearce (1983) achieved effective separation of the major whey proteins using a reversed-phase C_6 column and even succeeded in separating genetic variants of β-lg. De Frutos *et al* (1987) using a C-4 reversed phase column separated BSA, α-la and β-lg B and A in about 6 min.

Ion-exchange chromatography has also been used to analyze whey proteins. The best resolution and shortest analysis times have been obtained using anionic Mono Q FPLC columns with a pH 6-7 buffer and a NaCl gradient, but buffers used for the separation of whey proteins do not contain urea conversely to casein separation (Humphrey and Newsome, 1984; Andrews *et al,* 1985). Utilization of the phenyl-superose HR 5/5 bonded phase has made the separation of proteins possible on the basis of hydrophobicity. Effective hydrophobicity can be measured, despite its poor correlation with hydrophobicity as calculated from the Bigelow number. According to Chaplin (1986), the former is probably more closely related to the functional properties of the proteins than the latter.

De Frutos *et al* (1988) using hydrophobic interaction chromatography on a column of polyether phase bonded to silica support separated BSA, α-la and β-lg. With this technique it is not possible to separate genetic variants of β-lg, nevertheless it enables the recovery of proteins in their native state.

Dumay and Cheftel (1989) studied the behaviour of a concentrate of β-lg using different separation mechanisms: hydrophobic interaction, anion exchange and gel permeation. The results shown in figure 2 are similar to those previously described. Ion exchange chromatography gives the best separation of β-lg genetic variants A and B. Nevertheless, gel permeation chromatography might be useful for detecting soluble aggregates. Quantitative measurement of β-lg or α-la by hydrophobic interaction chromatography are quite precise, giving variation coefficients equal to or lower than 4–5%. On a Sepharose 12 column, variation coefficients are lower for β-lg and somewhat higher for α-la. Reten-

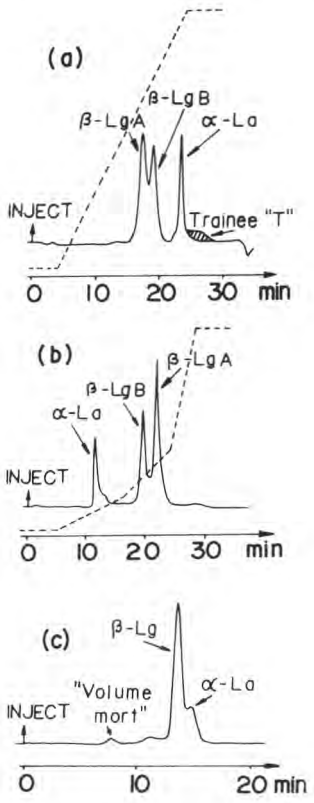

Fig 2. Chromatographic patterns of soluble proteins from β-lactoglobulin concentrate: a) hydrophobic interaction (Phenyl Superose column); b) anion exchange (Mono Q column); c) gel permeation (Superose 12 column). For elution conditions: see table I. [From Dumay and Cheftel (1989), *Sci Aliments* 9, 561, with permission]. *Profils de la fraction soluble du concentré de β-lactoglobuline obtenus par chromatographie a) d'interaction hydrophobe (colonne Phenyl Superose); b) d'échange d'anions (colonne Mono Q); c) de perméation de gel (colonne Superose 12). Conditions d'élution : voir tableau I.*

tion times for protein constituents remain relatively constant and with variation coefficients lower than 2% for 3–6 injections of the same protein solution.

Applications

Evaluation of the heat processing undergone by powdered skim milk is one of the practical applications of the HPLC separation of whey proteins. Kneifel and Ulberth (1985) suggested a gel permeation-based HPLC method to quantify the whey proteins without denaturing reconstituted powdered milk samples. This method was more sensitive than the American Dry Milk Institute, method (1971). Subsequently, Manji and Kakuda (1986) analyzed skim milks subjected to several different time-temperature treatment combinations, using FPLC to monitor the amount of thermal denaturation. The denaturation reaction appeared to follow a first-order kinetic equation in the case of α-la and second-order kinetic equations for β-lg A and B; at temperatures above 90 °C, denaturation of β-lg B took place slightly faster than that of β-lg A. Van den Bedem and Leenheer (1988) later described a high performance gel permeation chromatographic method suitable for classifying the type of heat processing used to produce powdered milk when low or extra-low heat treatments were employed.

Another application of whey protein separation by high performance gel filtration chromatography is to detect colostrum in milk. In comparison to whey, colostrum has a high immunoglobulin content, particularly Ig G Haasnoot *et al* (1986b) employed high performance gel filtration to detect the adulteration of milk with colostrum, and they established that Ig G levels in milk above 0.75 mg/ml were indicative of adulteration with colostrum.

PEPTIDE ANALYSIS

Different peptides exhibit a wide range of molecular weights and consequently distinct methodologies are used in the separation of these compounds. Reversed-phase chromatography on conventional columns with a small pore size is used for the smallest peptides (molecular weight lower than 3 000 Da). For larger peptides, reversed-phase columns with large pore sizes or gel permeation are suitable.

Table II summarizes the main papers dealing with peptide analysis in dairy products published in recent years. Most of the work has employed absorbance detectors operating between 205 and 230 nm, though 280 nm has also been utilized for the larger peptides. Reversed-phase chromatography has proved to be a reliable method with high resolving power for separating peptides from casein hydrolysates. Lemieux and Amiot (1989) fractionated peptides from phosphorylated and dephosphorylated casein hydrolysates on a TSK G 2 000 SW column and separated the fractionated peptides by RP-HPLC on a C_{18} column. The separation of phosphorylated and dephosphorylated hydrolysates yielded, respectively, 187 and 213 peptides, of which 99 and 116 were identified. Their study revealed that the peptide separation mechanism includes ionic interactions, hydrogen bonding, and peptide properties, in addition to overall peptide hydrophobicity.

Applications

Proteolysis of caseins, in particular proteolysis of the most hydrophobic caseins, *ie*, the β-caseins, is known to produce bitter peptides. There seems to be a relationship between hydrophobicity and bitterness, and therefore, RP-HPLC could be a useful

method for separating bitter from non-bitter peptides. Champion and Stanley (1982) used RP-HPLC on a C_{18} column to separate extracts of bitter peptides from Cheddar cheese coagulated with pepsin. A total of 71 substances, some of them bitter, were found, and the bitter fraction was reported to exhibit slightly higher mean hydrophobicity values and to present higher valine and leucine contents than the non-bitter fraction.

Leadbeater and Bruce-Ward (1987) using RP-HPLC after hydrolysis of β-casein with trypsin, separated the fragments into 16 peptides; identified them by means of external standards and N-terminal analysis using dansylation followed by partial Edman degradation. Retention of phosphoserine-containing peptides and large (> 1 500 Da) peptides was longer than expected from their mean hydrophobicity values and the authors therefore suggested that other factors, such as column pore size and peptide size, might also exert an influence on the separation results. However, there was a reasonable correlation between hydrophobicity and retention times for most individual peptides. Similar results were described by Carles and Ribadeau-Dumas (1986).

These methods, initially developed for the study of milk protein (β-casein) proteolysis, are currently applied to examine the action of the extracellular proteases of lactic acid bacteria on milk proteins. Monnet *et al* (1986) used RP-HPLC to determine the specificity of a proteinase from the cell wall of *Streptococcus lactis* on β-casein.

HPLC analysis of the water-soluble fraction can be useful in obtaining objective evaluation of cheese ripening. Using RP-HPLC, Pham and Nakai (1984) separated the water-soluble fraction in Cheddar cheeses at varying stages of ripening (fresh, young, ripe, and old) into 13 peaks, of which 6 increased, 2 decreased, and 4 remained unchanged with age. An additional peak appeared in the old cheese. The proportion of nitrogen compounds in the water-soluble fraction increased from 22 to 35% after 6 months of ripening (Park *et al,* 1980).

Gonzalez de Llano *et al* (1987a) analyzed the phosphotungstic acid soluble peptides from blue cheeses by gel filtration and HPLC.

Kaminogawa *et al* (1986) purified and identified the main low-molecular-weight peptides in Gouda cheese, confirmed by the formation of the same peptides by the protease fraction from *Streptococcus cremoris* on 23 N-terminal residues of α_{s1}-casein. The chromatograms for the cheese extracts varied according to ripening time, with peak height increasing with age.

Shimizu *et al* (1986) studied the functional properties (emulsifying capacity) of a 23-residue peptide hydrolyzed from α-casein by pepsin and purified by ion exchange chromatography.

Detection of cow's milk in ewe's or goat's milk and cheese is another application of the HPLC analysis of peptides. Tobler *et al* (1983) used HPLC to examine the difference between the caseins in the milks of various species. Goat's and cow's milk cheese caseins were hydrolyzed with trypsin, and the peptides thus obtained were separated by reversed-phase HPLC. The chromatograms were reproducible and distinct for the caseins of each species. Subsequently, Kaiser and Krause (1985) used HPLC to separate the tryptic peptides in cow's milk and goat's milk cheeses and cheeses made from mixtures of these milks. They reported that the quantitative detection limit could be as low as 1% cow's milk in goat's-milk cheese. The presence of cow's milk reduced the area of the 80-min peak characteristic of goat's milk.

Table II. HPLC application to the analysis of peptides in dairy products.
Application de la CLHP au dosage des peptides dans les produits laitiers.

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Objective*	*Reference*
TSK 2000 SW	0.1 M-KH_2PO_4, 0.01 M-K_2HPO_4, 0.15 M-Na_2SO_4,	205 nm	Size exclusion	Glycomacropeptide	Action of chymosin in milk	Van Hooydonk and Olieman (1982)
µ-Bondapak C18	a) H_2O b) methanol	280 nm	Reversed phase	Peptides	Separation of bitter peptides	Champion and Stanley (1982)
TSK 2000 SW	0.1 M-KH_2PO_4, 0.01 M-K_2HPO_4, 0,15 M-Na_2SO_4,	205 nm	Size exclusion	Glycomacropeptide	To detect rennet whey in milk powder	Olieman and Van den Bedem (1983)
Spherisorb S5 C8 Adsorbosphere C8 Vydak C18	0.1 M-Buffer phosphate pH 6.0	220 nm	Reversed phase	Peptides	To determine the maturity of cheese	Pham and Nakai (1984)
Lichrospher Si-500/2 CH8	a) 0.005 M-Ammonium acetate pH 7.25 b) Idem a) + 65% ethanol	220 nm	Reversed phase	Peptides	To detect mixtures of milk	Kaiser and Krause (1985)
TSK 2000 SW	0.1 M-Buffer-phosphate pH 6.0, 0.15 M-Na_2SO_4,	205 nm	Size exclusion	Glycomacropeptide	Proteolysis in raw milk	Mottar *et al* (1985)
µ-Bondapak C18	a) 0.01 M-potasium-phosphate, pH 6.5 b) Idem a) + 60% CH_3CN	220 nm	Reversed phase	Peptides	Separation of peptide mixture	Carles and Ribadeau-Dumas (1986)
RP-300 A	a) 0.02 % SDS, 0.02 % TFA, pH 5.5 b) Idem a) + 50% CH_3CN	210 nm	Reversed phase	Peptides	To detect mixtures of milks	Windemann *et al* (1986)
Zorbax-ODS	a) 0.1% TFA in H_2O b) 0.1 TFA in 60% CH_3CN	230 nm	Reversed phase	Peptides	Identification of low molecular weight peptides	Kaminogawa *et al* (1986)

Stationary phase	Eluent	Detection	Chromatographic mode	Separation of :	Objective	Reference
Finepak-SIL ODS	a) 0.1% TFA in H_2O b) 0.1% TFA in 40% CH_3CN	230 nm	Reversed phase	Peptides	Properties of a peptide α_{s1}CN–(f123)	Shimizu *et al* (1986)
Ultrasphere ODS	a) 0.1% TFA in H_20 b) 0.1% TFA in 30% CH_3CN	230 nm	Reversed phase	Peptides	Study of low molecular weight peptides	Gonzalez-Llano *et al* (1987a)
Apex C8	a) 0.1% TFA in H_20 b) 0.08% TFA in 50% CH_3CN	206 nm	Reversed phase	Peptides	Study of proteolysis	Leadbeater and Bruce-Ward (1987)
TSK 2000 SW	0.1 M-KH_2PO_4, 0.01 M-K_2HPO_4, 0.15 M-Na_2SO_4,	205 nm	Size exclusion	Glycomacropeptide	Fractionationation and characterization of GMP	Morr and Seo (1988)
Beckman-RPSD	a) 0.15 M-NaCl-HCl, pH 2.1 b) CH_3CN a) 0.01 M-TFA in H_2O b) 0.01 M-TFA in CH_3CN	210 nm	Reversed phase			
TSK G 2000 SW	0.1% TFA, 0.05 M-Buffer phosphate, 35% methanol	214 nm	Size exclusion	Peptides	Separation of peptides	Lemieux and Amiot (1989)
µ-Bondapak C18	a) 0.115% TFA in H_2O b) 0.1% TFA in 60% CH_3CN	206 nm	Reversed phase			
Ultrapore RPSC	a) 0.1% TFA in 15% CH_3CN b) 0.1% TFA in 30% CH_3CN	210 nm	Reversed phase	Glycomacropeptide	To detect rennet whey solids in milk and butter milk powders	Olieman and Van Riel (1989)
Protein Plus	a) 0.1% TFA in 15% CH_3CN: 2% isopropanol b) 0.1% TFA in 55% CH_3CN: 2% isopropanol	210 nm	Reversed phase			

The cleavage of the bond Phe 105-Met 106 of K-casein, which is responsible for the milk coagulation, liberates the glycomacropeptide (GMP) (fragment 106-169 of K-casein). This high-molecular-weight peptide can be recovered by ultrafiltration of cheese whey. The price of cheese whey being very low, it is used to adulterate powdered milk, and hence detection of this peptide could be used as an adulteration index.

Van Hooydonk and Olieman (1982) studied the action of chymosin in milk and quantified GMP by means of gel filtration HPLC. The method enabled them to determine the proteolytic activity of commercial rennets and rennet substitutes. Olieman and Van Den Bedem (1983) established the amount of whey in powdered skim milks by measuring the amount of GMP. The procedure was more sensitive than other tests available (determination of lactic acid, ash, sialic acid, etc) and offered the advantage of being unaffected by the procedure used to produce the whey. However, the interpretation of gel permeation chromatograms of acid buttermilk is sometimes complicated by the presence of components eluting closely after the glycomacropeptide (De Vilder *et al,* 1988). On the other hand, during prolonged cold storage of milk or butter-milk, psychrotrophic bacteria might proliferate and induce enzymatic proteolysis of caseins. This leads to false positive results. Recently Olieman and Van Riel (1989) developed a procedure which is more selective than the existing GPC method. It is based on the determination of GMP with HPLC on a reversed phase Protein Plus Column with a gradient of acetonitrile water containing 0.1% of trifluoroacetic acid. The sequential application of the 2 methods diminishes the chance of false positive results. The authors found no evidence that starters used for ripening cream produce enzymes which split K-casein at the same bond as chymosin. Some starters formed pseudo-GMP. Under practical circumstances it is also highly unlikely that psychrotrophic bacteria produce enzymes capable of splitting K-casein at the same bond as chymosin.

HPLC quantification of the degradation products of raw-milk proteins may furnish information on the shelf life of subsequently prepared UHT milk. Based on 2 earlier methods, Mottar *et al* (1985) applied HPLC to determine the specific proteolytic components which provide information concerning the presence and activity of Gram-negative psychrotrophic bacteria.

This information is important for monitoring milk products, especially UHT milk, since they produce heat-resistant proteolytic enzymes that can limit storage life. The method can be used to determine whether raw milk is suitable for the manufacture of UHT milk.

Morr and Seo (1988) carried out a more thorough evaluation of GMP (effective molecular weight and physico-chemical properties) and of methods for fractionating and quantifying this substance in dairy products.

ANALYSIS OF AMINO ACIDS AND AMINES

Although HPLC has been used for some time in the study of amino acids, the literature contains very few applications of this technique to dairy products.

Since the absorption maximum for amino acids falls in the same region of the spectrum (214 nm) as those of many other compounds, fluorescent derivates are often formed for detection purposes. The most frequently used derivatizing agents are dansyl chloride and orthophtaldehyde/

mercaptoethanol, and both reagents have been applied to the study of dairy products (Polo *et al*, 1985; Ramos *et al*, 1987; González de Llano *et al*, 1987b). Table III summarizes the most recent work on HPLC analysis of amino acids and amines in milk products. All separations have been performed using reversed-phase chromatography.

Polo *et al* (1985) studied free amino acids in artisanal and industrial Mahon cheese during ripening. For this purpose, amino acid derivatives were formed with dansyl chloride and separated on a Radial Pak C_{18} column in little more than 50 min.

Using the fluorescent derivative formed by the reaction of amino acids with orthophthaldehyde (OPA) in the presence of mercaptoethanol, Ramos *et al* (1987) studied the effect of freezing Cabrales cheese curds on final cheese composition. The free amino acid content in the cheeses produced from curds frozen for 4 months was lower than that in the control cheeses; in contrast, the amino acid content in the cheeses obtained from curds frozen for 8 months was similar to that in the control cheeses.

The reaction of amino acids with orthophthaldehyde/mercaptoethanol can be automated using an automatic injector and a mixing column. This technique has been employed to study the amino acid composition of the phosphotungstic acid-soluble peptides in blue cheese (González-Llano *et al*, 1987b) and the influence of enzyme-accelerated ripening of Spanish hard cheese on the free amino acid content (Fernández-Garcia *et al*, 1988). Fig 3 shows the chromatogram obtained in a Gamonedo cheese (González de Llano, 1989).

The fact that OPA/MCE does not react with secondary amino acids such as proline and hydroxy-proline and that dansyl chloride appears in the chromatogram as a big peak interferring with the detection of other amino acids, makes it necessary to look for other derivatizing reagents.

The derivatization with phenylisothiocyanate and 9-fluorenyl-metoxycarbonyl which are already used to detect amino acids in other food products, could also give good results in dairy products.

Cheese, like other fermented products, may contain biogenic amines produced by micro-organism-induced decarboxylation of certain amino acids. The detection and quantification of such amines were studied because of their toxic effects on some consumers and HPLC was applied in the determination of these nitrogen compounds in cheese.

Absorbance at 215-220 nm has been used for the detection of amines and their amino acid precursors in some experiments; however, derivative formation, as in the case of the amino acids mentioned above, is more common, because it facilitates more selective detection.

Antila *et al* (1984) published an excellent review of the literature dealing with amines in cheeses. In the same paper the authors also presented HPLC determinations of the histamine, tyramine, tryptamine, phenylethylamine, cadaverine, the putrescine contents in a total of 76 Finnish cheeses; these authors reported no relationship between cheese quality and amine content.

Chang *et al* (1985) used ion-pair chromatography with detection at 220 nm to determine the tyrosine, tyramine, histidine, histamine, tryptophan, and tryptamine content in cheese samples. Ion-pair chromatography with an ultraviolet detector was also employed by Van Boekel and Arentsen-Stasse (1987) to analyze the same compounds, along with phenylalanine and phenylethylamine, in cheeses.

Table III. HPLC application to the analysis of amino acids and amines in dairy products.
Application de la CLHP au dosage des acides aminés et amines dans les produits laitiers.

Stationary phase	*Eluent*	*Detection*	*Chromatographic mode*	*Separation of :*	*Derivatization*	*Reference*
μ-Bondapak C18	a) 0.02 N-acetic, CH_3CN b) 0.02 N-acetic, CH_3CN, methanol	Fluorescence	Reversed phase	Amines	Dansyl chloride	Antila *et al* (1984)
μ-Bondapak C18	0,02 M-butane- or, pentane- or, hexane- or, heptane- or, octane- or D-camphoric sulfonic acid sodium salt in 15% methanol: 85% H_2O; 10% CH_3CN: 90% H_2O; 20%CH_3CN: 80% H_2O, pH 3.0	220 nm	Reversed phase	Aromatic amino acids and their amines		Chang *et al* (1985)
Radial Pak C18	a) methanol, 0.01 M-Buffer phosphate pH 6.3, (15:85) b) Idem a) (35:65)	Fluorescence	Reversed phase	Amino acids	Dansyl chloride	Polo *et al* (1985)
Novapak C18	Methanol: 0.05 M-Na-phosphate (85:15), 1.5% PIC	Fluorescence	Reversed phase	Tyramine	o-Phthaldehyde	Reuvers *et al* (1986)
Nucleosil C18	a) 16g ninhydrin, 1.2g hydridantin, 2g SDS, 940 ml DMSO, 350 ml 2.8 M-sodium acetate (pH 5.0) 710 ml H_2O	546 nm	Reversed phase	Amines	Ninhydrin	Joosten and Olieman (1986)
Radial Pak C18	a) methanol: 0.01 M-Buffer phosphate, pH 7.3: THF (19:80:1) b) methanol: 0.01 M-Buffer phosphate, pH 7.3 (80:20)	Fluorescence	Reversed phase	Amino acids and amines	o-Phthaldehyde	Ramos *et al* (1987)
CPSpher C18	a) 0.01 M-Na heptano-sulfonate, 0.01 M-K-phosphate, pH 3 b) methanol o CH_3CN	215 nm 265 nm	Reversed phase	Aromatic amino acids and their amines		Van Boeckel and Arentsen Stasse (1987)

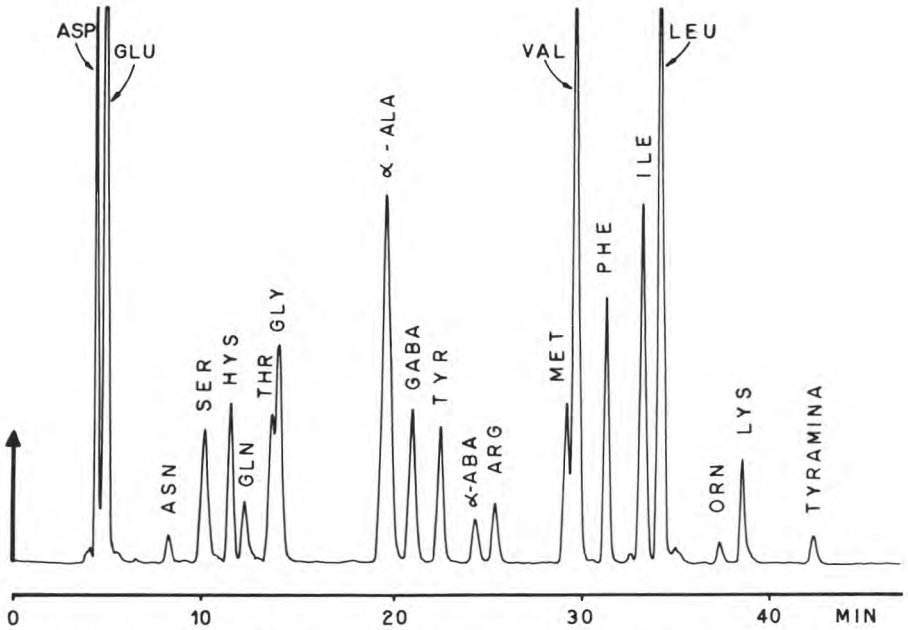

Fig 3. Chromatogram of OPA-amino acids of cheese. Column: Radial Pak C-18, 10 µm. Eluent A) Methanol: 10 mmol•l^{-1} sodium phosphate buffer, pH 7.3; tetrahydrofurane (19:80:1). Eluent B) Methanol: 10 mmol•l^{-1} sodium phosphate buffer, pH 7.3 (80:20) Lineal gradient : 0 min (0% B, 1.5 ml/min); 6 min (15% B, 1.5 ml/min); 11 min (15% B, 1.5 ml/min); 16 min (30% B, 1.5 ml/min); 20 min (40% B, 1.5 ml/min); 32 min (80% B, 1.3 ml/min); 38 min (80% B, 1.3 ml/min). Fluorescence detection (λexc = 340 nm, λem = 425 nm).

Chromatogramme des OPA-acides aminés d'un fromage. Colonne : Radial Pak C-18, 10 µm. Phase mobile A) Méthanol : phosphate de sodium 10 mmol•l^{-1}, pH 7,3 : tétrahydrofuranne (19:80:1) Phase mobile B) Méthanol : phosphate de sodium 10 mmol•l^{-1}, pH 7,3 (80:20) Gradient linéaire : 0 min (0% B, 1,5 ml/min); 6 min (15% B, 1,5 ml/min); 11 min (15% B, 1,5 ml/min); 16 min (30% B, 1,5 ml/min); 20 min (40% B, 1,5 ml/min); 32 min (80% B, 1,3 ml/min); 38 min (80% B, 1,3 ml/min). Detection par fluorescence (λexc = 340 nm, λem = 425 nm).

Reuvers *et al* (1986) put forward a faster method of separating tyramine in cheese: detection was achieved by measuring fluorescence after postcolumn reaction with orthophthaldehyde. Orthophthaldehyde was also used in an automatic derivative formation system prior to chromatographic separation, to determine the tyramine and histamine contents, together with the free amino acids, in cheese (Ramos *et al,* 1987; Fernández-García *et al,* 1988).

An unusual method for detecting amines was proposed by Joosten and Olieman (1986), using ninhydrin contained in the eluate itself as the derivatizing reagent and detecting the derivatives so formed at 546 nm.

CONCLUDING REMARKS

Taking into account the latest works published concerning the study of nitrogen compounds of dairy products, it can be concluded that HPLC and FPLC are gradually acquiring a very important role.

Although a great variety of chromatographic methods have been used for the study of nitrogen compounds, some of them present several advantages over the rest. Ion-exchange chromatography is the technique which gives the best protein separations. By this technique, it is possible to quantify the casein fractions and to identify some of the genetic variants of caseins (K and β-caseins) and whey proteins. Reversed phase chromatography has also been used to separate the genetic variants of whey proteins (β-lg).

Gel permeation chromatography and reversed phase chromatography with wide pore columns, allow the separation of large peptides. The separation of small peptides is carried out using conventional reversed-phase columns.

Separation of amino acids is usually carried out by HPLC using reversed-phase columns. The detection of amino acids requires the preparation of derivatives detectable by ultraviolet and fluorescent techniques. Dansyl chloride and orthophthaldehyde are the most commonly used derivatizing reagents. However, these compounds present some problems and will probably be substituted by other reagents in the near future.

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