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Review

Chromatographic and electrophoretic methods used for analysis of milk proteins

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ABSTRACT

Current knowledge of milk proteins and their behavior in dairy foods is based on early applications of chromatography and electrophoresis. Electrophoretic identification of the number and genetic variety of milk proteins inaugurated a research effort in which chromatographic techniques were successfully applied to the isolation of each milk protein, thus facilitating the characterization and further study of milk and dairy products. This review focuses on recent applications of chromatography for separations and analysis and on analytical applications of electrophoresis.

CONTENTS

1. INTRODUCTION

Milk is a basic food for humans providing essential nutritional components (proteins, fats, carbohydrates, minerals, and vitamins). Humans consume milk and milk products of many animal species: cow, zebu and yak (genus *bos*); water buffalo; goat; sheep; horse; donkey; reindeer; and camel [1]. Cows' milk (genus *bos*) is the most intensively studied. Bovine milk contains 3–3.5% (w/v) protein. Caseins are 2.4–2.8% of fluid milk; whey proteins are 0.5–0.7% [2]. Other classes [3] of protein present are MFGM protein, minor proteins, and enzymes.

Casein exists in milk as a casein micelle with an average diameter of about 140 nm [4]. The exact structure of the casein micelle and the forces responsible for its stability are still speculative and many models of the micelle have been proposed [5]. The casein micelle contains the four caseins: α_{s1}-, α_{s2}-, β- and κ-casein in the approximate ratios of 39:10:36:13 [6]. The primary sequence and phosphorylation sites of the four caseins are known, as well as some of the insertions, deletions and substitutions that comprise casein genetic variants [2]. The only glycosylated casein, κ-casein, contains various amounts and types of carbohydrates [3].

Proteolytic products of the four primary caseins are also present in milk. γ-Caseins (see ref. 2 for correct nomenclature) and some of the proteose-peptone components are β-casein fragments, created by the action of plasmin, the endogenous alkaline milk protease. λ-Caseins are probably α_{s1}-casein fragments also arising from plasmin cleavage. The glycomacropeptide and *para*-κ-casein are κ-casein fragments created by the action of chyomsin (rennet) [2].

Whey contains proteins soluble at pH 4.6 and

20°C, including β-LG (0.2–0.4%, w/v, milk), α-LA (0.06–0.17%, w/v, milk), BSA (0.04%, w/v, milk) and immunoglobulins (IgG, IgA, IgM; 0.04–0.09%, w/v, milk) [2]. Minor proteins are also present: lactoferrin, lactoperoxidase, enzymes, protein components of the MFGM, proteose–peptone components and glycomacropeptide. The primary sequences of α-LA, β-LG and BSA are known.

The MFGM proteins are not as well characterized, although several enzymes of the MFGM surface are known to be covalently linked to phosphatidylinsoitol [7]. Keenan *et al.* [8] reported up to 37 different polypeptides occurring in MFGM, of which seven are glycosylated.

Milk proteins are usually separated into classes before chromatographic or electrophoretic analysis. The casein micelles can be prepared from skim milk by ultracentrifugation or gel permeation chromatography; whole casein may be precipitated from skim milk at pH 4.6, a treatment that disrupts the micelles leaving whey proteins in solution. Whey proteins also remain in solution after rennet coagulation. In addition to gel filtration, ultrafiltration, microfiltration, reverse osmosis, and electrodialysis, a variety of complexing and precipitation techniques can be used to isolate whey proteins. MFGM proteins are isolated from washed cream and are separated from the fat by centrifugation.

Chromatographic techniques have been widely used for the isolation of milk proteins, and high performance methodology now forms the basis for several accurate methods of analysis. Electrophoresis has played a major role in the study of milk proteins and has been an integral part of research on the genetic variants of the major protein components of milk. Indeed, the designations of the caseins are derived from electrophoretic analysis. The minor casein components, γ_1, γ_2, γ_3, and *para*-κ-casein were discovered by electrophoresis. Research on the use of faster and more accurate and versatile chromatographic and electrophoretic techniques in milk protein analysis continues as does the evolution of such techniques. Capillary electrophoresis is being evaluated for milk proteins but nothing has been reported.

2. CHROMATOGRAPHY

2.1. Ion-exchange chromatography

Table 1 lists recent references, the type of column used, and the protein class that was separated. This list includes both anion and cation; preparative and high-performance chromatography.

2.1.1. Anion-exchange chromatography

2.1.1.1. Preparative separations

2.1.1.1.1. Caseins. DEAE-Cellulose is the most commonly used anion-exchange resin for separation of individual caseins. Four classical methods are used: Thompson [9], Mercier *et al.* [10], Davies and Law [11] and Andrews and Alichanidis [12]. Three of these methods [9,10,12] use ME in both the sample solvent and eluting buffers to reduce the disulphide bonds in κ- and α_{s2}-caseins. In addition, their eluting buffer (pH 7) contains 3.3 *M* urea. The major differences are in the NaCl gradients used to elute the caseins. Fig. 1 shows a typical DEAE-cellulose chromatogram of whole casein from bulk milk.

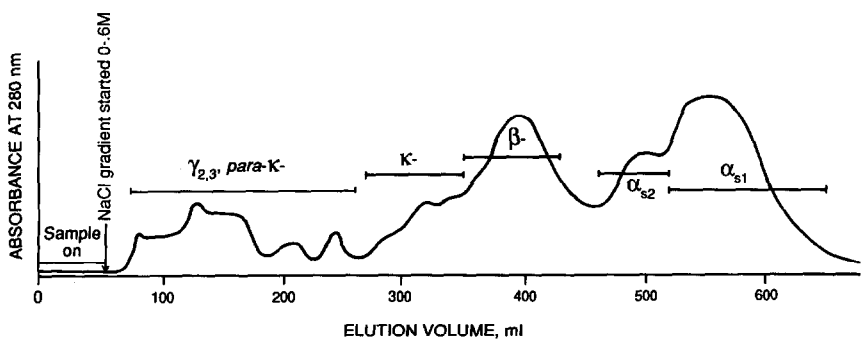

Fig. 1. DEAE-Cellulose chromatogram of whole casein. Eluent is 0.01 *M* imidazole, 3.3 *M* urea, 6 m*M* 2-mercaptoethanol pH 7, NaCl gradient from 0 to 0.35 *M*. Adapted from ref. 16.

TABLE 1

SUMMARY OF ION-EXCHANGE CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
Anion-exchange chromatography —preparative separations		
QAE ZetaPrep	Casein	15
DEAE-Sepharose	Casein	13, 14, 93
DEAE-cellulose	Casein[a]	9–12, 16, 25, 93, 107, 173–175
DEAE-cellulose	Proteose peptone	12
DEAE ZetaPrep	Casein peptides	176
QAE-Sephadex A-25	Casein peptides	177
Spherosil QMA	Whey	18
QAE ZetaPrep	Whey	23
DEAE-Selectacel	Whey	19
DEAE-cellulose	Whey	20, 21
DEAE-Toyopearl	Whey	22, 101
DEAE-Toyopearl	Minor proteins	87
DEAE-cellulose	Minor proteins[b]	178
Anion-exchange chromatography —analytical separations		
Mono Q	Casein	13–15, 25, 30, 26–28, 32, 46
Mono Q	Renneted casein	31
TSK DEAE-5PW	Casein	25, 33, 34
TSK DEAE-5PW	Casein[b]	35
Aquapore AX-300	Casein	16
Mono Q	Milk and cheese[a]	29
Mono Q	Casein peptides	177
Methyl iodine-PVIate	Casein peptides	62
Mono Q	Whey[a]	25, 26, 36–39
QPVI and KPVI[c]	Whey	40
TSK DEAE-5PW	Whey	25
Cation-exchange chromatography —preparative separations		
SP-ZetaPrep	Casein peptides	176
SP-Toyopearl	Minor proteins	44, 45
CM-Toyopearl	Minor proteins	43, 45
CM-cellulose	Whey[a,b]	42
Cation-exchange chromatography —analytical separations		
Mono S	Casein	26, 30, 46, 47
Mono S	Renneted casein	31, 48
Mono S	Whey	26
Mono S	Minor protein[a,b]	42
Mono S	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.
[b] Human milk proteins.
[c] See reference for column modification details.

Davies and Law [11] alkylated whole casein with iodoacetamide, after reduction with ME, and used an eluting buffer (pH 8.6) with 6 *M* urea.

A recent advance in casein separation methodology is the use of DEAE-Sepharose which allows higher flow-rates than does DEAE-cellulose. Barrefors *et al.* [13] used 3.3 *M* urea and ME in the buffers and obtained good separation of the caseins on DEAE-Sepharose. Christensen and Munksgaard [14] used 6.6 *M* urea and dithiothreitol in the buffers and reported good separation of the caseins except for α_{s1}- and α_{s2}-casein which could be separated on DEAE-Sepharose using 3.3 *M* urea and dithiothreitol in the buffer. Mass ion exchange has also been

used to separate caseins on a preparative level [15]. However, α_{s1}- and α_{s2}-caseins were not separated on a QAE ZetaPrep system with a pH 8, 4.5 *M* urea–ME buffer.

A single pass through a DEAE-cellulose column usually yields pure preparations of β- and α_{s1}-caseins [16]. The α_{s2}-casein fraction and the κ-casein fraction needed additional chromatography; α_{s2}-casein can be separated from contaminants, mainly β-casein and an unknown protein. The κ-casein fraction contains γ-caseins and *para*-κ-casein. Mercier *et al.* [10] separated κ-casein B, prepared by a precipitation method, into seven peaks by chromatography on DEAE-cellulose with a NaCl gradient.

2.1.1.1.2. Whey proteins. One technique used to isolate whey protein concentrate industrially is anion-exchange chromatography of sweet or medium acid whey, *i.e.*, whey from cheese such as Cheddar, Swiss or Mozzarella, manufactured with rennet (pH > 5.5) [17]. Proteins from the sweet whey are absorbed on Spherosil QMA, a silica-based strong anion exchanger [18]. Non-protein components, such as lactose, are eluted with water whereas the proteins require a pH < 4.5.

Anion-exchange chromatography is also used to isolate the major whey proteins and their genetic variants. β-LG A and B [19] and β-LG A and C [20] are separated using DEAE-cellulose at pH 5.8 with a NaCl gradient. α-LA and β-LG A and B were separated from dialyzed acid whey or ammonium sulphate treated whey by DEAE-cellulose chromatography [21] with a pH 7.2 Tris–HCl gradient. BSA was contaminated by an unidentified protein. Yoshida [22] used DEAE-Toyopearl columns at pH 6.8 and 8.5 and a linear NaCl gradient to separate whey fractions previously isolated from a Sephacryl S-200 column. At pH 6.8, β-LG A and β-LG B could be separated but α-LA was not retained on the anion-exchange column. At pH 8.5, α-LA separated from small quantities of β-LG and an unidentified protein. Imafidon and Ng-Kwai-Hang [23] used a QAE-ZetaPrep cartridge to separate residual α-LA from a 3% TCA supernate [24] β-LG preparation by eluting with pH 6.0 phosphate buffer and NaCl. Under these conditions α-LA did not bind to the ion exchanger; β-LG did but β-LG A did not separate from β-LG B.

2.1.1.2. Analytical separations

2.1.1.2.1. Caseins. Analytical (high-performance) liquid chromatography of caseins on strong anion-exchange columns, *i.e.*, Mono Q 50 mm × 5 mm I.D. [13,25–28], is based on techniques of DEAE liquid chromatography. Elution protocols evaluated include the use of pH 7 to 8.5 buffers that contain 3 to 8 *M* urea; NaCl gradients; and ME in the sample [25] and in both sample and buffer [13,26]; and alkylation of the casein [28]. Separation of α_{s2}-, α_{s1}- and α_{s0}-caseins presents the major difficulty. In addition to shallow salt gradients (2–5 m*M*/ml) [13,28] in the α_s region of the chromatogram, a concentration of urea just sufficient (3 to 4.5 *M*) [26] to dissociate the caseins allows for reproducible separation of these three caseins. Reported recoveries of proteins from Mono Q columns were greater than 95% [25,28]. Separation of β-casein B from other β-casein variants has been reported also [27].

Chromatography of milk on Mono Q allowed detection of cow's milk in goat's milk (1–2%) and in ewe's milk (2–4%) but goat and ewe milk could not be distinguished from each other [29]. In cheese extracts, the detection limit of cow's milk is 10% in 3-month-old Gouda type goat cheese using bovine α_{s1}-I-casein (a rennet peptide of α_{s1}-casein) as a marker [29]. Mono Q columns can be used to purify a κ-casein without the use of ME or alkylating agents [30], to identify κ-casein genetic variants and their glycosolated forms [31], to quantify individual caseins [32], and to confirm casein separation in a mass ion-exchange procedure [15].

Weak anion-exchange columns also are used to separate caseins [16,25,33,34]. For example, TSK DEAE-5PW column can separate the α_s-caseins with 3.3 and 4 *M* urea buffers and a linear NaCl gradient [33,34] as well as separate human caseins according to their degree of phosphorylation [35]. Separations on the TSK DEAE-5PW column take about twice as long as on the shorter Mono Q 5/5 column. Strange *et al.* [16] reported poor separation of α_{s1}- and α_{s2}-casein and extremely poor column stability for an Aquapore AX 300 column.

2.1.1.2.2. Whey proteins. Chromatography on the Mono Q column can be used to separate whey proteins [25,26,36–39]. Humphrey and Newsome [25] investigated buffer systems from pH 6 to 8.5 and found that pH 6 and a NaCl gradient separated α-LA, β-LG A, and β-LG B with 96% recovery from

dialyzed acid whey. The separation [36] and quantification [37] of α-LA, BSA, β-LG A and β-LG B in both sweet and acid wheys from reconstituted skim milk powder is done by using water at pH 6.6 for a buffer and eluting with a gradient to 0.7 *M* sodium acetate. Andrews *et al.* [26] separated the whey proteins, including BSA and immunoglobulins, at pH 7 with a NaCl gradient on the same type of column. Conditions were similar to those used for casein except no urea or ME was used. Addition of urea to buffers caused a loss in resolution with the whey proteins. Girardet *et al.* [38] found that, within the pH range 6 to 8, whey proteins eluted in the following order regardless of buffer system: immunoglobulins < α-LA < β-LG B < β-LG A. The resolution of BSA depends on pH and resolution of β-LG A and B depends on the steepness of the salt gradient. Laezza *et al.* [39] used a pH 7 NaCl gradient to separate and identify bovine and ovine whey proteins. Chaufer *et al.* [40] used retention of α-LA, BSA, β-LG, and lysozyme on a silica-based strong ion-exchange column to predict behavior with ultrafiltration membranes. Whey proteins also have been separated on a DEAE-5PW weak anion-exchange column with pH 7.2 NaCl gradient [41].

2.1.2. Cation-exchange chromatography

2.1.2.1. Preparative separations—whey and minor proteins

Cation-exchange liquid chromatography is used to isolate whey proteins and some of the minor proteins. Spherosil S, used in a manufacturing process [17], absorbs whey proteins from acidic whey (pH < 4.5) and then releases them when the pH is raised. Lactoferrin and lactoperoxidase can be separated from each other and acid whey proteins by chromatography on carboxymethyl cellulose at pH 7.8 [42]. Carboxymethyl-cellulose chromatography is also used to separate lactoperoxidase and lactoferrin in a whey fraction [43] and sulphopropyl cation-exchange chromatography is used to prepare lactoperoxidase from lactoferrin directly from colostral whey [44] and from sweet and acid whey [45].

2.1.2.2. Analytical separations

2.1.2.2.1. Caseins. A Mono S column, a cation exchanger, separates caseins at pH 3.5, 8 *M* urea and ME [26] with a NaCl gradient. Order of elution is: β-casein < κ-caseins and λ-caseins < α_s-caseins. α_{s2}-Casein did not separate from α_{s1}-casein. At pH 5–7, 6 *M* urea, a similar elution profile was reported [31]. Unreduced κ-casein separated from other caseins when eluted with 3.3 *M* urea, pH 3.5, octyl-glucoside (a non-denaturing detergent), and a NaCl gradient on a Mono S 10/10 column [30]. α_{s1}-Casein eluted before α_{s2}-casein at 6 *M* urea, pH 5, ME, and a very shallow NaCl gradient [46]. Genetic variants β-casein A^1, A^2, and B also could be separated [47].

2.1.2.2.2. Whey and minor proteins. High-performance cation-exchange liquid chromatography was used at low pH values to analyze the glycomacropeptide [48]. At higher pH values the same technique was used to compare human and bovine lactoperoxidase and lactoferrin as well as to confirm the separation of porcine lactoferrin from porcine colostral whey [49].

2.1.3. Recommendations

Preparative anion-exchange liquid chromatography with urea and ME is used to isolate relatively pure individual caseins in milligram to gram quantities. κ-Casein genetic variants and glycosylated moieties have also been isolated with this method. High-performance anion-exchange liquid chromatography is used for the rapid analysis of caseins. This technique separates only one of the known β-casein variants, but non-bovine caseins are readily separated from bovine caseins. However, casein peptides are poorly resolved compared to other chromatographic techniques. Both preparative and high-performance anion-exchange chromatography can separate α-LA, β-LG A and β-LG B. The simultaneous analysis of milk for whey proteins and caseins without prior separation has been unsuccessful because anion-exchange chromatography requires urea to dissociate the caseins.

Conditions for casein separation and analysis in cation exchange are not as well defined as in anion exchange. However, recent work shows that the determination of some β-casein variants is possible. Cation-exchange chromatography is useful for the separation of the minor proteins in milk.

2.2. Reversed-phase chromatography

Table 2 lists the proteins separated and columns

TABLE 2

REVERSED-PHASE ANALYTICAL (HIGH-PERFORMANCE) LIQUID CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
C_{18}	Casein	33, 50, 52
C_8	Casein[a]	13, 16, 53
C_4	Casein[a]	51, 54, 73
Phenyl	Casein	33
C_2–C_{18}	Casein peptides	179
C_{18}	κ-Casein peptides	48, 85
C_{18}	Casein peptides[a]	52, 55–59, 175
C_8	Casein peptides	60
C_4	Casein peptides	61
C_{18}	Cheese[a]	63–67, 69, 70
C_8	Cheese	67, 68
C_{18}	Whey proteins	50
C_8	Whey proteins	71
C_6	Whey proteins	72
C_4	Whey proteins[a]	51, 73–75
C_{18}	Whey peptides[a]	180, 181
C_4	Milk protein in meats	76
Ultrapore	Rennet whey in skim	77
Protein Plus	Milk powder	77
Phenyl	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.

used for reversed-phase high-performance chromatography of milk proteins.

2.2.1. *Analytical separations*

2.2.1.1. *Caseins*

Large-pore C_4, C_8 and C_{18} reversed-phase columns give excellent analytical separations of whole casein. The solvent system usually used is TFA–water–acetonitrile (0.1:70:30) with an acetonitrile gradient to 0.1:50:50. With a C_{18} column [33], all the caseins eluted in about 30 min with the order of elution being, κ-, α_{s2}-, α_{s1}- and β-casein. With a more complex acetonitrile gradient [50] and a C_{18} column, carbohydrate-free κ-casein separates into its A and B variants, α_{s1}-casein into its A, D and B/C variants (α_{s1}-casein B and C could not be separated) and β-casein into its A^1/C, A^2, A^3 and B variants. A variant designated β-casein X was also separated from bulk milk. Caseins separate in the same order on a C_8 column [16] except that α_{s1}-casein separates into two equal peaks and the β-casein variants are not as well resolved. Parris *et al.* [51] used a C_4 column, but no separation of casein variants was reported.

A C_{18} reversed-phase column, with pH 7.2 phosphate SDS buffer and propanol as eluting solvents, separates, in order of increasing retention time, α_{s1}-, β-, α_{s2}- and κ-casein [52]. One of the whole casein preparations analyzed showed two β-casein peaks with identical isoelectric focusing properties. However, one of the caseins had a leucine substituted for a proline, a mutation that would not be detected by electrophoretic analysis.

Reversed-phase columns, C_8 [53] and C_4 [54], have been used to confirm the existence of caprine α_{s1}-casein. Both of these columns gave the same order of elution, namely κ-, α_{s2}-, α_{s1}-, β-casein; the same order as found for bovine casein.

The proteins from skim milk [50] and from non-fat dry milk [51] have been analyzed without prior separation of caseins and whey proteins. On a C_{18} column [50], α-LA separated from α_{s1}-casein but α-LA had the same retention time as β-casein B; β-LG A and B were well resolved. On a C_4 column [51], α-LA appeared as a trailing shoulder on the α_{s1}-casein peak; but β-LG A and B were well resolved. In the same study, a complex of BSA, α-LA, β-LG, κ- and α_{s2}-casein, formed on heat treatment of the non-fat dry milk, eluted at the highest acetonitrile concentration of the chromatographic run. Fig. 2 shows the separation of caseins

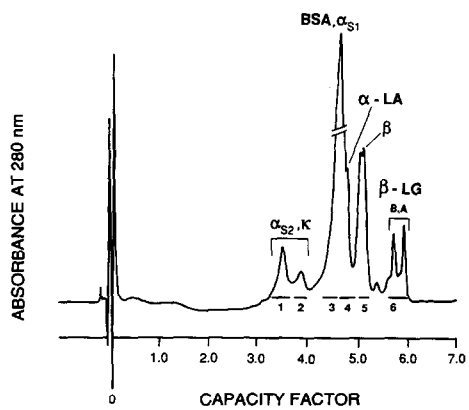

Fig. 2. C_4 Reversed-phase chromatogram of non-fat dry skim milk. Eluent is TFA–water–acetonitrile (0.1:70:30) to (0.1:40:60). Capacity factor is calculated from the position of peak maximum (t_R) and the dead time (t_0) of the column. Capacity factor equals $(t_R - t_0)/t_0$. Courtesy of N. Parris (unpublished data).

and whey proteins of a non-fat dry milk on a C_4 column.

2.2.1.2. Casein peptides

C_{18} columns in conjunction with acetonitrile gradients separate β-casein tryptic peptides at pH 2.1 [55] and pH 6.5 [56]. Tryptic and chymotryptic digests of α_{s1}-casein B are resolved with isocratic pH 2.1 95% acetonitrile [57]. Over 200 peptides were identified in casein hydrolysates [58,59] employing acetonitrile gradients at pH 2.1 and 6. C_8 chromatography [60] of a β-casein tryptic digest using pH 2.1 acetonitrile gradient resolved seven major peaks. C_4 chromatography at pH 2.1 of tryptic and chymotryptic digests of β-casein and α-LA was used to identify sites that are phosphorylated with casein kinase [61]. In comparison to size-exclusion and anion-exchange high-performance chromatography, reversed-phase C_{18} is superior in separation and resolution of peptides [62].

2.2.1.3. Cheese

The separation and identification of peptides present in cheese that contribute to its flavor, that result from aging, or that are due to type of cheese, source of cheese milk or type of rennet used in producing the cheese were studied by reversed-phase chromatography. A bitter extract of Cheddar contained 44 amino acids and/or peptides [63] whose elution order from a C_{18} column was influenced by both molecular mass and hydrophobicity. Chromatographic profiles of Gouda water extracts [64], using a C_{18} column and an acetonitrile gradient, showed three peptides, produced by lactic acid bacterial protease activity on α_{s1}-casein (f1–23), that increased with increasing cheese age. Similarly, profiles of acid precipitates of Danbo and Havarti cheeses [65] showed differences in degradation of α_{s1}- and β-casein with type of cheese, in age of cheese, source of the cheese milk and type of rennet. Water soluble and low molecular weight peptides of blue cheese [66], separated by C_{18} RP-HPLC with an acetonitrile gradient, increased as the cheese aged.

RP-HPLC was investigated as a tool for the evaluation and identification of cheeses. A C_8 column, using isocratic elution with 0.1 *M* phosphate, pH 6 was used to determine Cheddar cheese age [67]. A 65.9% correct prediction of age was achieved. A C_8 column using a ternary gradient system with an initial solvent of 0.1% TFA in water–acetonitrile–methanol (96.8:1.2:2) to a final ratio of solvents (56.3:30.3:13.4) with 0.1% TFA [68], and an internal standard of *p*-dimethylaminobenzaldehyde, could classify Cheddar, Edam, Gouda, Swiss and Parmesan correctly more than 90% of the time using multivariate analysis of HPLC data. Water extracts of Cheddar were used to evaluate the performance of four different types of C_{18} RP columns [69] and C_{18} RP-HPLC was used [70] to evaluate differences in cheese extraction procedures.

2.2.1.4. Whey proteins

Whey proteins can be separated and measured with reversed-phase columns. BSA, α-LA and β-LG were separated and identified [71] using a C_8 column and an isopropanol gradient. A C_6 column using an acetonitrile gradient [72] separated β-LG A, β-LG B, and partly, β-LG C as well as BSA and α-LA. Whey proteins from skim milk (BSA, α-LA, immunoglobulin G with minor β-casein fragment contaminants, β-LG B and β-LG A) [73], and β-LG in a model system [74], separated and were quantified on a C_4 column with an acetonitrile gradient and the amount of whey denaturation due to heat treatment determined. A short (5 cm) C_4 column was used to detect the presence of a bovine α-LA peak [75] in a 10% cow–90% ewe milk cheese. RP-HPLC can be used to detect the presence of casein and whey proteins in unheated meats [76] and rennet whey in skim and buttermilk powder [77].

2.2.2. Recommendations

Reversed-phase C_4, C_8 and C_{18} wide-pore (300 Å) silica-based columns separate the caseins, whey proteins and their various digests. Generally, resolution improves as hydrocarbon chain length of the column increases and quantitation is acceptable. Column chromatographic analysis of low-molecular-mass cheese peptides complements electrophoresis.

Caseins and whey proteins are soluble at pH < 3, especially in the presence of acetonitrile; therefore, neutral or alkaline conditions are not needed for solubility but the presence of urea and ME in the sample buffer when analyzing caseins improves resolution. Separation of milk proteins on reversed-phase columns is based on hydrophobicity of the proteins, ion exchange with the silica, as well as molecular mass effects.

Reversed-phase solvent systems are much simpler to use than those used in ion exchange. The urea used in ion-exchange buffers must be purified by passing through an ion exchanger to remove impurities before use and special equipment is needed for use with NaCl. Subsequent electrophoretic analysis from RP-HPLC is easier because non-volatile salts are absent.

2.3. *Size-exclusion chromatography*

Table 3 lists the recent contributions to the literature on the use of size-exclusion chromatography in the analysis of milk proteins.

2.3.1. *Preparative separations*

2.3.1.1. *Casein micelles*

Controlled pore glass beads (CPG-10 50 nm) were used to separate casein micelles from other milk proteins [78] and to separate them according to micelle size (CPG-10 300 nm) [78–82]. Before chromatography, milk samples are usually fixed with gluteraldehyde and the columns pretreated with 1% polyethylene glycol [79–82]. Buffers used for elution are Jenness–Koops [83] buffer pH 6.6 [79,80]; 5 m*M* calcium chloride, 50 m*M* NaCl, 20 m*M* imidazole pH 7 [81]; or, a synthetic milk serum containing 0.04% polyethylene glycol [78]. Fractionation of

TABLE 3

SUMMARY OF SIZE-EXCLUSION CHROMATOGRAPHY METHODS USED FOR BOVINE MILK PROTEINS

Column	Protein	Ref.
Preparative separations		
Controlled Pore Glass	Casein micelle	78–81, 182
Fractosil S-1000	Casein micelle	13, 84
Sephacryl S-200	Casein[a]	174
Sephadex G-25	Casein peptides	62
Sephadex G-50	Glycomacropeptide	85
Sephadex G-75	Proteose–peptone	12
Sephacryl S-200	Proteose–peptone	102
Sephadex G-10	Cheese	86
Sephadex G-50	Cheese	63
Sephacryl S-200	Whey proteins	22
Sephacryl S-200	Minor proteins	87, 88
Sephacryl S-300	Immunoglobulins	89
Fractogel TSK HW	Immunoglobulins	89
Sephacryl S-200	MFGM proteins	91
Sephadex G-200	MFGM proteins	90
Sephadex G-10	Minor proteins[b]	178
Analytical separations		
TSK 4000 SW	Casein micelles	34, 183
TSK 4000 SW	Artificial micelles[b]	35
dual TSK 2000 SW	Glycomacropeptide	93, 184
TSK 2000 SW	Casein peptides	59
dual TSK 2000 SW and TSK 3000 SW	Whey proteins[b]	99
TSK 3000 SW	Whey proteins	95, 96
Superose 12	Whey proteins[a]	26, 37, 100, 185
GPC-100	Whey proteins	71
TSK 4000 SW	Lactoferrin[a]	49

[a] Includes buffalo, caprine, ovine, porcine and/or rat.
[b] Human milk proteins.

casein micelles on large-pore silica gel is less successful than with CPG [81]. Sephacryl S-1000 with Jenness–Koops buffer is used to separate casein micelles [13,84].

2.3.1.2. Casein peptides

Andrews and Alichanidis [12] separated the proteose–peptone fraction into PP3, PP5 and PP8f on Sephadex G-75 and Zevaco and Ribadeau-Dumas [85] used Sephadex G-50 fine to purify the glycomacropeptides already separated by DEAE-cellulose. Lemieux and Amiot [58,59,62] used size-exclusion chromatography on Sephadex G-25 as the first step in the fractionation and identification of peptides in the hydrolysates of whole casein.

2.3.1.3. Cheese

Chromatography on Sephadex G-50 gave four fractions from an extract of bitter Cheddar cheese large enough for sensory analysis [63] and Sephadex G-10 chromatography isolated the peptides from the amino acids in a phosphotungstic acid soluble fraction of blue cheese [86].

2.3.1.4. Whey and minor proteins

Yoshida [22] used Sephacryl S-200 chromatography to isolate β-LG and α-LA from acid whey using strong acid conditions or high salt concentrations. Sephacryl S-200 chromatography was also used for isolation of xanthine oxidase and lactoferrin [87] and lactoperoxidase [88] from acid whey. Immunoglobulins and lactoferrin were isolated from colostral, cheese and acid wheys by size-exclusion chromatography on Sephacryl S-300 and on Fractogel TSK HW-55. Gel permeation on Sephacryl S-300 yielded immunoglobulin fractions of 99% purity from colostral whey, 83% purity from acid whey, and 92% purity from cheese whey [89].

Sephadex G-200 was used to separate the water soluble portion of the MFGM proteins [90] and to evaluate the efficiency of washing procedures used in the preparation of MFGM proteins [91].

2.3.2. Analytical separations

2.3.2.1. Casein micelles

Analytical (high-performance) liquid chromatography with a TSK GEL G4000SW column separated casein micelles, partially disaggregated by 6 *M* urea Jenness–Koops buffer but held in micelle form by colloidal calcium phosphate, from other milk proteins [34,35].

2.3.2.2. Casein peptides

Mottar *et al.* [92] used size exclusion on a TSK 2000 SW column (exclusion limit approximately M_r 100 000) to separate TCA-soluble peptides to identify milk suitable for UHT processing. Vreeman *et al.* [93] used two TSK 2000 SW columns in tandem to quantitate the glycomacropeptide. The TSK 2000 SW columns were conditioned with glycomacropeptide to avoid unwanted absorption of the glycomacropeptide during analytical runs. TSK 2000 SW columns were also used to separate casein polypeptides [58].

2.3.2.3. Whey and minor proteins

Whey proteins have been separated using the following size-exclusion columns: a 30 cm TSK 3000 SW (exclusion limit M_r 350 000) in tandem with a 30 cm TSK 2000 SW [94]; a 30 cm TSK 3000 SW [95]; and a 60 cm TSK 3000 SW [96]. Denaturation of whey proteins in commercial whey protein isolates and concentrates [97,98] was determined by size-exclusion chromatography on a 30 cm TSK 3000 SW column. Human whey proteins (immunoglobulins, lactoferrin, BSA, α-LA and lysozyme) were separated with dual 50 cm TSK 3000 SW and 50 cm TSK 2000 SW columns [99]. Superose 12 columns, with a reported separation range of M_r 1000–300 000 have been used to separate bovine immunoglobulins, BSA, β-LG and α-LA [26]. A comparison of caprine and bovine whey proteins by Superose 12 showed similarities between bovine β-LG and caprine β-LG and bovine α-LA and caprine α-LA. An unidentified lower-molecular-mass protein in caprine whey was the major difference found [100].

2.3.3. Recommendations

Preparative size-exclusion chromatography on CPG or Sephacryl S-1000 is the only effective way to separate casein micelles by size. One of the major difficulties with this technique is detection and evaluation of the micelle size. Size exclusion does not separate the caseins because their molecular masses are similar, but is used in preliminary separation of casein hydrolysates or of cheese extracts before

peptide mapping. Size exclusion is also valuable in the isolation of minor proteins from whey.

Analytical (high-performance) size-exclusion chromatography is useful for the separation of casein submicelles and whey proteins. However, it does not separate genetic variants of β-LG and the similarity in size between species of whey proteins probably will not permit species identification by this approach.

2.4. Hydrophobic chromatography

Table 4 lists the less commonly used types of chromatography, the specific supports and the proteins they are used for.

2.4.1. Preparative separations —minor proteins

Preparative hydrophobic chromatography is usually done to purify the minor proteins present in acid or cheese whey. Butyl Toyopearl 650M was used to purify minor proteins from acid whey [43,87,88,101]. Hydrophobic chromatography on Phenyl-Sepharose CL 4 B helped characterize the proteose–peptone fraction of milk by strongly absorbing the PP3 fraction confirming its possible origin in the MFGM [102]. Hydrophobic chromatography may be useful for separation of MFGM proteins. Lindahl and Vogel [103] exploited the hydrophobicity of calcium free α-LA to prepare α-LA from cow, human, goat, sheep and horse milks by hydrophobic chromatography. The separation was rapid and efficient. Sephadex LH-20 was used to separate bitter peptides from extracts of Gouda cheese by isocratic elution with 70% aqueous *n*-propanol [104].

TABLE 4

SUMMARY OF HYDROPHOBIC AND AFFINITY CHROMATOGRAPHY METHODS USED FOR MILK PROTEINS

Column	Protein	Ref.
Hydrophobic chromatography —preparative separations		
Phenyl Sepharose CL-4B	Proteose–peptone	102
Sephadex LH	Cheese	104
Phenyl Sepharose	α-Lactalbumin[a,b]	103
Butyl Toyopearl 650M	Minor proteins	43, 87, 88, 101
Hydrophobic chromatography —analytical separations		
Phenyl 5 PW	α_{s1}-Casein	106
Phenyl-Superose	Casein and whey	105
Affinity chromatography —preparative separations		
Thio-Sepharose 4B	Casein	108
Hydoxyapatite	Proteose–peptone	102
Sephadex G-25-Cu	Casein peptides	62
Cu^{2+}-Chelating Sepharose	Immunoglobulins	110
DNA-Agarose	Lactoferrin[a]	49
Cibacron Blue F3GA	Lactoferrin	44
Concanavalin A-agarose	MFGM protein	186
Affinity chromatography —analytical separations		
Hydroxyapatite	Casein	33
TSK chelate-5 PW/Cu^{2+}	Lactoferrin[a]	49

[a] Includes buffalo, caprine, equine, ovine, porcine and/or rat.
[b] Human milk proteins.

2.4.2. Analytical separations —caseins

Caseins were separated using a Phenyl-Superose column. Order of elution was β-, α_{s2}- and γ-, κ- and α_{s1}-casein [105]. Semipreparative hydrophobic chromatography on a Spherogel TSK-G Phenyl 5 PW column was used to purify α_{s1}-casein by removing traces of β- and α_{s2}-casein and degradation products [106]. Hydrophobic chromatography seems to be an attractive way to isolate caseins because they are extremely hydrophobic proteins but little research has been done.

2.5. Affinity chromatography —preparative and analytical separations

2.5.1. Caseins

Hydroxyapatite is classified as an affinity technique for caseins because their phosphate groups are assumed to interact with calcium and elution is carried out by increasing phosphate buffer concentrations. Hydroxyapatite was used to purify large amounts of bovine and buffalo κ-casein from whole casein [107]. Caseins eluted in the order of increasing number of phosphates: κ-casein (1P), β-casein (5P), and α_s-caseins. α_{s1}-Casein (8–9P) did not separate from α_{s2}-casein (11–13P). High-performance hydroxyapatite chromatography on whole caseins showed similar results except that the κ-casein A and B separated to some extent and the γ-caseins eluted in the solvent peak [33].

Ovine caseins were separated into two fractions on activated thiol Sepharose 4B after reducing the κ- and α_{s2}-caseins with dithiothreitol by binding to the support thiol groups. The bound caseins were eluted with cysteine [108].

2.5.2. Minor proteins

Lactoferrin and immunoglobulins can be isolated from acid and cheese wheys by metal chelating chromatography on a Sepharose 6B support with immobilized copper (Cu^{2+}) [109]. Activity of immunoglobulins was improved by alteration of the elution scheme [110].

Lactoferrin was purified by DNA affinity chromatography of urea-treated colostral whey [49]. Purity of the isolated lactoferrin was checked by HPLC on TSK gel chelate-5PW loaded with Cu^{2+}, phenyl reversed-phase, cation exchange on Mono-S and size-exclusion on TSK4000SW.

3. ELECTROKINETIC TECHNIQUES

Electrophoresis has been universally adopted for research on proteins since the introduction of polyacrylamide as a support [111,112]. The technique has progressed from separations based only on charge/mass (PAGE) to those based on molecular mass (SDS-PAGE) or isoelectric point (isoelectric focusing, IEF). Combinations of techniques providing 2D electrophoresis are commonplace. The recent introduction of precast ultrathin minigels and an automated system for performing electrophoresis, staining, and destaining (PhastSystem; Pharmacia-LKB, Piscataway, NJ, USA, and Uppsala, Sweden) has reduced analysis time from several days to a few hours and improved detection limit to 1 ng of protein.

3.1. Electrophoresis under non-denaturing conditions

The procedures of Groves for PAGE and urea-PAGE in alkaline or acid buffers [113] are still used with modification. In PAGE, proteins are negatively or positively charged, depending on buffer conditions, and migrate with mobilities related to their charge/mass ratio. For caseins, the addition of urea prevents aggregation and, at acid pH, also prevents precipitation. Urea is not considered a denaturant because the tertiary structures of caseins are more unordered than typical globular proteins. The advantages for milk proteins are that bands are well separated and genetic variants and differences in degree of phosphorylation can be readily detected. Information on relative mass is absent, however, and standards of pure caseins and/or whey proteins should be included on a gel. Moreover, there are no clues to the identity of unknown bands. Urea-PAGE has been adapted for use with the PhastSystem [114] (Fig. 3). Applications of PAGE and urea-PAGE are listed in Table 5.

TABLE 5

PAGE AND UREA-PAGE OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Milk proteins	Characterize proteose–peptone fraction	102
Milk proteins	Quantitate caseins, whey proteins	115, 116
Milk proteins	Quantitate caseins, whey proteins; compare with cellulose acetate	117
β-Casein	Proteolysis by plasmin yields γ-caseins	187
α-Casein	Determine chymosin cleavage sites	121
β-Casein	Determine cleavage sites of chymosin and pepsins	122
Bovine, buffalo casein	Comparison after rennet and other enzymes	188
Ovine casein	Determine heterogeneity	189
Whey proteins	Characterize effect of Ca^{2+} on mobility	190, 191
Buffalo whey	Characterize whey proteins	192
Cheddar cheese extracts	Effect of proteinase negative starter cultures on flavor	120
Mozzarella cheese extracts	Protein breakdown during refrigerated storage	193
Caprine milk	Detect bovine milk addition	194, 195
Ovine cheese extracts	Detect bovine milk addition	123
Ovine, caprine cheese extracts	Detect bovine milk addition	124

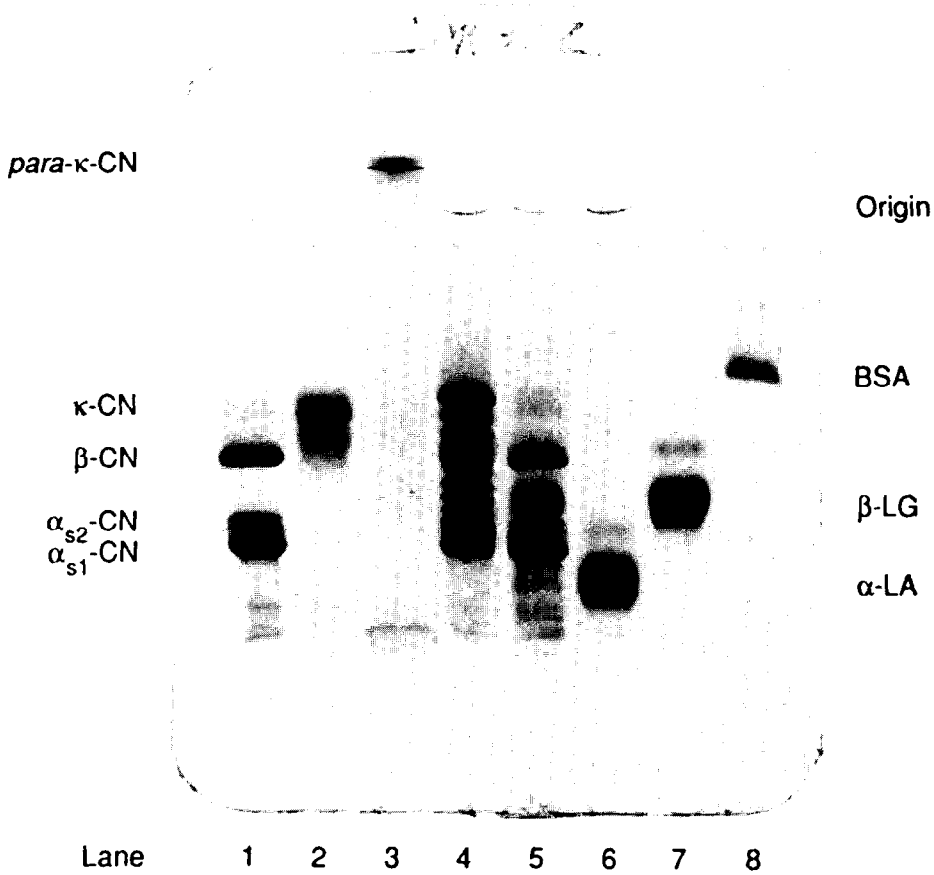

Fig. 3. Urea-PAGE analysis of bovine caseins and whey proteins on 8 to 25% gradient PhastGels and Coomassie Blue R250 staining. Samples contained 2-mercaptoethanol; buffer did not. Lanes: 1 = whole casein; 2 = κ-casein; 3 = *para*-κ-casein; 4 = 40% dephosphorylated whole casein; 5 = skim milk; 6 = α-LA; 7 = β-LG; 8 = bovine serum albumin. From ref. 114.

3.1.1. Caseins and whey proteins

After comparing polyacrylamide-agarose gels in urea-PAGE with analytical anion-exchange chromatography, Collin *et al.* [115] concluded that electrophoresis provides the greatest amount of information for quantitating bovine milk caseins, despite the 6% variability in the uptake of Coomassie Blue R250 by each casein. PAGE without urea gave better results for identifying genetic variants of β-LG [116]. Deshmukh *et al.* [117] also observed differences in absorption of aniline blue black (Amido black) among the caseins after PAGE and concluded that cellulose acetate electrophoresis was better for quantification of milk proteins.

3.1.2. Cheese

Fox [118] has reviewed many applications of electrophoresis to the analysis of cheese. Grappin *et al.* [119] reviewed studies using PAGE to analyze casein breakdown arising from the action of plasmin, starter culture proteinases and rennet. Detailed diagrams showing relative mobilities of the major peptide products are an excellent information source. In studying flavor development in Cheddar cheeses made with proteinase-negative or proteinase-positive starter cultures, urea-PAGE analysis showed that there was no correlation between flavor and casein breakdown [120]. Model studies of α_{s1}- and β-casein cleavages by chymosin [121,122] have been

important contributions to current knowledge of ripening mechanisms.

3.1.3. Detecting adulteration

The addition of bovine milk to cheese milk used for cheeses made from ewe milk [123] and from ewe and goat milks [124] was quantitated by PAGE. In both instances, the presence of bovine β-LG A and B at specific distances from the origin provided the necessary proof of adulteration. Both PAGE and IEF were equally sensitive for detecting adulteration of ewe cheese by addition of bovine and/or goat milk [125].

3.2. Electrophoresis in the presence of sodium dodecyl sulphate

The discovery that SDS binds to proteins at a ratio of 1.4 g per gram of protein [126] initiated a new class of electrophoretic separations. When disulphide bonds are broken by ME, SDS binding alters the protein chain completely. Although several structures have been proposed for SDS-protein complexes, the empirical result is that migration of the complexes in an electric field depends on mass alone. The well-known plot of log molecular mass *vs.* mobility permits estimation of protein molecular masses more easily than analytical chromatography or analytical centrifugation [127,128]. Table 6 lists specific applications.

3.2.1. Caseins and whey proteins

An SDS-PAGE method for quantitating caseins and whey proteins in processed milk powders or fluid milk, developed by Basch *et al.* [129], is based on the Laemmli procedure [130]; thiourea and 30% hydrogen peroxide were substituted for TEMED and ammonium persulphate, respectively (Fig. 4). Meisel and Carstens [131] concluded that SDS-PAGE is highly appropriate as a screening method

TABLE 6

SDS-PAGE OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Non-fat dry milk, whey protein concentrate	Quantitate caseins and whey proteins in processed milk	129
Caprine milk	Characterize proteose–peptone fraction	137
Porcine milk, colostrum	Characterize casein and whey proteins	196
Whole cascin	Detect proteolytic action of *Streptococcus lactis* strains	197
α-, β-Casein	Anomalous mobility of α-casein	132
α-Casein	Plasmin cleavage sites	134
α-, κ-Caseins	Effect of plasmin	133
β-Casein	Identity of plasmin peptides with proteose–peptone fraction	12, 135, 136
κ-Casein	Association behavior	198
α-LA	Characterize effect of Ca^{2+} on electrophoretic behavior	191
Lactoferrins	Compare bovine, ovine and caprine	199
Cheese extracts	Proteolysis of Parmesan, Cheddar, Port Salut and Brie	139
Cheddar, stirred-curd cheese extracts	Effect of frozen storage	143
Low-fat Cheddar cheese extracts	Compare effects of starter culture additions	141
Cheddar cheese extracts	Effect of pasteurization on protein breakdown	140
Mozzarella cheese extracts	Compare breakdown in low-fat and high-fat cheeses	142
Dairy products	Quantitate casein and whey proteins	131
Buttermilk powder	Detect adulteration by NFDM	144
Meat products	Detect casein, whey proteins	200

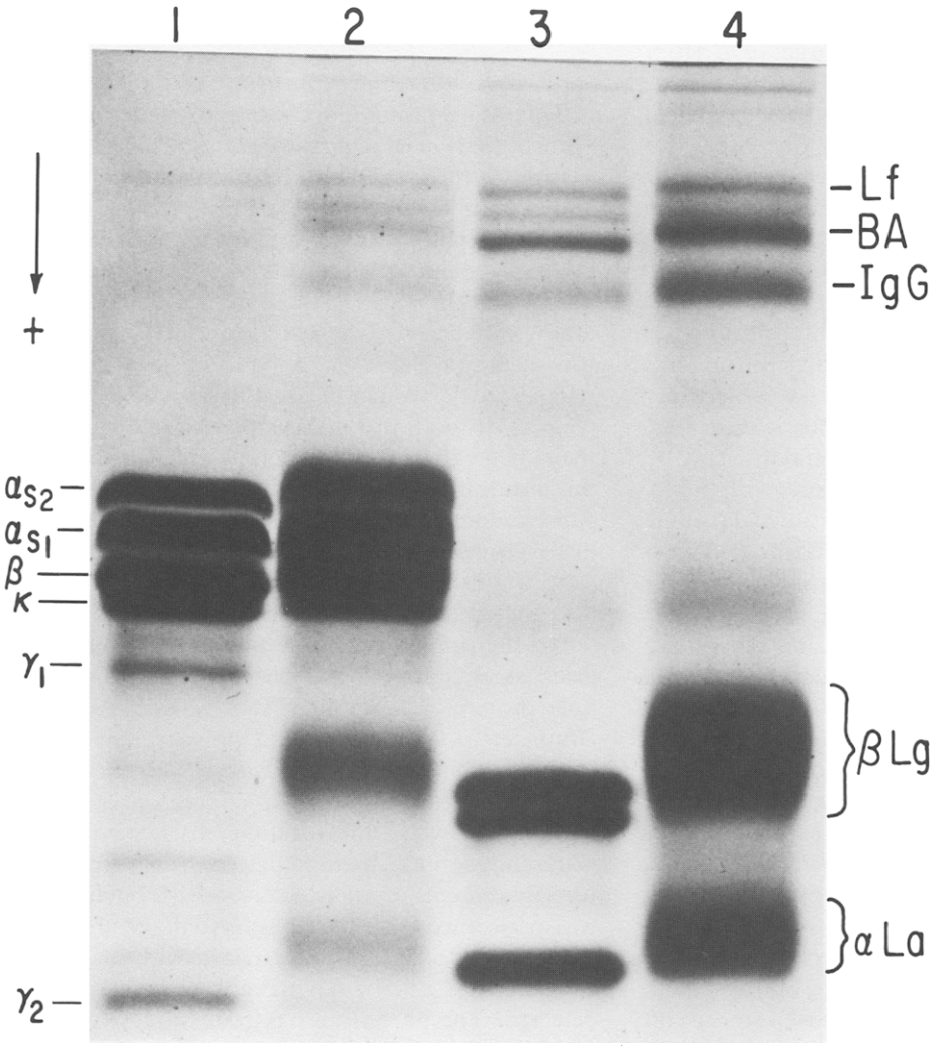

Fig. 4. SDS-PAGE comparison of laboratory-prepared whole caseins (lane 1) and whey (lane 3) with processed dairy products (non-fat dry milk, lane 2, and whey protein concentrate, lane 4). Gels were 18 × 14 cm and 3 mm thick and stained with Coomassie Blue R250. Lf = Lactoferrin; BA = bovine serum albumin; IgG, heavy chain immunoglobulin G; β-Lg = β-lactoglobulin; α-La = α-lactalbumin; α_{s2}, α_{s1}, β, κ, γ_1 and γ_2 = various caseins. From ref. 204.

for determining whey protein and casein contents of milk products.

Although proteins separate in SDS-PAGE according to mass, a somewhat anomalous behavior has been observed in the retardation of α_{s1}-casein relative to the slightly larger β-casein. Creamer and Richardson [132] found that although both α_{s1}- and β-caseins bound 1.3 g of SDS per gram of protein, α_{s1}-casein had an unexpectedly large hydrodynamic size. SDS-PAGE of pure α_{s1}-casein treated with plasmin indicated the formation of three lower-molecular-mass bands ranging from 20 500 to 10 300 [133]. Similarly, sites of plasmin attack on α_{s2}-casein were studied by Visser *et al.* [134] in the isolated protein.

The proteose–peptone fraction has been extensively investigated with SDS-PAGE [12,102,135,136]. Nearly all of this heterogenous mixture in bovine

milk can now be accounted for by plasmin-mediated proteolysis of β-casein, although Paquet *et al.* [102] have suggested that a higher-molecular-mass portion may be a fragment of MFGM. Molecular masses of the proteose–peptone components of goat milk, determined by SDS-PAGE, were similar to those of bovine [137]. This has been confirmed and extended to ewe milk, as well [138].

3.2.2. Cheese

SDS-PAGE provides valuable information for ripening studies when coupled with densitometric analysis of decreasing band intensity with time. Although casein breakdown can also be measured with PAGE, the increasing number and intensity of lower-molecular-mass bands observed with SDS-PAGE during ripening provides additional data and suggests further research. Characteristic patterns were found in SDS-PAGE comparisons of Parmesan, Cheddar, Port Salut and Brie cheeses [139]. Lau *et al.* [140] used SDS-PAGE to show that pasteurization can induce whey-casein interactions that could inhibit access of proteolytic enzymes in Cheddar cheese during ripening, thus influencing flavor development. The potential of *Micrococcus freudenreichii* or *Pediococcus pentosaceus* to accelerate ripening of low-fat Cheddar cheese at different temperatures was tested with SDS-PAGE after 6 months of ripening [141]. Tunick *et al.* [142] used SDS-PAGE to monitor breakdown of caseins and peptide formation in low-fat Mozzarella cheese during 6 weeks of storage. Basch *et al.* [143] also used SDS-PAGE to evaluate long-term frozen storage of traditional and stirred-curd Cheddar cheeses in terms of quality for process cheese manufacture.

3.2.3. Detecting adulteration

The availability of molecular mass data, in addition to separation of components, makes SDS-PAGE a method of choice for monitoring activities, especially when adulteration is suspected. For detecting adulteration of buttermilk powder, Holsinger *et al.* [144] developed an SDS-PAGE procedure based on the presence of three specific MFGM bands characterized earlier by Basch *et al.* [91]. The method can be used with the PhastSystem.

3.3. Isoelectric focusing

3.3.1. Caseins and whey proteins

Separating proteins according to their isoelectric points (p*I*) is particularly appropriate for analyzing caseins, as there are many genetic variations among and within species. Analysis of bovine β-casein genetic variants is simplified by use of IEF [145]; otherwise, both alkaline and acid PAGE would be needed to differentiate A variants from B, C and D and A^1, A^2 and A^3 from each other. Rapid methods for identifying genetic polymorphism in bovine milks were developed for the PhastSystem [146,147] (Fig. 5). In both procedures, precast IEF gels were incubated with urea and ampholytes before use. IEF on ultrathin (50 μm) polyacrylamide gels was a superior method for detecting heat-induced changes in whey proteins [148].

3.3.2. Detecting adulteration

An IEF method for determining the content of dried skim milk, casein, whey proteins, and total milk protein in compound feeds was developed by Braun *et al.* [149]. Ultrathin polyacrylamide gels containing urea were used with α_{s1}-casein B as a marker for casein and α-LA B plus β-LG A as markers for whey. Substitution of whey solids for skim milk could be readily detected. IEF for monitoring adulteration of ewe cheese with bovine milk was performed with the PhastSystem on urea-containing polyacrylamide gels cast with a special assembly [150]; 0.5% bovine milk content could be detected. The procedure has been successfully applied to detection of bovine milk in Roquefort cheese [151], even after 5 months of ripening.

IEF detection of bovine milk in ewe milk or cheese (as low as 0.5%) was enhanced by adding plasmin to generate γ_2-casein *in vitro* [152]. Monitoring *para*-κ-casein as a marker in an IEF procedure for detecting bovine milk in ewe hard cheese was successful [153], but false positive responses occurred in ripened Roquefort because of a peptide migrating with bovine *para*-κ-casein. Additional applications appear in Table 7.

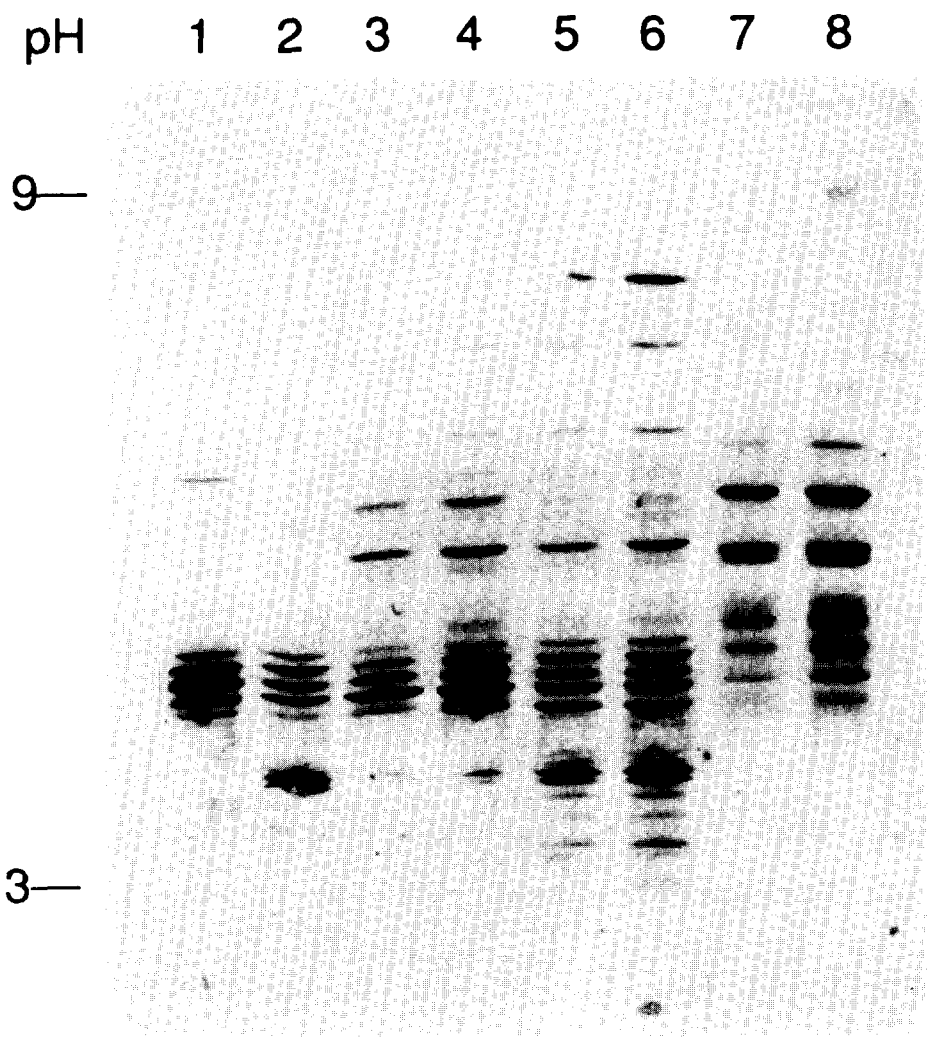

Fig. 5. IEF of bovine caseins on PhastGels using modifications described by Vegarud *et al.* [146]. The ampholyte solution (Serva) contained one part pH 4.0–6.0 and two parts pH 5.0–7.0 (v/v). Lanes: 1 = α_{S2}-casein; 2 = α-casein complex; 3 and 4 = crude β-casein; 5 and 6 = whole casein; 7 and 8 = κ-casein B. Courtesy of E. D. Wickham and H. M. Marrell, Jr. (unpublished data).

3.4. Two-dimensional electrophoresis

The combination of IEF, usually in gel rods, followed by SDS-PAGE on a slab gel (2D electrophoresis), provides the greatest differentiation among casein and whey components of milk and other dairy products. In a modified 2D electrophoresis method for whey proteins [154], IEF over a range of pH 3 to 8 was followed by PAGE without SDS in a gradient gel. However, the gradient did not compensate for the absence of denaturant, and a cluster of spots resulted. Bovine milk proteins were analyzed by IEF in the presence of a non-ionic detergent and urea with a pH range from 3 to 10; SDS-PAGE on a 14% acrylamide slab gel followed [155]. The method was used to show that high-speed centrifugation of raw skim milk results in removal of a large portion of α_S-caseins; β-casein was unchanged. The same workers used capillary tubes for IEF and a minigel for the slab to obtain enhanced

TABLE 7

IEF OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Caseins, whey proteins	Identify genetic polymorphism	146
Caseins	Identify genetic variants	145
Caseins, whey proteins	Phenotype genetic variants	147
Milk proteins	Characterize proteose–peptone fraction	102
Whey proteins	Characterize heat-induced changes	148
β-LG B, H	Characterize β-LG H	201
Lactoferrins	Compare bovine, ovine and caprine	199
Bovine, ovine milk cheeses	Characterize γ_2-, γ_3-, and *para*-κ-caseins	145, 153
Ovine cheese extracts	Detect bovine milk addition	150, 151, 202
Ovine cheese extracts	Detect bovine, caprine milk addition	125
Ovine cheese	Detect bovine milk addition by enhancing γ_2-casein content	152
Feed materials	Detect caseins and whey proteins	149

resolution in much less time [156]. To investigate the occurrence of α_{S1}-casein in goat milk, a "reverse" 2D electrophoresis procedure was used [157]. The first dimension was electrophoresis on either starch–urea or polyacrylamide–agarose. This was followed by IEF on polyacrylamide. The 2D electrophoresis procedure provided the best resolution of caseins. Additional applications appear in Table 8.

3.5. Detection of protein bands on polyacrylamide gels

Methods for staining gels seem to be more numerous and diversified than the variety of procedures for electrophoresis. Two recent reviews are recommended [158,159]. Coomassie Blue R250 has been the most widely used general stain because its sensitivity is about 10 times greater than that of aniline blue black (Amido black), it binds more uniformly to proteins, and the colors of stained bands do not change as much in stored gels [160]. Unfortunately, Coomassie blue does not obey Beer's law at high concentrations and this can lead to problems in densitometry [161]. The advent of silver stains has increased sensitivity up to 100-fold [162], but the increased ability to analyze dilute samples and observe all components of a sample can be a

TABLE 8

2D ELECTROPHORESIS OF MILK PROTEINS

Proteins are bovine unless other species indicated.

Sample	Application	Ref.
Caseins	Effect of ultracentrifugation	155
Caseins	Effect of *Pseudomonas fluorescens*	156
Bovine, ovine and caprine milk	Characterize hydrophobic components of proteose–peptone fractions	138
Caprine caseins	Compare starch–urea, agarose–PAGE, IEF methodology	157
Caprine casein	Characterize α_{S1}- and α_{S2}-casein polymorphism	203
Ovine casein	Determine heterogeneity	189
Whey proteins	Determine p*I* values	154

mixed blessing. For regulatory applications, high levels of sensitivity are desirable, whereas protein purifications must now meet the more stringent requirement imposed by silver staining. Poehling and Neuhoff [163] have discussed the relative advantages of silver and Coomassie Blue stains.

A special technique for silver staining of PhastGels involves manual use of the Gelcode system (Pierce, Rockford, IL, USA) to give silver stained bands in several colors [164]. Similarly, in another silver stain modification [165], glycosylated proteins appear as colored bands while non-glycosylated proteins are black or grey. In addition, with double-staining techniques, Coomassie Blue R250 can be used after the silver stain (Bio-Rad, Richmond, CA, USA) to provide even greater differentiation, or a Coomassie-stained gel can be decolored and restained with silver [166]. Glycosylated proteins bind Coomassie Blue R250 very poorly but can be detected with the traditional periodic acid–Schiff stain [167], although the color intensity of the dye is low. The technique has been adapted for detection of glycoproteins on PhastGels after PAGE, SDS-PAGE or IEF [7,168,169].

The newest methodology involves the use of metal salts, such as cupric chloride and zinc chloride, which yield clear bands on a colored background [170]. The technique is especially appropriate when proteins will be recovered from gels or subjected to immunoblotting, as fixing is not required before staining and the metal salt binds reversibly [171, 172]. Copper-based reverse stains are available from Bio-Rad and BDH (Poole, UK).

4. ABBREVIATIONS

BSA	Bovine serum albumin
CPG	Controlled pore glass
CM	Carboxymethyl
2D	Two-dimensional
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
LA	Lactalbumin
LG	Lactoglobulin
ME	2-Mercaptoethanol
Ig	Immunoglobulin
MFGM	Milk fat globule membrane
M_r	Relative molecular mass
NFDM	Non-fat dry milk
P	Phosphate bound to casein
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
PP3	Proteose–peptone component 3
PP5	Proteose–peptone component 5
PP8f	Proteose–peptone component 8 fast
QAE	Diethyl-(2-hydroxypropyl)aminoethyl
RP	Reversed-phase
SDS	Sodium dodecyl sulphate
SP	Sulphopropyl
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
UHT	Ultra-high temperature

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