Journal of Chromatography, 483 (1989) 319–329
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 770

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF THE EXTENT OF PROTEOLYSIS IN GOUDA CHEESE[a]

W. HAASNOOT*, P. STOUTEN and D. P. VENEMA
State Institute for Quality Control of Agricultural Products (RIKILT), Bornsesteeg 45, 6708 PD Wageningen (The Netherlands)
(First received April 3rd, 1989; revised manuscript received June 22nd, 1989)

SUMMARY

To determine the extent of proteolysis in Gouda cheese, a study was made of the possibility of using high-performance liquid chromatography as an alternative to nitrogen measurements on cheese extracts. Separations of the components present in water and trichloroacetic acid extracts and urea solutions of four Gouda-type cheeses, each of different maturity, were performed on an anion exchanger, a cation exchanger, a reversed-phase column and two gel filtration columns in series. Anion-exchange chromatography of urea solutions was selected for the further investigation of cheese proteolysis. After analysing 27 Gouda-type cheeses, it was concluded that the ratio of γ-caseins to β-casein peak areas can serve as an indicator of the maturity of cheese ripened for up to about 10 months, whereas the ratio of α_{s1}- to $\alpha_{s1\text{-I}}$-casein peak areas seems to be a suitable indicator for very young cheeses (<4 weeks).

INTRODUCTION

Dutch regulations require a minimum ripening time of 28 days at a minimum temperature of 12°C before Gouda and Edam cheeses may be sold[1]. For more aged cheeses there are no regulations. Cheeses being sold at retail outlets are divided into categories or age classes on basis of their ripening time[2] and the retail price is dependent on the category, *i.e.*, the younger the cheese, the cheaper it is.

To verify the ripening time or age, every cheese is labelled with a cheese mark containing a code indicating the production date and factory. However, with prepacked cheese it is often not possible to check whether the declaration of category is correct. The piece of cheese offered will only occasionally bear the cheese mark.

Recent investigation of methods of accelerating the ripening of cheese emphasize the need for objective methods to determine the degree of ripening because in such instances the category (class) will no longer be related to the ripening time as it is with traditionally produced cheeses.

The International Dairy Federation (IDF) has also expressed an interest in

[a] Presented at the *3rd FPLC Symposium, Maarssen, March 14, 1989.*

0021-9673/89/$03.50

determining the maturity of cheese by chemical, physical or sensory indicators[3]. The chemical parameters, particularly those reflecting protein breakdown (proteolysis), appear to be the most promising[4–8]. A widely used technique for assessing proteolysis involves solubilization of cheese in various solvents, such as water or trichloroacetic acid (TCA), with subsequent determination of the nitrogen content of the extracts[2,4–7]. However, with these nitrogen indices, only the smaller breakdown products are measured; no information is given about individual components and their assessment is time consuming. The measurement of proteins or more specific protein breakdown products might give a better correlation with ripening time.

A rapid automatic separation technique for determining such products would be attractive. For this reason, we have investigated the suitability of fast protein liquid chromatography (FPLC) on wide-pore columns with various stationary phases.

Urea solutions of four Gouda cheeses, of different maturity classes, were analysed to investigate differences in protein composition. To monitor changes in the smaller protein breakdown products, water and TCA extracts were analysed. The identities of the eluted peaks were, if possible, established by polyacrylamide gel electrophoresis (PAGE) or by FPLC with standard proteins and amino acids.

EXPERIMENTAL

Materials

Four Gouda-type cheeses, of the maturity classes young (1–2 months), young-matured (2–4 months), matured (4–7 months) and old (> 10 months) were obtained from a local market. Gouda block-cheeses ($n = 27$) of known age (between 40 and 300 days) were obtained from the Netherlands Government Purchasing Office and were taken from six different wholesalers.

Chemicals

The α_{s1}-casein standard was purchased from Merck (Darmstadt, F.R.G.) and the β-casein standard was kindly provided by the Netherlands Institute of Dairy Research (NIZO, Ede, The Netherlands). Tryptophan and tyrosine were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents and chemicals were of analytical-reagent grade from Merck.

Apparatus

The FPLC-system (Pharmacia, Uppsala, Sweden) consisted of two Model P-500 pumps, a Model LCC-500 liquid chromatography controller, a Model UV-1 single-path monitor (280 nm), a Model REC-482 two-channel recorder, a Model ACT-100 automatic injector and a Model FRAC-100 fraction collector. A Pye Unicam (Cambridge, U.K.) Model LC3 variable-wavelength UV detector was used for measurements at other wavelengths.

Polyacrylamide gel electrophoresis (PAGE) was performed with a Bio-Rad Labs. (Richmond, CA, U.S.A.) protein cell and an LKB (Bromma, Sweden) Model 2103 power supply.

Gel filtration chromatography

Two TSK 3000SW and 2000SW (300 mm × 7.5 mm I.D.) gel filtration columns (Toyo Soda, Tokyo, Japan) were used in series. The eluent consisted of 0.1 *M* sodium sulphate and 0.01 *M* sodium dihydrogenphosphate (pH 6.8) with or without 6 *M* urea. The eluent flow-rate was 0.5 ml/min and the injection volume was 50 μl.

Reversed-phase chromatography

A Pharmacia PRO RPC column (50 mm × 10 mm I.D.) was used. Eluent A was 0.1% trifluoroacetic acid (TFA) in water and eluent B was 0.1% TFA in acetonitrile, with a linear gradient from 0 to 30% B over 27 ml. The eluent flow-rate was 0.5 ml/min and the injection volume was 25 μl.

Cation-exchange chromatography

A Pharmacia Mono S HR 5/5 column (50 mm × 5 mm I.D.) was used. Eluent A was 20 m*M* citric acid (pH 3.2) with or without 6 *M* urea and eluent B was the same buffer with the addition of 0.5 *M* sodium chloride, with a linear gradient from 0 to 50% B over 50 ml, then to 100% B over 5 ml and, after 2 ml, to 0% B. The eluent flow-rate was 1 ml/min and the injection volume was 25 μl.

Anion-exchange chromatography

A Pharmacia Mono Q HR 5/5 column (50 mm × 5 mm I.D.) was used. Eluent A was 20 m*M* Tris–HCl (pH 8.5) with or without the additionof 6 *M* urea and eluent B was the same buffer with the addition of 1 *M* sodium chloride, with a gradient from 0 to 100% B as indicated in Fig. 5B. The eluent flow-rate was 0.5 ml/min and the injection volume was 50 μl.

Polyacrylamide gel electrophoresis

Electrophoresis was performed according to De Jong[9] using a 7% acrylamide gel containing 4.5 *M* urea. The gels were stained with Coomassie Brilliant Blue R250.

Sample preparation

Samples of about 1 kg were taken from the cheeses at least 4 cm from each side. After removing about 0.5 cm of the rind from the top and bottom, the cheese sample was ground according to Dutch standard NEN 3752.

Water extracts of cheese (in 0.037 *M* calcium chloride) were prepared according to the method described by Noomen[10]. The 0.037 *M* calcium chloride extract was adjusted to pH 7.5 and, after centrifugation (20 min at 28 000 *g*), the supernatant was filtered through a 0.45-μm filter (Gelman, Ann Arbor, MI, U.S.A.).

TCA extracts were prepared according to Venema *et al.*[2]: to 30 ml of the above water extract, 10 ml of 48% (m/v) TCA solution were added, a precipitate was allowed to form overnight at room temperature and, after centrifugation (10 min at 16 000 *g*), the supernatant was filtered (0.45-μm filter).

Urea solutions of cheese were made according to Haasnoot *et al.*[11]: to 2 g of ground cheese, 20 ml of 20 m*M* Tris-HCl (pH 8.5), containing 6 *M* urea, were added and the mixture was homogenized by means of an Ultra-Turrax (Janke and Kunkel, Staufen, F.R.G.) for 1 min. After centrifuging at 10 000 *g* for 10 min, the upper layer (fat) was removed and the supernatant filtered through a 0.45-μm filter.

RESULTS AND DISCUSSION

Four Gouda-type cheeses, each of different maturity, three extraction solvents and four separation techniques were investigated. Water and TCA extracts of the cheeses were analysed in order to study changes in the "smaller" protein breakdown products, such as (poly)peptides and amino acids. Such extracts were also used in earlier work to determine the soluble nitrogen-to-total nitrogen ratios and their correlation with the ripening time of Edam and Gouda block-cheeses[2].

The nitrogen contents of the four cheeses and the percentage of total nitrogen-soluble material in the extracts are given in Table I. Only part of the nitrogen present in cheese was soluble in water and a smaller part in TCA. The 12% TCA fraction is a widely used indicator of the maturity of Cheddar cheese and, according to Reville and Fox[5], this fraction contains the smaller peptides and amino acids.

Urea solutions were analysed in order to determine differences in protein composition. To dissolve a maximum amount of casein, the concentration of urea in the sample solution should be at least 6 M[11].

Gel filtration chromatography

Fig. 1 shows the separation of the components present in the urea solutions. Peak fractions were collected, analysed by PAGE and the components identified, if possible, by comparison with the two protein standards (α_{s1}- and β-caseins) and by comparison with electrophoretic separations described in the literature[12,13]. The principal cheese proteins, *i.e.*, α_{s1}-and β-casein (molecular weight, MW = 23 612 and 23 980 daltons, respectively[13]) and their degradation products $\alpha_{s1\text{-}I}$- and γ_1-casein (MW 20 800 and 20 500 daltons, respectively[13]) were found in peak 1. The area of this peak decreased slowly with increasing ripening time. The area of peak 2 increased with ripening time. This peak contained several components, including one with a higher and another with a lower electrophoretic mobility than γ_1-casein, which may correspond to γ_2- and γ_3-casein (MW 11 822 and 11 557 daltons, respectively[13]). An increase in the concentrations of γ_2- and γ_3-casein, identified as breakdown products of β-casein, during the ripening of cheese has been reported by Creamer[12].

Although the retention volumes of peaks 3 and 4 corresponded to those of standards of the aromatic amino acids tyrosine and tryptophan, respectively, these peaks could also contain small aromatic amino acids containing peptides, which were

TABLE I

NITROGEN CONTENT OF THE FOUR GOUDA-TYPE CHEESES AND THEIR WATER AND TCA EXTRACTS

Cheese class	*Total nitrogen in cheese (%)*	*Nitrogen (% of total)*	
		Soluble in water	*Soluble in TCA*
Young	3.92	12.5	9.4
Young-matured	3.96	21.7	14.4
Matured	4.04	24.5	17.3
Old	4.60	39.3	31.5

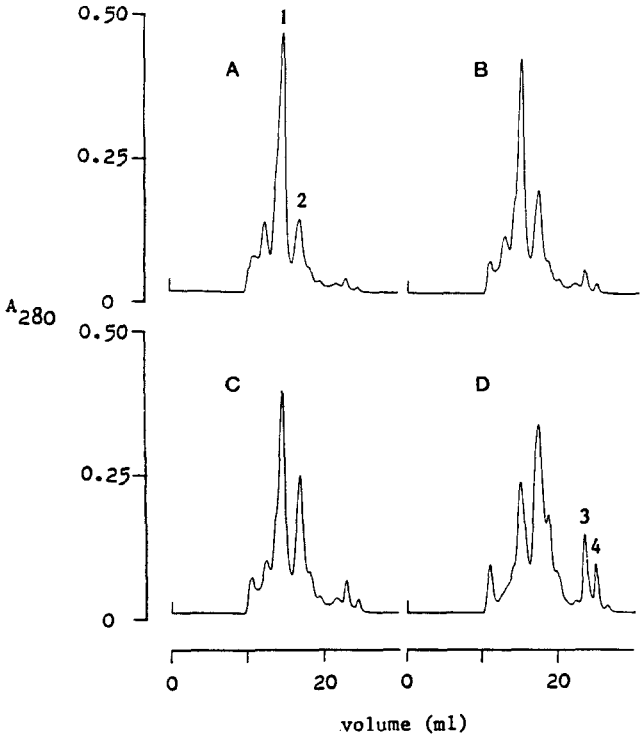

Fig. 1. Gel filtration chromatograms of urea solutions from four Gouda-type cheeses, each of a different maturity class: (A) young; (B) young-matured; (C) matured; (D) old.

not detectable by PAGE. As shown in Fig. 1, the concentration of these peaks increased with ripening time. Other peaks present in the gel filtration chromatograms could not be identified.

Gel filtration chromatography of urea solutions gives information about both the primary proteolysis, *i.e.*, formation of high-molecular-weight peptides, and the secondary proteolysis, *i.e.*, formation of smaller breakdown products such as amino acids or small peptides.

The gel filtration columns were not suitable for the separation of the components present in the water and TCA extracts. Two major peaks, with retention volumes corresponding to standards of the aromatic amino acids tyrosine and tryptophan, were detected at 280 nm, whereas detection at 220 nm resulted in a number of poorly separated components eluting between 18 and 25 ml.

Reversed-phase chromatography

The chromatograms of components present in the water extracts of the four cheeses are shown in Fig. 2. Many unidentified components were observed in the chromatogram of the older cheeses. Most of these components increased with ripening time. In the TCA extracts, almost all of these compounds are absent, indicating that they correspond to higher-molecular-weight peptides[6]. Pham and Nakai[14] ap-

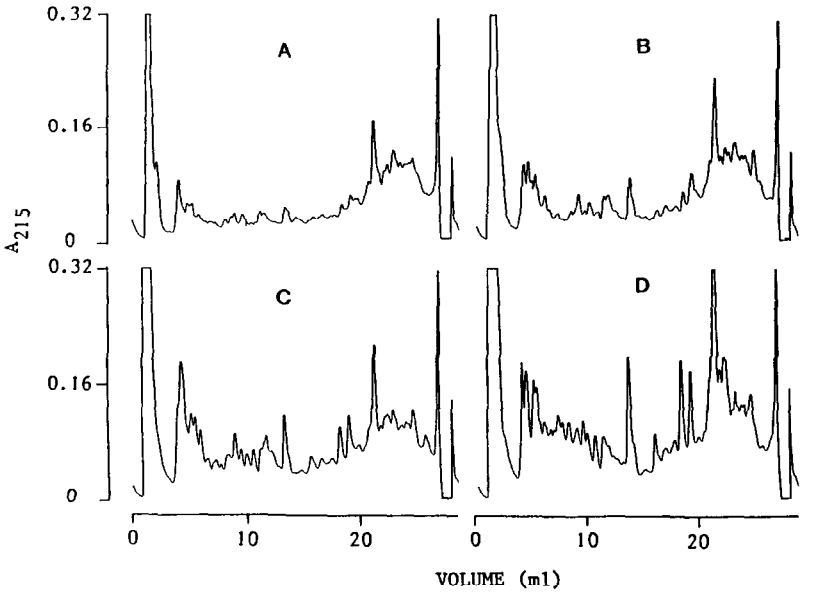

Fig. 2. Reversed-phase chromatograms of water extracts from the same cheeses as in Fig. 1.

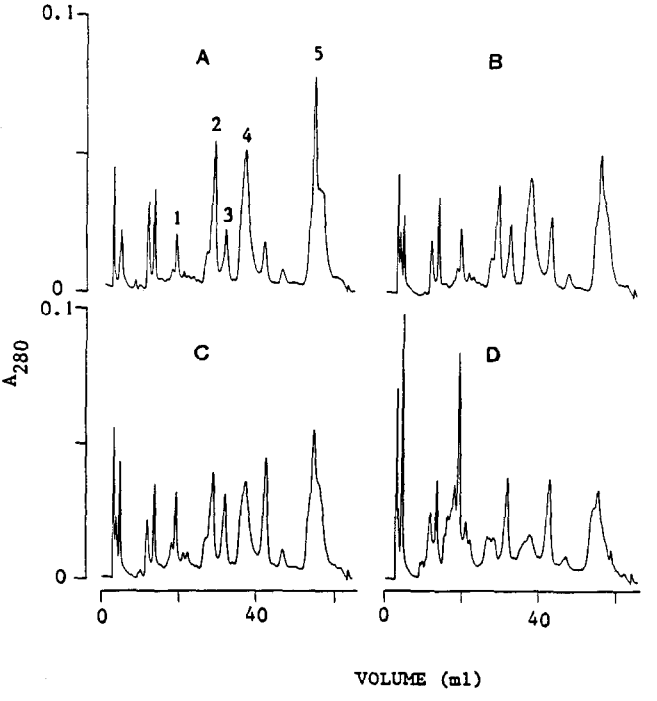

Fig. 3. Cation-exchange chromatograms of urea solutions from the same cheeses as in Fig. 1.

plied the same separation technique to water extracts of Cheddar cheese, and selected thirteen peaks for classifying cheeses into four maturity classes by stepwise discriminant analysis. However, in our opinion, a serious disadvantage of this technique is that the classification of cheeses is based on unidentified peaks.

Reversed-phase chromatography of urea solutions resulted in poor separation of the cheese proteins and their major breakdown products.

Cation-exchange chromatography

The chromatograms of urea solutions of the four cheeses are shown in Fig. 3. The indicated peaks were collected and analysed by PAGE. The γ-caseins, breakdown products of β-casein, were found in peaks 1 and 3. Peak 3 contained γ_1-casein and two other unidentified components. Both peaks 1 and 3 increased with ripening time. Peak 2 consisted of β-casein and some unidentified components. $\alpha_{s1\text{-}I}$-Casein was the main component in peak 4. Peaks 2 and 4 decreased with ripening time. Peak 5 contained α_{s1}-casein and, as this protein is present in low concentrations in ripened cheese, this peak must contain more components, although not detected by PAGE.

In the water and TCA extracts of the cheeses, only a few unidentified peaks were observed, the areas of which increased during ripening.

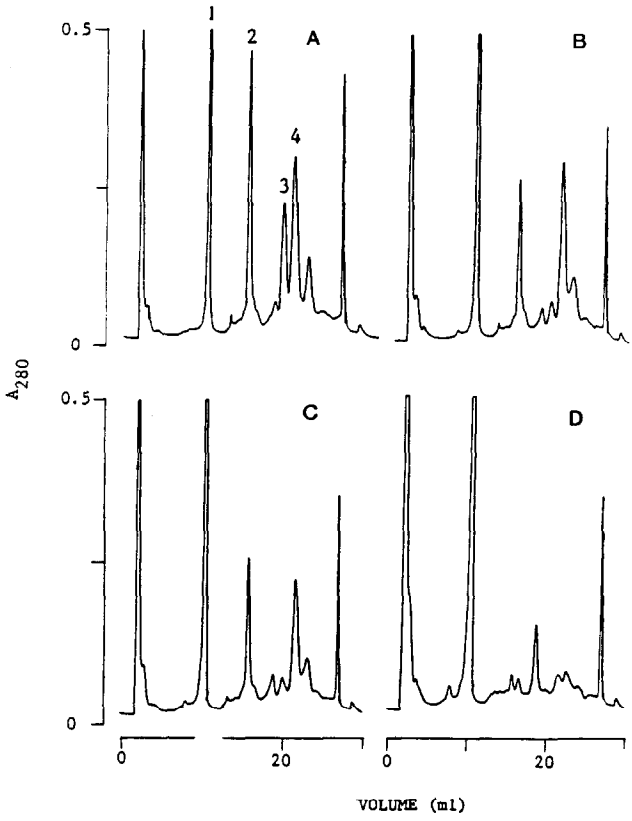

Fig. 4. Anion-exchange chromatograms of urea solutions from the same cheeses as in Fig. 1.

Anion-exchange chromatography

The chromatograms of cheese proteins present in urea solutions are shown in Fig. 4. Fractions were analysed by PAGE. Peak 1 contained γ-caseins (γ_1, γ_2 and γ_3). Peaks 2, 3 and 4 contained β-, α_{s1}- and $\alpha_{s1\text{-}I}$-casein, respectively. A gradual decrease in β-casein and an increase in γ-caseins with ripening time was observed. In young cheese, only a small amount of α_{s1}-casein was present, confirming that during the first month of ripening this protein is extensively degraded into $\alpha_{s1\text{-}I}$-casein[15]. The latter is degraded further during the ripening process. Amino acids eluted at the void volume of the column.

As with cation-exchange chromatography, the separation of water and TCA extracts resulted in only a few unidentified peaks, the areas of which increased with ripening time.

Grappin *et al.*[6] considered PAGE to be a very useful technique for the study of cheese ripening. As shown in Fig. 5, anion-exchange chromatography (AIEC) is a good alternative to PAGE for separating cheese proteins; in addition AIEC, with on-line detection, is much faster and more suitable for automatic operation.

The other chromatographic techniques assessed did not produce a better resolution of cheese proteins than AIEC. However, the proteins in old cheeses are (almost) completely degraded and for these cheeses a different technique, *e.g.*, the separation of identified peptides or amino acids by reversed-phase chromatography, is necessary.

As we were mainly interested in measuring proteolysis in cheeses in the maturity classes young, young-matured and matured[2], AIEC of the proteins present in urea solutions was selected for further investigation in cheese proteolysis. In this preliminary study, we analysed 27 Gouda block-cheeses of known age (40–300 days) ob-

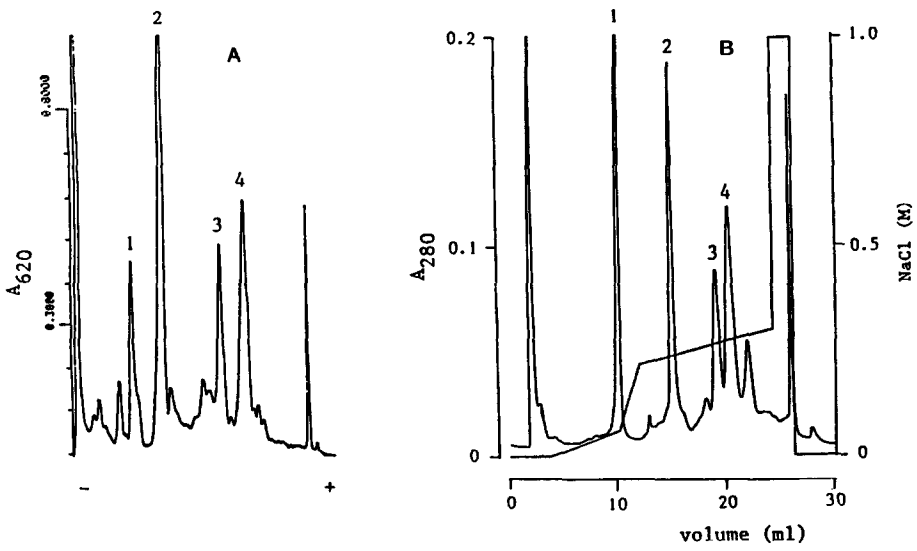

Fig. 5. Comparison of PAGE and AIEC for analysing a urea solution from a young Gouda-type cheese. (A) Densitogram; (B) chromatogram. 1 = γ-Caseins; 2 = β-casein; 3 = α_{s1}-casein; 4 = $\alpha_{s1\text{-}I}$-casein.

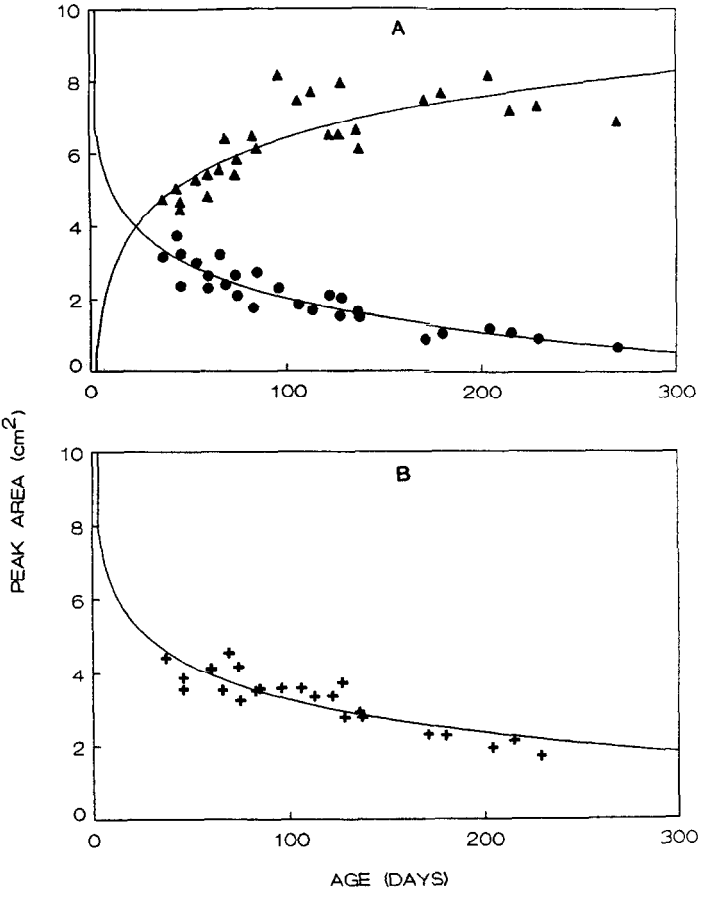

Fig. 6. Casein peak areas *versus* age of 27 Gouda-type cheeses analysed by anion-exchange chromatography. (A) γ-Caseins (▲) and β-casein (●); (B) α_{s1}-casein.

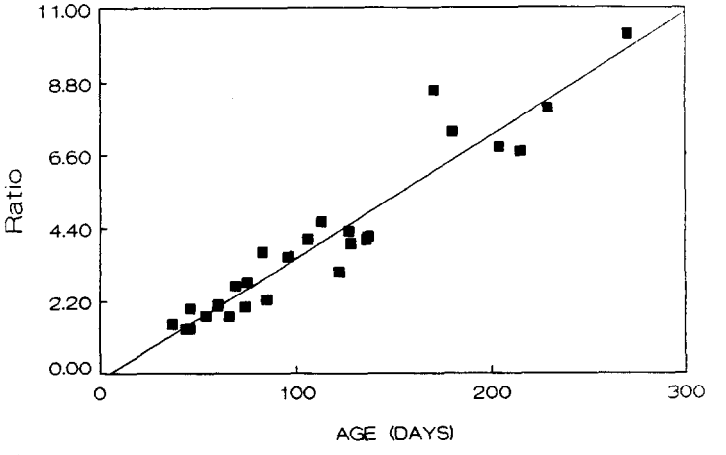

Fig. 7. Ratio of γ- to β-casein peak areas *versus* age of 27 Gouda-type cheeses analysed by anion-exchange chromatography.

TABLE II

CORRELATION COEFFICIENTS BETWEEN THREE RIPENING INDICES AND WITH THE AGE OF 27 GOUDA-TYPE BLOCK-CHEESES OBTAINED FROM SIX WHOLESALERS

Values in parentheses are the nine corresponding correlation coefficients for nine cheeses obtained from one wholesaler.

Parameter	*Ratio of γ-casein to β-casein peak areas*	*Ratio of water-soluble N to total N*	*Ratio of TCA-soluble N to total N*
Age	0.9542 (0.9935)	0.9200 (0.9827)	0.9620 (0.9932)
Ratio of γ-casein to β-casein peak areas	1	0.9337 (0.9774)	0.9645 (0.9867)
Ratio of water-soluble N to total N	0.9337 (0.9774)	1	0.9661 (0.9933)
Ratio of TCA--soluble N to total N	0.9645 (0.9867)	0.9661 (0.9933)	1

tained from six different wholesalers. Peak areas of the proteins were plotted against the age and the results are shown in Fig. 6.

A plot of the ratio of γ-caseins to β-casein peak areas against age (Fig. 7) yielded a correlation coefficient of 0.954. An advantage of determining the ratio of these peak areas is that variations in, for instance, sample preparation, injection volume and detector sensitivity are avoided. For the same cheeses, water-soluble nitrogen-to-total nitrogen and TCA-soluble nitrogen-to-total nitrogen ratios were determined and the correlation coefficients calculated (see Table II). The correlation of the ripening indices with age improved on selecting nine cheeses obtained from one wholesaler (see Table II), which can be explained by the fact that the production and storage conditions will vary between wholesalers.

Although the indices reflect different compounds present in the cheese, the correlation between the ratio of protein peak areas and the ratio of TCA-soluble nitrogen-to-total nitrogen was high (see Table II). Therefore, the ratio of γ-caseins to β-casein peak areas can serve as an alternative to the nitrogen indices.

CONCLUSIONS

Although the major proteins and their breakdown products, present in urea solutions of cheese, were too similar in size to be completely separated by gel filtration chromatography, this technique gave information about both the primary proteolysis, *i.e.*, the formation of high-molecular-weight peptides, and the secondary proteolysis, *i.e.*, the formation of smaller breakdown products such as amino acids or small peptides.

Reversed-phase chromatography was the most suitable technique for the separation of water-soluble breakdown products; however, a disadvantage of this technique is that the classification of cheeses is based on unidentified peaks.

Anion-exchange chromatography was selected as the most suitable technique for further investigation of cheese proteolysis. This method is a good alternative to the separation of cheese proteins by PAGE. The latter technique can be used to identify the fractionated proteins and peptides. In a preliminary study, it was shown that the ratio of γ-caseins to β-casein peak areas can serve as an indicator of age for cheeses ripened up to about 10 months, and the ratio of α_{s1}-casein to $\alpha_{s1\text{-}I}$-casein peak areas might be a suitable indicator for very young cheeses (<4 weeks).

REFERENCES

1 *Landbouwkwaliteitswet: Uitvoeringsvoorschriften Melk- en Zuivelprodukten; Landbouwkwaliteitsbeschikking Kaasprodukten, No. J 7974*, Koninklijke Vermande BV, IJmuiden, 1982.
2 D. P. Venema, H. Herstel and H. L. Elenbaas, *Neth. Milk Dairy J.*, 41 (1987) 215.
3 N. F. Olson, *Chemical Indices of Maturation in Cheese*, International Dairy Federation, Brussels, F-DOC 115, 1985.
4 N. F. Olson, R. C. Lindsay and P. Grappin, *Chemical Indices of Cheese Maturation*,International DairyFederation, Brussels, F-DOC 95, 1983.
5 W. J. Reville and P. F. Fox, *Ir. J. Food Sci. Technol.*, 2 (1978) 67.
6 R. Grappin, T. C. Rank and N. F. Olson, *J. Dairy Sci.*, 68 (1985) 531.
7 T. C. Rank, R. Grappin and N. F. Olson, *J. Dairy Sci.*, 68 (1985) 801.
8 S. I. Shalabi and P. F. Fox, *Ir. J. Food Sci. Technol.*, 11 (1987) 135.
9 L. de Jong, *Neth. Milk Dairy J.*, 29 (1975) 162.
10 A. Noomen, *Neth. Milk Dairy J.*, 31 (1977) 163.
11 W. Haasnoot, D. P. Venema and H. L. Elenbaas, *Milchwissenschaft*, 41 (1986) 642.
12 L. K. Creamer, *J. Dairy Sci.*, 58 (1974) 287.
13 H. E. Swaisgood, in P. F. Fox (Editor), *Developments in Dairy Chemistry—1, Proteins*, Applied Science Publ., Barking, 1982, p. 19.
14 A. Pham and S. Nakai, *J. Dairy Sci.*, 67 (1984) 1390.
15 F. M. W. Visser and A. E. A. de Groot-Mostert, *Neth. Milk Dairy J.*, 31 (1977) 247.