Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants

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Original article

Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants

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Abstract – Hydrophilic and hydrophobic peptides in the water-soluble fraction of semi-hard, vacuum packed ovine cheeses made with calf, microbial and cardoon coagulants were monitored for 90 d by reversed-phase HPLC. Hydrophobic peptides of the cheeses were further analysed by size exclusion HPLC to determine their molecular sizes and the bitterness of the cheeses was evaluated by a trained sensory panel. Cheese made with cardoon coagulant had the highest levels of both hydrophilic and hydrophobic peptides. Cheeses made with calf and microbial coagulants had comparable levels of hydrophilic peptides, but the former had higher levels of hydrophobic peptides. The concentration of bitter peptides (those with a molecular size of 165–6500 g·mol^{-1}) was highest in cheese made with microbial coagulant and lowest in cheese made with calf rennet. Cheese made with microbial coagulant was perceived to be the most bitter by the sensory panel, followed by calf and cardoon coagulant cheeses. Sensory bitterness score was significantly ($P < 0.05$) correlated with total bitter peptides and the ratio of bitter peptides to total peptides, but not with total hydrophobic peptides.

Peptide / bitterness / hydrophobic / hydrophilic / ovine cheese

Résumé – Formation de peptides amers pendant l'affinage de fromages au lait de brebis fabriqués avec différents coagulants. La formation de peptides hydrophiles et hydrophobes contenus dans la phase aqueuse de fromages de brebis à pâte demi-dure fabriqués avec des coagulants d'origine bovine, microbienne ou végétale, a été suivie pendant 90 j en CLHP. Les peptides hydrophobes des fromages ont de plus été analysés par CLHP exclusion de taille pour déterminer leur taille moléculaire, et l'amertume des fromages a été évaluée par un jury d'analyse sensorielle entraîné. Le fromage fabriqué avec le coagulant de chardon possède les plus hautes teneurs en peptides hydrophiles et hydrophobes. Les fromages fabriqués avec les coagulants d'origine bovine et microbienne ont des concentrations comparables en peptides hydrophiles, mais celui fait avec le coagulant microbien a des teneurs en peptides hydrophobes plus importantes. La concentration en peptides amers (ayant une masse molculaire de 165–6500 g·mol^{-1}) était plus élevée dans les fromages realisés avec le coagulant d'origine microbienne et était inférieure dans le cas des fromages fait avec le coagulant de veau. Le fromage fabriqué avec le coagulant d'origine microbienne a été perçu comme le plus amer. L'amertume a été corrélée de manière significative ($P < 0.05$) avec les peptides amers totaux et le rapport peptides amers sur peptides totaux, mais pas avec les peptides hydrophobes totaux.

Peptide / amertume / hydrophobie / hydrophilie / fromage de brebis

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1. INTRODUCTION

Bitter taste is one of the most common quality defects of cheese and is caused by the production of bitter peptides from casein hydrolysis during cheese-making, especially at the ripening stage [13, 30]. Bitter peptides isolated from casein hydrolysate and cheese are those with a high proportion of aromatic amino acids residues and a high average hydrophobicity [23], most of which arise from the breakdown of α_{s1}- and β-casein [17]. Although the formation of bitter peptides in cheese is mostly attributed to the proteolytic activity of bacteria, especially some of the non-starter bacteria, other proteases such as rennet and other coagulants could also play a role [11]. Rennet can produce bitter peptides from both α_{s1}- and β-casein [12], which, if retained in a high concentration, can cause marked bitterness in cheese [32]. Furthermore, milk coagulant could conceivably influence the perception of bitterness in cheese in two other ways: the first is through the production of casein breakdown products, which are subsequently used as substrates by bitter peptide-producing bacteria [11]. The second is through the production of flavour peptides and amino acids, which may have a masking effect on the bitter taste of cheese.

The water-soluble fraction of cheese is a major contributor to cheese flavour [21], whereas the water-insoluble fraction is thought to be largely flavourless [22]. Separation of the water-soluble fraction by reversed-phase high performance liquid chromatograph (RP-HPLC) into hydrophilic (early elution) and hydrophobic (late elution) fractions has formed the basis of several indices for predicting bitterness intensity in cheese. Gaya et al. [16] and Lau et al. [20], for example, suggest that the ratio of hydrophobic to hydrophilic peptides is correlated with bitter taste in cheese. Fractions from Cheddar cheese separated by RP-HPLC showed the bitter fractions to be hydrophobic peptides, whereas the savoury fractions were hydrophilic peptides and amino acids [10]. Gomez et al. [17] suggest that the level of hydrophobic peptides determined at 280 nm is a reliable indicator of bitterness for semi-hard cheeses. However, the relationship between the size of the hydrophobic peptides and the bitterness of cheese has not been examined in detail in these studies.

Extracts of the wild thistle plant, cardoon (*Cynara cardunculus* L.), has traditionally been used as a milk coagulant for the manufacture of several cheese varieties in Southern Europe [28, 29]. The coagulant has gained renewed interest in recent years, partly due to its plant origin, as consumers are increasingly concerned about the use of calf rennet out of health and ethical concerns. However, use of cardoon coagulant has been found to lead to the development of bitterness in aged ovine milk cheese [1]. Furthermore, Carrera et al. [5] reported that the level of hydrophobic peptides, which was related to bitter taste, in 24 h cheese made using cardoon coagulant was more than 70-fold the level in cheese made using chymosin. However, using encapsulated cardoon coagulant accelerated the development of flavour intensity in cheese without enhancing bitterness [26].

The objective of the present study is twofold: (1) to examine the role of the size of hydrophobic peptides in determining the bitterness of ovine milk cheese; and (2) to compare the bitterness intensity of ovine milk cheese made using cardoon and commercial (calf and microbial) coagulants. The peptide profile of the water-soluble fractions of the cheeses was examined by RP-HPLC over a ripening period of 90 d and the molecular size of the hydrophobic peptides was analysed by size exclusion-high performance liquid chromatography (SE-HPLC). The bitterness intensity of the cheeses aged for various times were assessed by a trained sensory panel, and the results were compared with various peptide indices obtained from the chromatographic methods.

2. MATERIALS AND METHODS

2.1. Ovine milk, coagulant and starter culture

Ovine milk was collected from Awassi Aust. Pty. Ltd., Cowra, NSW, Australia. The milk was cooled to 4 °C immediately and transported to the Charles Sturt University cheese factory. Ovine milk cheeses were made using three types of coagulant: calf, microbial and cardoon. The calf and microbial coagulants used were commercial preparations with the trade name of Naturen^{TM} and Microlant^{TM}, respectively, and were supplied by Chr. Hansen Pty. Ltd. (Bayswater, Victoria, Australia). Naturen^{TM} was a rennet preparation manufactured from the extract of the fourth stomach of calves while Microlant^{TM} was manufactured by submerged fermentation of a strain of the fungus *Rhizomucor miehei.* The plant coagulant used was a water extract from the flowers of the thistle plant cardoon (*Cynara cardunculus* L.), partially purified by fractional precipitation with ammonium sulphate. The cardoon flowers were collected from a single paddock in Werribee, Victoria, Australia, and the extraction and purification procedures have been described elsewhere [8]. The starter culture used was a commercial product, Flora Danica^{TM}, also supplied by Chr. Hansen. This is a mesophilic cheese starter containing a mixture of *Lactococcus lactis* subsp. *lactis, L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc cremoris*.

2.2. Cheese-making

Two batches of Peccorino style cheese were made from standardised milk (4.83 ± 0.97% fat, 5.03 ± 0.38% protein and 15.49 ± 1.03% total solids) in two sequential periods using a mini-cheese-making system with a vat capacity of 80 L. For each batch, the milk was pasteurised at 72 °C for 30 s and the pasteurised milk was divided into three portions, which were coagulated with cardoon, calf and microbial coagulant respectively. The general procedure for making Peccorino cheese described by Battistotti and Corradini [4] was followed. Briefly, pasteurised milk was cooled to 37 °C and starter culture was added at a rate of 20 g per 100 L milk. After 30 min, rennet was added at a rate of 20 mL per 100 L milk for Naturen^{TM}, 10 mL per 100 L for Microlant^{TM}, and 400 mL per 100 L for the cardoon extract, respectively. The dosage of the two commercial coagulant preparations used was based on manufacturer's recommendations, while the dosage of the cardoon extract was determined experimentally to be equivalent to calf rennet in clotting properties (clotting time and curd firmness). Details of this determination have been described [34]. The curd formed was cut to 1.5–2 cm squares and allowed to stand for 5 min until the whey began to separate out. The vat was then raised to 44 °C and held for 15 min until the curd granules were reduced to the size of corn grains (about 0.8–1 cm^3). After a further 10–12 min, the whey was removed and the curd granules were moulded manually into three oval-shaped hoops with each fresh cheese weighing about 1 kg. The cheeses were pressed at 300 kPa for 24 h, and then brined in a saturated NaCl solution containing 0.15% $CaCl_2$ at 13–16 °C for 24 h. After removal from the brine, the cheeses were vacuum-packed in plastic bags (Cryovac Pty Ltd, Victoria, Australia) made of composite plastic film with an oxygen transmission rate of 0.08 $cm^3 \cdot m^{-2} \cdot 24\ h^{-1} \cdot kPa^{-1}$ (at 23 °C and 75% RH) and a moisture vapour transmission rate of 0.06 $g \cdot m^{-2} \cdot 24\ h^{-1} \cdot kPa^{-1}$ (at 38 °C and 90% RH). The cheeses were allowed to mature at 13–16 °C for 90 d. One cheese of each type was taken at random at 7, 30, 60, 90 d of age and was analysed by HPLC for peptide profiles and by sensory evaluation by a trained panel for bitterness.

2.3. HPLC analysis of water-soluble fractions of cheese

Water-soluble fractions of the cheeses were extracted according to the procedure of Kuchroo and Fox [19]. Aliquots (1 mL) of the extracts were added to 3 mL of a

100:1 (v/v) mixture of solvents A and B (described below). The mixtures were filtered through 0.45 μm cellulose acetate filters (Millipore Ltd, Sydney, Australia) and the filtrates were retained for analysis by RP-HPLC.

HPLC was performed with a Waters 2690 Separation Module run by the Millenium^{32} software. The module was an integrated HPLC system consisting of two Waters 6000A solvent delivery pumps and an U6K universal chromatography injector with an auto sampler and a column holder and heater. Separation was achieved on a Pharmacia 5 μm 4RP-C8 column (250 mm × 4 mm i.d.). Detection was performed with a Waters 996 photodiode array detector set at 214 and 280 nm, and the data were processed by the Millenium^{TM} software installed on an IBM PC.

2.3.1. RP-HPLC

The RP-HPLC procedure used was based on the gradient elution method of Singh et al. [27]. Solvent A was a 0.05% trifluoroacetic acid (TFA) solution in de-ionised water. Solvent B was a solution of 0.035% TFA in a 90:10 (v/v) mixture of de-ionised water and acetonitrile. Prior to use, the solvents were filtered through a 0.45 μm nylon membrane filter, and de-gassed by sonication for 5 min. Prior to sample injection, the column was equilibrated at room temperature with 0.05% TFA for 5 min at a flow rate of 1 mL·min^{-1}. After injection of a sample (30 μL), elution began with 100% solvent A for 5 min, followed by a linear gradient to 50% solvent B over 30 min, 50% solvent B for 3 min, 60% solvent B over 2 min, and 60% solvent B for 30 min.

Hydrophobic fractions were defined as the fractions that were eluted after the linear gradient to 50% solvent B [27]. Hydrophobic fractions were pooled, their volumes measured, and then freeze-dried using a Christ Alpha 1-4 Freeze-dryer (Braun Biotech International, Melsungen, Germany). The freeze-dried samples were used for SE-HPLC assay to determine their molecular size.

2.3.2. SE-HPLC

The HPLC instrumentation was the same as described above. The procedure used was based on the method of O'Sullivan [24]. On the day of analysis, solutions of the freeze-dried samples (see above) were prepared in the mobile phase (0.1% TFA in a 36/64 mixture of acetonitrile and water v/v), and filtered through 0.45 μm cellulose acetate filter. Separation was achieved on a Pharmacia HR 10/30 column (300 mm × 10 mm i.d.), which was protected by a Phenomanex 6 μm guard column (75 × 7.8 mm i.d.). Elution was carried out isocratically at 25 °C with 0.1% TFA in a 36:64 mixture of acetonitrile and water (v/v) at a flow rate of 0.5 mL·min^{-1}, and the absorbance of the eluate was recorded at 214 nm and 280 nm.

SE-HPLC was also performed on protein size standards (Sigma-Aldrich, Sydney, Australia). The standards were first made up into individual stock solutions in the mobile phase at a concentration of 2 mg·mL^{-1}, with the exception of phenylalanine which was at a concentration of 1 mg·mL^{-1}. The working standard mixture was prepared by diluting in 0.75 mL mobile phase each of 0.1 mL egg white lysozyme (molecular mass 14300 g·mol^{-1}), aprotinine (6500 g·mol^{-1}), insulin chain B (2300 g·mol^{-1}) and phenylalanine (165 g·mol^{-1}) stock standards. The standard mixture was filtered through a 0.45 μm-PTFE filter (Millipore Ltd, Sydney, Australia) before injection.

2.4. Sensory evaluation of cheese

The cheeses were subjected to sensory evaluation by a trained panel of 12 people. Most (11) of the panellists were chosen from the School of Wine and Food Sciences and one from the university winery for their familiarity and experience with sensory analysis. The panellists were trained according to the methods described by Barcenas et al. [2, 3]. The test was carried out at the university's sensory evaluation laboratory, and cheeses aged for 30, 60 and 90 d were

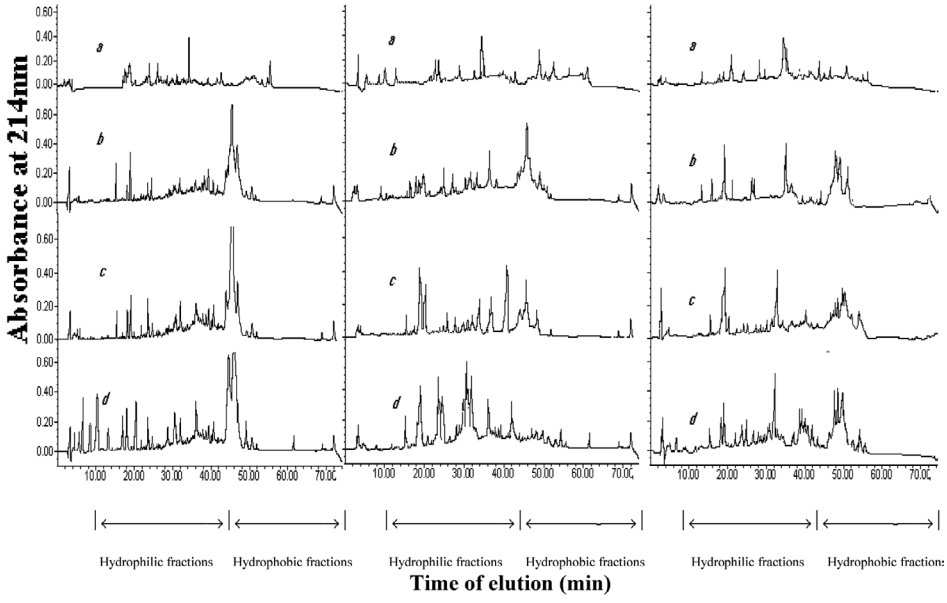

Figure 1. RP-HPLC profiles of water-soluble fractions of cheeses made from ovine milk with cardoon (left), microbial (middle) and calf (right) coagulant. The cheeses were ripened for 7 (a), 30 (b), 60 (c) and 90 (d) d, respectively.

evaluated for bitterness on a 1–10 scale (10 being most bitter). Cheese samples were cut into bite pieces of standard size (1.25 × 1.1 × 1.1 cm), and the cut samples were placed in plates and kept at a constant temperature of 20 °C for at least 1 h before serving.

2.5. Statistical analysis

The data collected were subjected to one way analysis of variance (ANOVA) and means of treatments showing significant differences were subjected to Fisher's Least Significant Difference test.

3. RESULTS

3.1. Peptide profiles of water-soluble fractions of ovine milk cheeses

Figure 1 shows the RP-HPLC peptide profiles of the water-soluble fractions (WSF) of ovine milk cheeses made with cardoon, calf and microbial coagulant and ripened for 90 d. Table I summarises the total peak areas of the hydrophilic and hydrophobic peptides, as well as their ratios in the various cheese samples.

Milk coagulant had a significant effect ($P < 0.05$) on the levels of hydrophobic and hydrophilic peptides, and on their ratio in the WSF of cheeses determined at both 214 and 280 nm (Tab. I). At 214 nm, cheese made with cardoon extract had the highest levels of all three parameters throughout the ripening period. Microbial coagulant cheese had the lowest levels of hydrophobic peptides and the ratios of hydrophobic to hydrophilic peptides during later stages of ripening (30–90 d), while the levels of hydrophilic peptides in cheeses made with microbial and calf rennet were similar. The levels of peptides in the cheeses measured at 280 nm were markedly (about 100-fold) lower than those measured at 214 nm. At 280 nm, cheese made with cardoon coagulant also had the highest levels of hydrophobic

Table I. Peak areas of hydrophilic and hydrophobic peptides of cheeses made from ovine milk with cardoon, calf and microbial coagulant determined by RP-HPLC at 214 nm and 280 nm.

Detection wavelength (nm)	Ripening time (d)	Hydrophobic peptides			Hydrophilic peptides			Hydrophobic / Hydrophilic		
		Cardoon	Calf	Microbial	Cardoon	Calf	Microbial	Cardoon	Calf	Microbial
214	7	170	110	120	280	260	230	0.61	0.42	0.52
	30	880	410	310	610	560	510	1.44	0.73	0.61
	60	1280	590	460	1570	1070	1290	0.82	0.55	0.36
	90	1120	670	220	1760	1290	1320	0.64	0.52	0.17
280	7	5.0	1.2	3.3	8.8	4.3	5.8	0.57	0.28	0.57
	30	7.1	3.9	5.3	16.9	8.1	12.1	0.42	0.48	0.44
	60	18.1	7.4	12.6	26.9	16.2	20.7	0.67	0.46	0.61
	90	18.9	8.4	14.9	31.9	17.9	23.6	0.59	0.47	0.63

Data were mean values of three measurements with standard deviation for the values ranging from 21 to 50 for data obtained at 214 nm and 0.12 to 0.82 for data obtained at 280 nm.

Table II. Peak area and molecular size of hydrophobic peptides of cheeses made from ovine milk with cardoon, calf and microbial coagulant determined by SE-HPLC at 214 nm.

Type of coagulant	Peaks	Estimated molecular size ($kg \cdot mol^{-1}$)	Peak area of peptides for cheese aged for		
			30 d	60 d	90 d
Cardoon	1	> 14.3	15.5 ± 0.2	13.7 ± 0.2	11.3 ± 0.3
	2	14.3–6.5	11.8 ± 0.4	26.8 ± 0.3	31.4 ± 0.9
	3	6.5–2.3	2.6 ± 0.2	5.2 ± 0.4	5.3 ± 0.2
	4	2.3–0.165	1.7 ± 0.3	0.4 ± 0.1	–
	5, 6	< 0.165	3.6 ± 0.2	5.2 ± 0.4	2.2 ± 0.1
	Total hydrophobic peptides		35.2 ± 1.3	51.3 ± 1.4	51.2 ± 1.5
Microbial	1’	> 14.3	23.5 ± 0.9	22.8 ± 0.3	29.4 ± 1.0
	2’	14.3–6.5	3.8 ± 0.4	2.8 ± 0.5	0.7 ± 0.2
	3’, 4’ 5’	6.5–2.3	9.4 ± 0.5	9.6 ± 0.5	5.8 ± 0.3
	6’	< 0.165	1.2 ± 0.1	1.4 ± 0.1	–
	Total hydrophobic peptides		37.9 ± 1.9	36.6 ± 1.4	36.2 ± 1.5
Calf	1*, 2*	> 14.3	22.4 ± 1.2	20.4 ± 0.9	27.9 ± 2.9
	3*, 4*, 5*	6.5–2.3	6.2 ± 0.4	2.6 ± 0.4	2.0 ± 0.2
	6*, 7*	< 0.165	1.7 ± 0.2	0.2 ± 0.1	2.3 ± 0.2
	Total hydrophobic peptides		28.8 ± 1.5	23.2 ± 1.4	32.2 ± 3.3

–: Peaks did not appear or were too small to be integrated.

and hydrophilic peptides while the levels of these two parameters were lowest in cheese made with calf rennet.

The levels of hydrophobic and hydrophilic peptides and their ratios changed significantly ($P < 0.05$) during ripening. The level of hydrophobic peptides in cardoon and microbial coagulant cheeses determined at 214 nm increased between 7 and 60 d of age, then declined between 60 and 90 d, while that of calf rennet cheese increased over the entire ripening period. The level of hydrophilic peptides in all three types of cheese increased throughout the 90 d of ripening, while the ratio of hydrophobic to hydrophilic peptides increased between 7 and 30 d of age, then declined thereafter. When the detection was made at 280 nm, the levels of hydrophobic and hydrophilic peptides in all three types of cheese increased over the entire 90 d of ripening. The ratio of hydrophobic to hydrophilic peptides, however, did not follow a consistent trend.

3.2. Molecular size of hydrophobic peptides in ovine milk cheeses

The molecular size of the hydrophobic peptides in the cheeses was estimated by comparing their retention times with those of protein size standards when eluted by SE-HPLC. Figure 2 shows the SE-HPLC profiles of the hydrophobic peptides of ovine milk cheeses made with the three different coagulants, while Table II summarises the area and estimated molecular size of the major peaks. Depending on the type

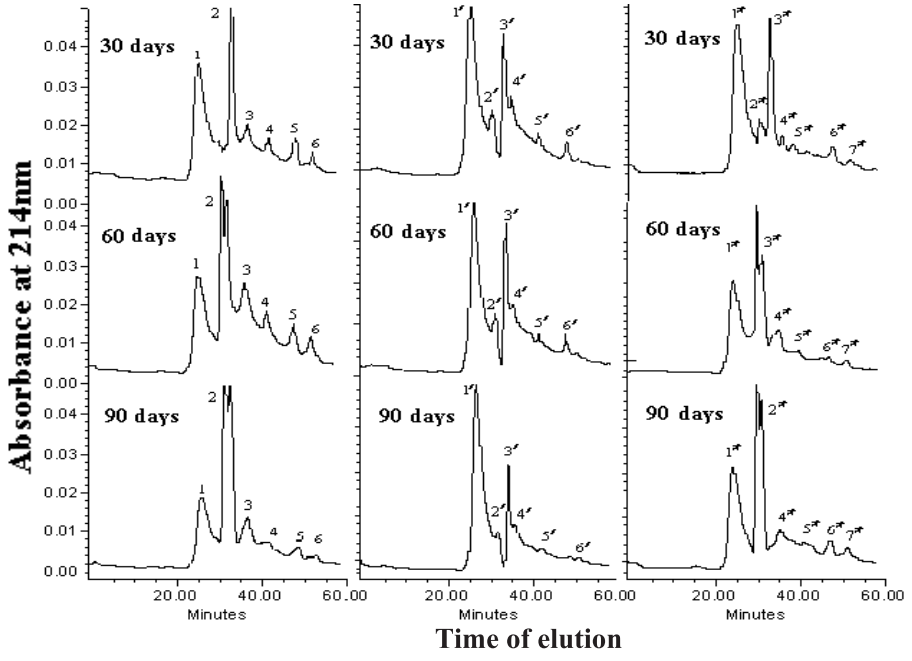

Figure 2. SE-HPLC profiles of hydrophobic fractions of cheeses made from ovine milk with cardoon (left), microbial (middle) and calf (right) coagulant. The cheeses were ripened for 30, 60 and 90 d, respectively.

of coagulant used, six or seven peaks appeared in the chromatograms. Although the elution profiles and retention times of the peaks in the different chromatograms were similar, retention time alone was considered insufficient to determine whether these peaks were derived from the same peptides. To emphasise this point, peaks in the chromatograms derived from different cheeses were labeled differently.

Six peaks appeared in the chromatograms of the hydrophobic fraction of cheese made with cardoon coagulant, of which, peaks 1 and 2 were predominant. These two peaks contained peptides with molecular mass greater than 14.3 and 6.5 kg·mol^{-1}, respectively. The molecular sizes of peptides eluted in peaks 3 and 4 fall within the range 165–6500 g·mol^{-1} while those of peaks 5 and 6 were smaller than 165 g·mol^{-1}. Six peaks also appeared in the chromatograms of the hydrophobic fraction of cheese made with microbial coagulant. Peaks 1' and 3' were predominant while the other four peaks were much smaller in comparison. Of the six peaks, three (3'–5') fall within the peptide range of 165–6500 g·mol^{-1}. The hydrophobic peptides in cheese made with calf rennet showed 7 peaks; of which, 1* and 2* were predominant. Three of the seven peaks (3*–5*) fall within the peptide range of 165–6500 g·mol^{-1}.

The total peak area of all hydrophobic peptides of cheese made with cardoon was significantly greater than that of calf or microbial coagulant cheese at later stages of ripening (60–90 d). However, this was largely a result of the two large peaks, 1 and 2. As far as the peptides in the range of 165–6500 g·mol^{-1} were concerned, highest levels were found in microbial coagulant cheeses, followed by cardoon coagulant cheese while calf rennet cheese had the lowest levels, especially at later stages of ripening.

Table III. Mean bitterness scores of ovine milk cheeses as evaluated by a trained panel.

Type of coagulant	Cheese aged for		
	30 d	60 d	90 d
Cardoon	2.8 ± 0.1[a]	2.9 ± 0.2[a]	2.6 ± 0.1[d]
Calf	3.2 ± 0.2[b]	3.1 ± 0.1[b]	2.6 ± 0.2[d]
Microbial	3.5 ± 0.3[c]	3.7 ± 0.3[c]	2.9 ± 0.2[a]

[a–d] Values in the same column or row without a common superscript differ significantly ($P < 0.05$).

The SE-HPLC eluate of the hydrophobic fractions of ovine milk cheeses was also measured at 280 nm (chromatograms not shown), and the profiles showed a similar trend to those recorded at 214 nm, except that the number of peaks for microbial and calf rennet cheeses was fewer when measured at 280 nm than at 214 nm. Also, the peptides of all the cheeses exhibited much lower absorbance at 280 nm than at 214 nm and, consequently, the peak areas of the hydrophobic peptides calculated were much smaller than those at 214 nm (data not shown).

3.3. Bitterness of cheeses

Table III shows the average bitterness scores of the cheeses made using different coagulants given by a trained panel of 12 panelists. The coagulants used for the manufacture of the cheeses significantly ($P < 0.05$) affected their bitterness scores. Cheese made with cardoon extract received the lowest bitterness scores, followed by calf rennet cheese, while cheese made with microbial coagulant received the highest bitterness scores. The bitterness scores of all cheeses remained unchanged between day 30–60, but decreased significantly ($P < 0.05$) at later stages of ripening (60–90 d).

Bitterness scores correlated significantly ($P < 0.05$) with peptides in the 165–6500 g·mol^{-1} range and with the ratio of these peptides to total peptides determined at 214 nm. None of the other indices including hydrophobic peptides, hydrophilic peptides or their ratio determined either at 214 nm or 280 nm was significantly ($P > 0.05$) correlated with panel bitterness scores.

4. DISCUSSION

Results presented in this study show that the type of coagulant used in the manufacture of ovine milk cheeses affected their peptide profiles. In particular, cheese made with cardoon extract was found to give a greater amount of both hydrophilic and hydrophobic peptides than cheese made with either calf or microbial coagulant. Similar findings have also been reported by Sousa and Malcata [28] on ovine milk cheese made with cardoon and calf rennet. In a previous communication [7], we reported that the levels of water-soluble nitrogen in cheese made with cardoon extract were higher than those in cheeses made with the other two types of coagulant, which was consistent with results obtained in the present study.

Previous studies have established that, in general, hydrophobic peptides with molecular masses between 300 and 6000 g·mol^{-1} taste bitter, while peptides outside this molecular mass range exhibit little or no bitterness [9, 23, 25, 33]. Our results appeared to confirm this theory. Thus, although cheese made with cardoon extract gave the highest levels of hydrophobic peptides, it was however lower in the concentrations of bitter peptides than microbial coagulant cheese, while the latter showed the highest bitter peptide levels among the cheeses made with the three types of coagulant. This explains the highest bitterness scores awarded to microbial coagulant cheese by the panel. This, however, does not explain the lower bitterness scores received by cardoon coagulant cheese than calf rennet

cheese although the former had higher levels of bitter peptides than the latter. A possible hypothesis can be put forward to explain this apparent discrepancy. It is possible that the bitter taste of cardoon coagulant cheese was masked to a greater degree than that of the calf rennet cheese by other cheese flavours, especially those contributed by low molecular weight hydrophilic peptides and free amino acids. As shown in Table I, and also in our previous study [7], the use of cardoon extract as a coagulant resulted in cheeses with higher levels of water-soluble nitrogen. Many of these water soluble peptides and free amino acids are known to have a sweet or savoury taste and give aromatic flavours [18, 25]. It is also worth noting that the difference in bitterness scores between cardoon and calf rennet cheeses were, though statistically significant, not very big.

A number of peptide indices have been proposed as indicators of bitterness intensity of cheese. These include major peaks eluded by reversed-phase HPLC detected at 280 nm [6], hydrophobic peptides, and the ratio of hydrophobic to hydrophilic peptides detected at both 214 and 280 nm [17, 20]. In the present work, bitterness scores of cheeses given by a trained sensory panel did not correlate with the level of hydrophobic nor hydrophilic peptides. Strong proteolysis, such as that occurred in cardoon coagulant cheeses, resulted in higher levels of both hydrophobic and hydrophilic peptides, but lower perceived bitterness scores. The ratio of hydrophobic to hydrophilic peptides did not significantly ($P > 0.05$) correlate with the bitterness score either. This is probably due to the fact that these indices do not take into account the fact that only a small proportion of the hydrophobic peptides taste bitter. This study appears to show that those peptides are, in all likelihood, size-dependent.

Previous studies have shown that the water-soluble fraction of cheese is a major contributor to cheese flavour [21], whereas the water-insoluble fraction is thought to be largely flavourless [23]. For this reason, the present study only focused on the water-soluble fraction of cheese. However, preparation of the water-soluble fraction using the Kouchroo and Fox method [19] has a diluting effect with a consequent decrease in ionic strength. As a result, the solubility of the hydrophobic peptides may have changed and their extractability affected [14, 15]. It might be more appropriate in future studies to use cheese juice to examine proteolysis.

In summary, although cheese made using cardoon coagulant gave higher levels of peptides (both hydrophilic and hydrophobic) than the cheeses made with other two types of coagulant, its levels of bitter peptides were not high, and the cheese was actually lower in bitterness intensity as perceived by a trained panel, probably due to flavour modification by other cheese components. Notwithstanding the fact that proteolytic activity of bacteria, especially some of the non-starter bacteria, is mainly responsible for the production of bitter peptides, this study appears to confirm that milk coagulant can play a role in determining the bitterness in cheese.

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