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The effect of trichloroacetic acid on water-soluble fractions from Fynbo cheese

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Abstract

The effect of 4% trichloroacetic acid (TCA) on peptide separation of the water-soluble fraction was analysed during Fynbo cheese ripening. The water-soluble fraction and 4% TCA-soluble sub-fraction were analysed by RP-HPLC. The information of the chromatograms was successfully summarized in 2 dimensions, accounting for 84.7% of data variation using principal component analysis. Peaks that eluted after 30 min in the chromatographic run were most affected by the TCA treatment. Some differences in the amino acid composition were observed between peaks with different signs of the PC2 loading. The TCA extraction and RP-HPLC profiles of the soluble compounds may help in the peptide analysis during Fynbo cheese ripening.

Keywords: Cheese; Ripening; Water-soluble fraction; Trichloroacetic acid; RP-HPLC

1. Introduction

Proteolysis is the principal and most complex biochemical event occurring during the maturation of the majority of ripened cheese varieties (Katsiari, Alichanidis, Voutsinas, & Roussis, 2001). During cheese ripening, a part of the casein is converted into water-soluble nitrogenous compounds, such as peptides and amino acids (Fox, 1989). These peptides have different solubilities in water and other solvents. Therefore, extractions with different solvents and subsequent quantification of nitrogenous compounds in the cheese extract are used to study the extent of proteolysis in cheese (Polychroniadou, Michaelidou, & Paschaloudis, 1999).

Water is the most common extractant. The extract contains whey proteins, medium and small-sized peptides, amino acids and other low molecular weight compounds (Polychroniadou et al., 1999). Different precipitants can be used to obtain sub-fractions of this extract, e.g., trichloroacetic acid (TCA).

Many researchers have applied selective precipitation by TCA to subfractionate peptides in the water-soluble fraction of different cheese types (Freitas, Fresno, Prieto, Malcata, & Carballo, 1997; Katsiari, Alichanidis, Voutsinas, & Roussis, 2000; Katsiari et al., 2001; Laborda, 2000; Pavia, Trujillo, Guamis, & Ferragut, 2000; Venema, Herstel, & Elenbaas, 1987). The influence of different parameters, such as the time and temperature of incubation, and the original extraction method, on the 12% TCA-soluble nitrogen fraction of cheese was evaluated by Polychroniadou et al. (1999). The authors reported that any combination of time and temperature had little effect on the level of 12% TCA-soluble nitrogen obtained from a cheese extract. However, the type of components of the extracts depended on the cheese to water ratio used for extraction and on the cheese pH. The contribution of each factor was

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dependent on the cheese variety and the extent of proteolysis. In general, the different TCA fractions are analysed by the Kjeldahl method to determine their nitrogen content, but there is very little information about the chromatographic profiles of those extracts.

Fynbo is a semihard cheese of either regular or low fat content, commonly salted for 10 h at 12 °C in 20% NaCl brine, and ripened for 30 days. Zorrilla and Rubiolo (1997) studied the effect of partial replacement of NaCl by KCl on α_{s1}-casein degradation during ripening. The influence of NaCl replacement and ripening temperature on the chromatographic profiles of water-soluble nitrogen fractions (Laborda & Rubiolo, 1999) and on the electrophoretic profiles of casein fractions (Sihufe, Zorrilla, & Rubiolo, 2003) were also determined. Moreover, the results obtained for the control cheeses allowed characterisation of the primary casein degradation and peptide formation during the ripening of Fynbo cheese. As a result, a better understanding of the ripening process was achieved. Taking into account the large number of peptides of the water-soluble fraction, an analysis of this fraction may help to thoroughly examine the different peptides and contribute to a better study of the key peptides.

Our objectives in this work were to study the effect of 4% TCA on the RP-HPLC chromatograms of the water-soluble fraction and to examine different peptides of the chromatographic profiles by PCA during ripening of Fynbo cheese.

2. Materials and methods

2.1. Materials

Unsalted low-fat Fynbo cheeses (782.9 ± 27.4 g weight, 11.5 ± 0.3 cm dia, 6.1 ± 0.2 cm height), manufactured on the same date from the same cheese vat, were brought from a local factory to our laboratory. Their initial composition was: 49.34 ± 0.29% (w/w) moisture, 29.72 ± 1.75% (w/w) protein, 12.56 ± 0.15% (w/w) fat; and the pH was 5.15–5.35. Zorrilla (1993) and Zorrilla and Rubiolo (1997) did not observed significant differences in data obtained for different cheese vats. Nevertheless, one cheese vat was used in this study to reduce the number of cheeses assayed (Sihufe et al., 2003). Seven cheeses were salted for 10 h at 12 °C in a solution of 190 g NaCl l^{-1}. Brine also contained 0.55% Ca^{2+} to prevent softening of the cheese rind (Geurts, Walstra, & Mulder, 1972). After brining, each cheese was wiped and packed under vacuum in a heat-shrinkable plastic bag. During ripening, the cheeses were stored at 12 °C. Although Fynbo cheese is traditionally ripened at a factory for 30 days, this study was extended for a further 60 days to consider changes that may occur during the shelf life of the product. Different cheeses were sampled at 1, 5, 10, 20, 30, 60, and 90 days. Slices of 1.5 cm thickness were cut parallel to the flat surface from the centre and cylindrical cores of 4.8 cm diameter were cut from those slices. Samples were used to obtain the water-soluble fraction.

2.2. Aqueous extraction and fractionation with TCA

Water-soluble fraction extraction was performed as suggested by Kuchroo and Fox (1982). Grated cheese (20 g) mixed with 30 ml, water was homogenised using an ULTRA-TURRAX® T25 (IKA® Werke, Janke & Kunkel GmbH & Co KG, Staufen, Germany) homogeniser for 2 min. The homogenate was held at 40 °C for 1 h, pH was adjusted to 4.6, and the suspension was centrifuged at 4800 rpm and 5 °C for 30 min (Biofuge 28RS; Heraeus Sepatech, Osterode, Germany). After centrifugation, the upper layer of fat was removed. The supernatant was filtered through Whatmann No. 42 (Maidstone, England) paper and diluted to 100 ml, constituting the water-soluble fraction (WSF). Aqueous extraction was carried out in duplicate.

Various concentrations of TCA have been used to fractionate peptides in the water-soluble fraction – 2%, 4%, 8%, and 12% (w/v) – depending on the fractionation required. In this case, the concentration of 4% TCA was interesting in that it precipitated a significant amount of peptides (2% TCA precipitated a few peptides, while 8% or 12% TCA precipitated a large number of peptides). A sub-fraction of WSF soluble in TCA (TCA-WSF) was obtained by mixing 1 ml of WSF with 1 ml of 8% TCA solution. After 1 h at room temperature, the precipitate was removed by centrifugation at 3570 rpm for 10 min. The supernatant constituted the TCA-WSF.

The WSF and TCA-WSF were filtered through 0.2-μm disposable filters (Alltech Associates, Inc., Deerfield, IL, USA) and 100 μL were analysed by RP-HPLC.

2.3. RP-HPLC analysis

A chromatograph with a gradient programmer model, 2360, a V[4®] variable wavelength absorbance detector (Isco, Inc., Lincoln, NE, USA) and a SynChropak RPP (250 × 4.6 mm) C_{18}, 300 Å column (SynChrom, Inc., Lafayette, IN, USA) at 30 °C were used. The chromatographic method reported by McSweeney, Olson, Fox, Healy, and Højrup (1993) was followed. Separations were carried out at a flow rate of 1 ml min^{-1} using solvent A (0.1% trifluoracetic acid in water) for 5 min, linear gradient from 0% to 50% of solvent B (0.1% trifluoracetic acid in acetonitrile) over 30 min and isocratic step at 50% B for 15 min. Detection was at 220 nm. Data were processed with the Chem Research Data System Program version 3.0.2. 1994 (Isco, Inc., Lincoln, NE, USA). One chromatogram was obtained from each fraction extracted.

2.4. Amino acid composition of peaks

The determination of the amino acid composition of a peak consisted of two steps: complete hydrolysis of the substrate to liberate the residues, followed by chromatographic analysis and quantification of the liberated amino acids. Peaks were collected manually from successive chromatographic runs. Before hydrolysis, acetonitrile and water were removed by evaporation under vacuum and lyophilisation, respectively. Samples were hydrolysed in a Knauer Protein Hydrolyzer with Knauer Air Oven (Knauer-Vertretung Schweiz, Berlin, Germany), using 6 N HCl, sequanal grade, of constant boiling (Pierce, Rockford, IL, USA) at 110 °C for 24 h. After hydrolysis, each sample was dissolved in water and the amino acid composition was determined by RP-HPLC, as described by Verdini, Zorilla, and Rubiolo (2002).

2.5. Statistical analysis

Pripp, Shakeel-Ur-Rehman, McSweeney, and Fox (1999) showed that the multivariate statistical analysis of chromatographic profiles was a more objective and powerful approach for evaluating proteolysis in cheese, than visual assessment of the chromatograms. Principal component analysis (PCA) is a multivariate statistical technique based on the linear combination of the measured variables to produce derived variables called principal components (PC). In this case, PCA was performed to summarise the large amount of data obtained from the RP-HPLC chromatograms, using Minitab (Minitab Inc., State College, PA, USA).

3. Results and discussion

RP-HPLC chromatograms of the WSF and TCA-WSF from Fynbo cheeses at different ripening times were obtained. Typical chromatograms of the WSF and TCA-WSF corresponding to 5 days of ripening, are shown in Fig. 1. Twenty-one peaks with significant chromatographic areas were analysed in both fractions. The main effect of 4% TCA was observed in the zone

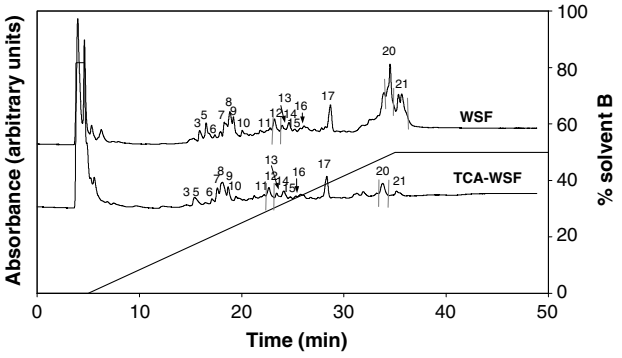

Fig. 1. RP-HPLC chromatograms of the WSF and TCA-WSF from a Fynbo cheese of 5 days of ripening. Peaks which were recognized and used as variables in the multivariate statistical analysis are indicated by numbers.

Table 1
Mean value with standard deviation of the chromatographic areas ($\times 10^4$/100 g cheese) of the 21 peaks analysed in the WSF from Fynbo cheese for different ripening times

Peak	Ripening time (days)						
	1	5	10	20	30	60	90
1	1.18 ± 0.84	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± ND	0.00 ± 0.00
2	1.01 ± 0.26	3.43 ± 0.52	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	11.9 ± ND	0.00 ± 0.00
3	2.19 ± 0.27	4.58 ± 0.29	8.29 ± 0.71	5.89 ± 8.33	17.7 ± 0.22	24.4 ± ND	32.6 ± 2.24
4	1.16 ± 0.20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± ND	0.00 ± 0.00
5	4.19 ± 0.23	6.20 ± 0.05	12.5 ± 0.47	25.6 ± 2.81	29.1 ± 0.16	39.3 ± ND	25.4 ± 0.48
6	1.72 ± 0.36	2.21 ± 0.12	4.20 ± 0.78	4.33 ± 1.68	4.68 ± 0.38	19.2 ± ND	6.65 ± 0.66
7	5.69 ± 0.52	4.34 ± 0.04	6.37 ± 1.02	18.1 ± 4.34	12.6 ± 0.40	28.9 ± ND	42.0 ± 1.18
8	5.48 ± 2.43	19.0 ± 2.21	30.7 ± 5.50	41.5 ± 8.57	30.7 ± 1.12	52.2 ± ND	46.8 ± 3.49
9	2.75 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	10.8 ± 15.2	0.00 ± 0.00	0.00 ± ND	0.00 ± 0.00
10	2.92 ± 1.10	4.44 ± 0.67	4.41 ± 2.75	11.1 ± 4.25	28.0 ± 7.11	48.3 ± ND	24.7 ± 13.6
11	3.07 ± 0.26	5.29 ± 2.20	11.5 ± 3.06	26.2 ± 10.8	13.8 ± 0.10	28.4 ± ND	38.5 ± 9.04
12	7.49 ± 0.31	10.6 ± 0.45	15.6 ± 4.42	22.1 ± 0.83	14.4 ± 3.78	45.6 ± ND	48.3 ± 7.71
13	2.38 ± 0.39	5.57 ± 1.02	9.20 ± 5.02	15.5 ± 3.10	6.14 ± 0.01	17.0 ± ND	64.0 ± 0.26
14	5.03 ± 0.67	8.25 ± 1.40	10.5 ± 0.59	21.5 ± 6.66	13.7 ± 10.1	36.5 ± ND	56.5 ± 8.43
15	2.17 ± 1.30	3.40 ± 0.21	3.74 ± 0.76	14.0 ± 7.90	13.7 ± 1.36	14.5 ± ND	20.5 ± 0.04
16	4.42 ± 0.54	3.83 ± 0.47	10.1 ± 0.49	12.1 ± 1.56	11.2 ± 0.41	40.6 ± ND	36.1 ± 1.39
17	9.59 ± 0.68	12.8 ± 0.17	16.4 ± 0.31	24.0 ± 3.65	18.9 ± 3.01	34.7 ± ND	22.8 ± 0.20
18	4.27 ± 0.39	0.00 ± 0.00	2.11 ± 0.67	6.86 ± 3.77	11.7 ± 1.40	12.8 ± ND	20.5 ± 0.21
19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.54 ± 4.14	15.4 ± 8.77	16.9 ± ND	0.00 ± 0.00
20	44.6 ± 1.78	40.1 ± 6.60	46.1 ± 3.23	52.9 ± 10.0	33.9 ± 2.62	106 ± ND	103 ± 18.8
21	23.9 ± 0.12	31.0 ± 0.67	27.6 ± 2.27	31.8 ± 11.0	5.21 ± 1.24	47.1 ± ND	58.1 ± 2.66

Peaks correspond to those indicated in Fig. 1.

Table 2
Mean value, with standard deviation, of the chromatographic areas ($\times 10^4$/100 g cheese) of the 21 peaks analysed in the TCA-WSF from Fynbo cheese for different ripening times

Peak	Ripening time (days)						
	1	5	10	20	30	60	90
1	2.04 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	1.33 ± 0.29	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.49 ± 9.18	15.4 ± 1.50
3	1.99 ± 0.43	2.98 ± 2.45	8.08 ± 0.28	6.57 ± 9.29	12.5 ± 0.27	27.0 ± 1.57	23.9 ± 1.69
4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	4.00 ± 1.00	8.99 ± 4.04	12.6 ± 0.39	20.9 ± 3.68	26.0 ± 4.40	34.1 ± 1.39	24.9 ± 0.91
6	1.19 ± 0.18	3.43 ± 2.32	2.82 ± 3.99	5.10 ± 1.17	5.56 ± 4.75	11.2 ± 2.47	9.82 ± 0.61
7	4.02 ± 1.04	9.02 ± 4.19	7.88 ± 0.25	14.9 ± 4.06	11.6 ± 4.09	29.9 ± 2.67	38.8 ± 14.3
8	6.12 ± 1.13	16.5 ± 1.90	28.6 ± 12.2	39.2 ± 14.5	21.5 ± 2.56	51.5 ± 1.40	44.9 ± 0.10
9	2.34 ± 0.19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10	2.76 ± 0.86	5.65 ± 1.98	5.30 ± 2.22	10.2 ± 7.84	22.1 ± 0.80	29.6 ± 5.71	28.8 ± 0.63
11	3.00 ± 0.03	4.43 ± 0.80	14.2 ± 3.40	18.9 ± 4.02	13.0 ± 6.16	18.8 ± 7.95	35.9 ± 12.1
12	5.46 ± 1.83	10.8 ± 1.95	14.6 ± 4.76	23.9 ± 0.72	12.6 ± 8.04	26.8 ± 5.33	36.7 ± 5.22
13	1.98 ± 0.66	4.71 ± 0.93	9.27 ± 0.77	12.1 ± 6.51	15.4 ± 8.84	22.0 ± 4.00	44.8 ± 12.2
14	3.26 ± 2.12	8.92 ± 3.27	11.8 ± 0.24	21.9 ± 0.62	9.12 ± 2.79	35.8 ± 20.0	39.7 ± 19.1
15	2.97 ± 0.06	3.72 ± 0.47	2.45 ± 3.46	8.85 ± 3.47	6.36 ± 4.71	22.8 ± 0.29	12.9 ± 1.35
16	2.67 ± 0.87	3.27 ± 0.68	7.61 ± 2.10	13.2 ± 4.07	15.6 ± 0.21	30.6 ± 3.22	29.9 ± 4.03
17	6.51 ± 0.32	11.7 ± 0.67	13.6 ± 0.42	18.7 ± 1.82	16.8 ± 8.01	23.9 ± 1.83	8.46 ± 1.11
18	3.91 ± 0.64	3.02 ± 1.92	4.27 ± 1.18	6.10 ± 0.58	4.99 ± 5.10	18.2 ± 2.89	9.20 ± 1.58
19	1.94 ± 0.49	3.60 ± 1.66	4.25 ± 0.68	6.34 ± 0.56	0.00 ± 0.00	2.48 ± 3.51	7.14 ± 0.98
20	8.98 ± 2.93	6.58 ± 4.36	6.88 ± 2.73	8.00 ± 1.48	14.6 ± 2.05	29.7 ± 5.46	19.7 ± 5.68
21	2.87 ± 1.52	3.36 ± 2.18	2.79 ± 0.75	2.91 ± 0.22	0.00 ± 0.00	3.04 ± 2.20	8.87 ± 2.85

Peaks correspond to those indicated in Fig. 1.

associated with the more hydrophobic peaks (retention time higher than 30 min). These results agree with those obtained by Yvon, Chabanet, and Pélissier (1989), who studied the properties of 75 peptides obtained from different digests of α_{s1}-, β- and κ-casein. In that case, the authors concluded that the peptide retention time in RP-HPLC was better correlated with the peptide solubility in TCA solutions, which can be considered as an experimental measurement of peptide hydrophobicity.

Tables 1 and 2 show the mean values of the chromatographic areas per 100 g cheese of the 21 selected peaks for WSF and TCA-WSF, respectively. The majority of the peak areas increased with ripening time as expected. The information obtained from the chromatograms was successfully summarised in 2 dimensions, accounting for 84.7% of the data variation using PCA. Fig. 2 shows the plot of samples in the plane defined by the two principal components (PC1 and PC2). The source of variation explained by PC1 (64.9% VAR) can be related to the ripening time; clearly, the scattering of samples increases as ripening time increases. On the other hand, the fractionation with 4% TCA can be considered as the cause of variation entailed to PC2 (19.8% VAR).

Fig. 3 shows the distribution of peaks in the PC loading plot. Peaks with higher PC1 and PC2 loadings were 3, 5, 7, 8, 10, 11, 12, 13, 14, 16, 20, and 21. Most of the PC1 and PC2 loadings were negative. Among the peaks with positive PC2 loading, the peaks 20 and 21 had the highest values. The separation of peaks 20 and 21 from

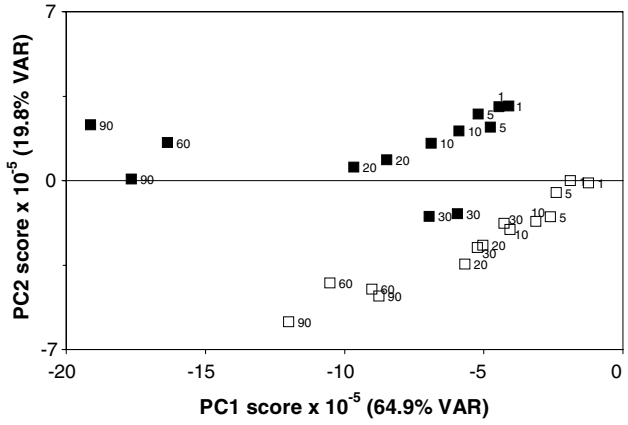

Fig. 2. Score plot from PCA with data from RP-HPLC chromatograms of the WSF (■) and TCA-WSF (□) from Fynbo cheese. Numbers indicate the ripening time of samples.

the rest of the peaks can be explained by considering the important effect of TCA on the peptide profiles of the WSF. In this case, the effect of 4% TCA was evident from the first day of ripening (e.g., peak 20, Fig. 4), while the TCA effect was hardly observed for the other peptides (e.g., peak 8, Fig. 5).

The identification of peptides has the potential to increase the understanding of the ongoing process during cheese ripening. Scarce information about the peptides that characterise Fynbo cheese proteolysis is available. In this case, an attempt at isolation and study of some peptides was carried out by acid hydrolysis of collected

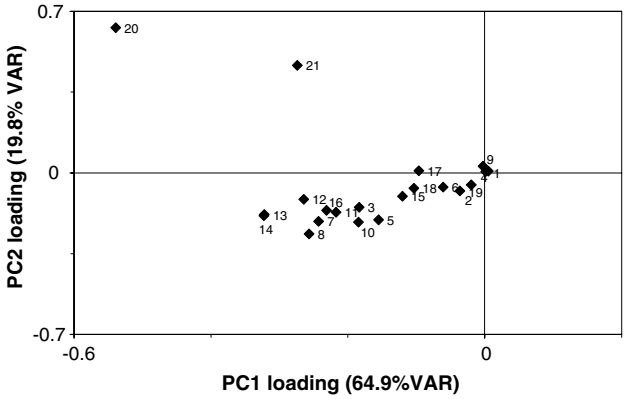

Fig. 3. Loading plot from PCA with data from RP-HPLC chromatograms of the WSF and TCA-WSF from Fynbo cheese. Numbers correspond to peaks shown in Fig. 1.

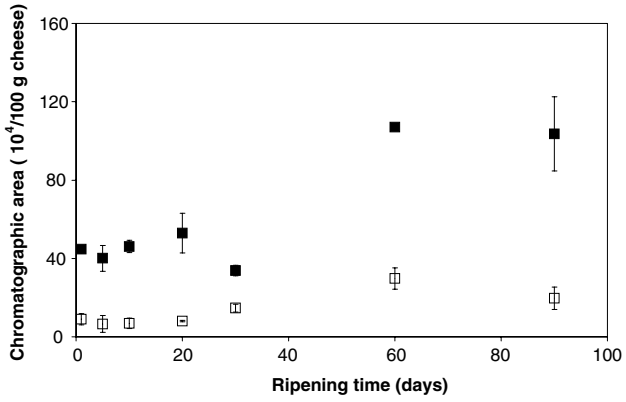

Fig. 4. RP-HPLC area of peak 20 in WSF (■) and TCA-WSF (□) during ripening of Fynbo cheese.

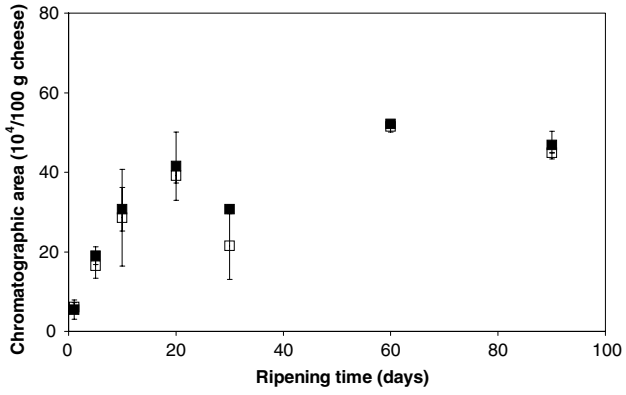

Fig. 5. RP-HPLC area of peak 8 in WSF (■) and TCA-WSF (□) during ripening of Fynbo cheese.

peaks. Peaks 12 and 20 were collected as representative of the peak groups with negative and positive PC2 loading, respectively.

Hydrochloric acid hydrolysis generally yields over 95% recovery for 10 amino acids: aspartic and glutamic acids, glycine, alanine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine (Gehrke, Wall, Absheer, Kaiser, & Zumwalt, 1985). The presence of these amino acids in peaks 12 and 20 was determined (Table 3). Differences in some amino acids that constitute both peaks can be observed. A hydrophobicity value or Δf – free energy of transfer of the side chains of amino acids – exists for each amino acid (Habibi-Najafi & Lee, 1996). These authors proposed calculating an average hydrophobicity value (Q) for a peptide as:

$$Q = \sum \Delta f / n, \quad (1)$$

where n is the number of amino acid residues. Values of Q for peaks 12 and 20 were calculated using values of Δf for each amino acid proposed by Habibi-Najafi and Lee (1996) and data of Table 3, resulting in Q (peak 12) = 821 and Q (peak 20) = 968. The higher value of Q for peak 20 can be useful for explaining the major effect of TCA on this peak, according to other results reported by different authors, which showed a correlation between peptide hydrophobicity and a lower solubility in TCA (Yvon et al., 1989).

TCA extraction and its RP-HPLC analysis may help in determining some characteristics of the peptides produced during Fynbo cheese ripening. The RP-HPLC profiles of the soluble compounds in 4% TCA may be used to control the regular process conditions or to study the proteolysis when some modifications in the elaboration process are tested.

Table 3
Amino acid composition corresponding to hydrolysed of peaks 12 and 20

	Nearest integer (mol aa/mol peptide)	
Amino acid	Peak 12	Peak 20
Asp	3	2
Glu	6	5
His	0	0
Gly	5	2
Ala	3	1
Tyr	0	0
Phe	2	1
Leu	2	2
Lys	0	1

4. Conclusions

The effect of 4% TCA solution on the water-soluble fraction of Fynbo cheese during ripening was evaluated by RP-HPLC and by PCA of the chromatographic data. The information obtained was successfully summarized in 2 dimensions, accounting for 84.7% of the data variation. The first and second principal components were related to the ripening time and to the treatment with 4% TCA, respectively. The effect of the acid was clearly evident for the peptides most retained by the

chromatographic column. Differences in the amino acid composition and in average hydrophobicity values were observed between peaks with different signs of PC2 loading. RP-HPLC profiles of the TCA-WSF can be useful for studing the different peptides produced during Fynbo cheese ripening, which could lead to a more detailed knowledge of the proteolytic reactions. Moreover, the peptide analysis may be used to control Fynbo cheese production under regular conditions or for comparisons when some modifications in the manufacturing process are tested.

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