Article No. fs970342 Lebensm.-Wiss. u.-Technol., 31, 297–301 (1998)

Research Note

Ripening of Emmental Cheese Wrapped in Foil with and without Addition of *Lactobacillus casei* subsp. *casei*. IV. HPLC Separation of Water-soluble Peptides

U. Bütikofer*, E. Baumann, R. Sieber and J. O. Bosset

Swiss Federal Dairy Research Station, Liebefeld, CH-3003 Berne (Switzerland)
(Received July 24, 1997; accepted October 10, 1997)

*The present work describes the high-performance liquid chromatographic (HPLC) separation of water-soluble peptides of eight quarters of raw milk Swiss Emmental cheese loaves which were packaged at 3 months in plastic wrap. The loaves were manufactured with (*n = 4*) and without (*n = 4*) the addition of* L. casei *subsp.* casei *to the usual starter culture. Samples were analysed at 3, 6, 9 and 12 months of ripening. The water-soluble peptides were separated by HPLC and analysed using a UV-detector at 210 nm. More than 100 peaks could be resolved. Analysis of variance and principal component analysis of the peak heights led to the following conclusion: 15 peaks increased and seven peaks decreased during the ripening period from 3–12 months. Cheese produced with* L. casei *subsp.* casei *showed three larger and two smaller peaks than those produced without this adjunct. The corresponding peptide composition was not determined.*

Keywords: Swiss cheese; Emmental; HPLC peptide mapping; *Lactobacillus casei* subsp. casei; cheese ripening

Introduction

Several authors have reported on the separation of peptides produced during cheese ripening, by polyacrylamide gel electrophoresis (PAGE), capillary zone electrophoresis (CZE), anion- and cation-exchange chromatography and high-performance liquid chromatography (HPLC) (1–8). A comprehensive review on methods for characterization of proteolysis in cheese during ripening was recently published by McSweeney and Fox (9). Urea–PAGE was used for the partial resolution of the water-insoluble peptides (10), and HPLC for the separation of bitter peptides in Cheddar cheese (11) or casein peptides from cows' and ewes' milk cheese (12). The age of Cheddar cheese could be approximated by use of statistical methods including only ten peaks separated by HPLC of a water-soluble extract (13). In all these studies, a great number of peaks were present in spite of a probably not exhausted resolution of all peptides present. From the 120-g/L trichloroacetic acid (TCA)-soluble nitrogen fraction of Parmigiano-Reggiano cheese samples, 39 oligopeptides, mainly phosphopeptides, could be demonstrated (14). Gonzales *et al.* (15) identified four peptides from the 50-g/L phosphotungstic acid-soluble fraction of blue cheese. Bican *et al.* (16) identified six basic peptides from Emmental cheese, four peptides originated from α_{s1}- and two from α_{s2}-casein. The diafiltration retentate of the water-soluble extract of Cheddar was resolved by Singh *et al.* (17) into eight fractions. In the first two fractions they found 51 peptides and in the next two fractions a further 80 peptides were identified completely or partly (18); most of these peptides were from β-casein (17, 18). Gouldsworthy *et al.* (19) proposed a new analytical tool for investigating peptides with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer. These authors identified 16 peptides in commercial mature Cheddar cheese: eight came from α_{s1}-casein, seven from β-casein and one from α_{s2}-casein. Furthermore, it is also possible that during cheese ripening, peptides with opioid activity might be formed. However, β-casomorphins were absent in extra-sharp Cheddar, Swiss (aged 60 d), Blue, Brie and Limburger cheeses (20). In continuation of the previous series of publications (21–23) on the use of *Lactobacillus* (L.) *casei* subsp. *casei* in the ripening of Emmental cheese wrapped in plastic foil, the aim of the present work was to study the formation of peptides over a ripening period from 3–12 months.

Materials and Methods

Cheese sampling procedure

Selection of the cheese loafs and sample preparation were described by Bachmann *et al.* (21). Emmental cheeses from eight different factories (A–H) were chosen at 3 months. Half of the cheese samples were

*To whom correspondence should be addressed.

0023-6438/98/030297 + 05 $30.00/0

produced with an addition of *L. casei* subsp. *casei*. The loaves were cut into four parts and packed in a plastic film (co-extruded PE/PVDC/PE plastic film, art. nr. BK1, Grace A.G., Kriens, Switzerland). Ripening from 3–12 months was performed in a cellar at 11–12 °C. The cheeses were analysed at 3 (unpackaged cheese), 6, 9 and 12 months of ripening. The samples were grated and then frozen at –20 °C. All samples were extracted at the same time and analysed within 2 d.

Chemicals and equipment

HPLC-grade acetonitrile was purchased from Gebr. Mächler (Basel, Switzerland, no. 8706). Trifluoroacetic acid (TFA) was obtained from Fluka (Buchs, Switzerland, no. 91699). Deionized water was produced by Milli-Q-Equipment. The 0.45 μm membrane filter RAWP was supplied by Millipore (Volketswil, Switzerland). A Polytron homogenizer from Kinematica (Zürich, Switzerland) was used.

Sample preparation

Grated cheese (10 g) was dispersed in 50 mL water using the Polytron homogenizer (twice for 1 min at 10000 rpm within 3 min). The homogenates were stored for 1 h at 40 °C and then centrifugated at 3000 *g* for 30 min at 4 °C. The extracts were filtered through glass-wool and then through a 0.45 μm membrane filter.

High-performance liquid chromatography (HPLC)

HPLC was performed using a HP 1090M system (Hewlett Packard, Zürich, Switzerland) fitted with a UV detector HP 1040A. Samples (20 μL) of aqueous extracts were chromatographed at an oven temperature of 45 °C. The precolumn was a KS 11/6/4 Nucleosil 300-5 C_{18} and the column an ET 250/8/4 Nucleosil 300-5 C_{18} (Macherey-Nagel, Oensingen, Switzerland). A two-components eluant was used: 1 g/L TFA in water was used as mobile phase A and a mixture (600 mL acetonitrile + 400 mL phase A) as mobile phase B. The flow rate was 1000 mL/min. Detection was performed at 210 nm (reference at 450 nm). The gradient was from 0–10 min, 0% phase B, from 10–90 min, 0–80% phase B and from 90.1–100 min, 80–100% phase B.

Statistical analysis

Small changes in the retention time were checked with overlaid printed chromatograms. Mean values and standard deviations were then calculated for all peak heights with a threshold of 10 mAU. Student's *t*-test was applied to find significant differences between the groups. All calculations were performed with SYSTAT for Windows Version 5.0 (24).

Results and Discussion

The HPLC chromatograms showed a great number of water-soluble peptides (**Fig. 1**). More than 100 peaks could be integrated. Bican and Spahni (4) found at least 100 peptides in Emmental, Gruyère and Appenzell cheeses, and according to McSweeney and Fox (9), see also (17) and (18) more than 200 peptides have been isolated and identified from water-soluble extract of Cheddar.

The heights of the peptide peaks over the ripening period considered are shown in **Table 1**. Changes in the size of the peaks may be used as an index of protein breakdown. From the 121 recognized peaks, only 85 were finally selected for the statistical evaluation. Several hundreds were probably covered and could not be resolved without multidimensional chromatography. It has been shown by two-dimensional separation of tryptic digests of horse cytochrome (25) and porcine thyroglobulin (26) by HPLC and a further CZE separation that most peaks consisted of up to 10 compounds. Several authors have shown that the peptide system in Emmental cheeses is extremely complex, even more complex than in other cheese varieties (4, 7, 14).

The evolution of the proteolysis, based on peptide mapping at various stages of ripening, has already been studied for Emmental, Gruyère, Appenzell (4), Parmigiano-Reggiano (14), Blue cheese (15) and for several artisan cheeses of Asturias (7). None of these studies,

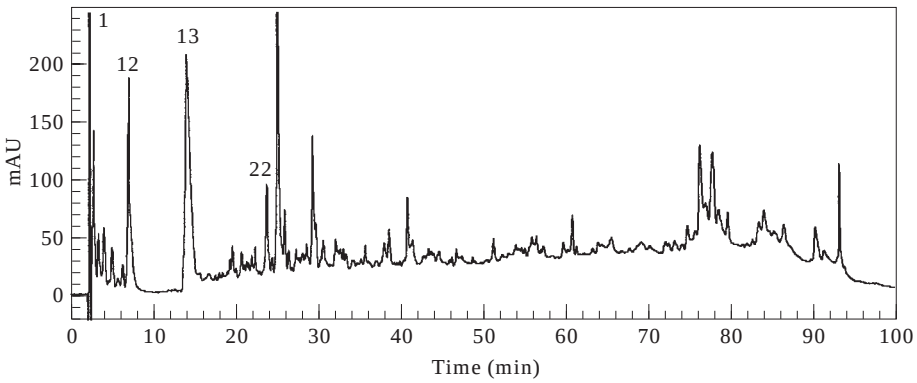

Fig. 1 HPLC separation of a water-soluble extract (sample G9) of a 9-month-old Swiss Emmental cheese with addition of *Lactobacillus casei* subsp. *casei* to the starter peptides culture. (Peak numbers: 1 = various free amino acids; 12 = tyrosine; 13 = phenylalanine; 22 = tryptophane, see text)

however, considered each individual peak over the complete ripening period, which was the main aim of the present work.

The sum of all peak heights increased by about 28% between the third and the twelfth month of ripening. In comparison, the corresponding increase in the water-soluble fraction was approximately 39%, i.e. in the same order of magnitude (21). Several free amino acids co-eluted within the first peak. Free tyrosine (peak 12), phenylalanine (peak 13) and tryosine (peak 22) eluted as characteristic markers in the first part of the chromatogram. The increase of peak 1 over the considered ripening period was 107%, in fair agreement with the increase in the total concentration of free amino acids (120%) (21). In soft cheeses, these three aromatic amino acids increased quickly after 15 d of ripening (7).

Further assays carried out on the HPLC chromatograms of 120-g/L TCA-soluble nitrogen extract (instead of the aqueous extract used in the present work) showed only peaks with elution times of less than 25 min. The same was found by Addeo *et al.* (14) in the TCA-soluble fraction of Parmigiano-Reggiano cheese. The sum of the corresponding 23 first peak heights increased by approximately 66%, in agreement with the 73% increase of the TCA-soluble fraction (21).

Peaks 1, 3, 4, 5, 6, 12, 13, 19, 22, 30, 31, 36, 75, 86 and 87 increased over the ripening period. Three peaks (nos 76, 104, 108) remained constant. Seven others (nos 15, 33, 35, 107, 119, 120 and 121) present at 3 months were hydrolysed and consequently decreased over the same time. Moreover, peaks 42 and 115 increased until 9 months and then decreased up to 12 months; peaks 65 and 68 increased from 3 to 6 months and decreased later.

Cheeses produced with addition of *L. casei* subsp. *casei* to the starter culture (**Table 1**) exhibited at least at two different ages significantly higher (nos 2, 3 and 23) and lower (nos 7 and 11) peaks. Eleven Cheddar cheeses after 3-months ripening, each manufactured with a different single-strain starter, were indistinguishable in the peptide pattern of their insoluble fraction (10).

Table 1 Changes of peptides during the ripening of Emmental cheese (peak heights)

Peak number[a]	Retention time (min)	Cheese age (months)							
		3		6		9		12	
		–LC	+LC	–LC	+LC	–LC	+LC	–LC	+LC
1	2.55	578 (191)[b]		799 (246)		1043 (205)		1196 (261)	
2	2.93	22 (25)	83 (10)	20 (23)	77 (4)	43 (5)	70 (7)	33 (22)	72 (4)
3	3.10	52 (7)	68 (7)	53 (4)	68 (3)	67 (10)		76 (16)	
4	3.55	20 (17)		35 (16)		51 (9)		60 (11)	
5	3.63	14 (16)		26 (22)		44 (19)		57 (9)	
6	4.29	37 (19)		59 (12)		95 (15)		106 (15)	
7	5.00	43 (2)	31 (6)	48 (3)	30 (2)	55 (5)	39 (6)	54 (3)	40 (5)
11	6.64	46 (30)	3 (6)	79 (45)	6 (12)	105 (57)	11 (13)	119 (65)	21 (5)
12	7.35	91 (27)		113 (27)		128 (37)		120 (42)	174 (3)
13	14.67	209 (31)		224 (21)		261 (19)		277 (22)	
15	17.24	15 (2)		7 (5)		7 (6)		7 (6)	
19	21.34	11 (18)		12 (8)		25 (5)		25 (6)	
22	24.20	89 (20)		113 (28)		148 (21)		167 (29)	
23	24.99	20 (2)		20 (2)		20 (3)	26 (1)	21 (6)	30 (4)
30	31.51	1 (3)		2 (3)		6 (4)		10 (2)	
31	33.33	0 (0)		0 (0)		9 (6)		12 (2)	18 (4)
33	34.78	46 (19)		44 (13)		39 (6)	28 (7)	25 (13)	
35	36.26	184 (26)		152 (49)		117 (57)		91 (54)	
36	37.38	9 (5)		14 (9)		21 (6)		23 (5)	
42	41.70	14 (7)		22 (5)		32 (12)		28 (14)	
65	55.06	8 (11)		25 (12)		25 (9)		19 (10)	
68	56.65	1 (4)		10 (9)		3 (6)		0 (0)	
75	61.38	10 (7)		16 (5)		19 (4)		18 (5)	
76	61.98	12 (8)		12 (6)		13 (3)		10 (5)	
86	69.72	5 (8)		13 (6)		19 (6)		22 (7)	
87	70.12	19 (4)		20 (3)		25 (4)		25 (5)	
104	80.44	40 (6)		42 (6)		38 (6)		43 (7)	
107	82.36	33 (6)		28 (5)		18 (5)		18 (5)	
108	83.53	19 (12)		19 (9)		16 (4)		18 (4)	
115	92.84	73 (30)		85 (6)		100 (4)		93 (34)	
119	94.45	19 (9)		9 (5)		2 (3)		0 (0)	
120	95.10	10 (12)		0 (0)	8 (6)	0 (0)		0 (0)	
121	95.97	39 (13)		23 (2)		11 (5)		7 (4)	
Sum		3529 (254)		3794 (384)		4277 (480)		4516 (518)	

[a]Eight cheese samples were measured at each age, four with *Lactobacillus casei* subsp. *casei* (+LC) and four without *L. casei* (–LC). Only peaks that changed during ripening or differing with the adjunct are listed.
[b]Mean (standard deviation).

Principal component analysis on the co-variance matrix was performed on the same data set (**Table 2**). The first principal component highlights the dominant influence of cheese age (**Fig. 2**), which alone explains 78% of the total variance. The second principal component discriminates between the cheese of factory H, which underwent a much slower proteolysis probably owing to a significantly higher copper content (see part I of this series, 21), and cheeses from the other factories. Earlier experiments (27) confirm this observation. The release of large peptides was much slower up to 6 months. The fourth principal component accounted for only 2% of the total variance, but more clearly discriminated cheese loaves with and without addition of *L. casei* subsp. *casei* (**Fig. 3**). The third principal component had in this case no significance.

Conclusions

The present study revealed new information on the breakdown of the caseins of Swiss Emmental cheese over a 3- to 12-month ripening period. Difficulties were encountered: (a) an insufficient separation of the numerous amino acids and peptides released over this ripening period, and (b) a difficulty of interpretation of the changes of the peptide mapping without mass spectrometry.

HPLC peptide mapping indicated numerous changes over the ripening period considered. More than 121 peaks could be used for statistical analysis. A discrimination analysis indicated that many peaks increased, some remained constant, and others decreased over the period considered. Five peaks significantly discriminated between cheeses with and without addition of *L. casei* subsp. *casei*. The technique used in this study allows only a semi-quantitative determination of peptides. A quantitative study would need the tedious collection of the different peaks and their further sequencing, combined with amino-acid analysis and mass spectrometry (19). In the near future, new mass-sensitive detectors which could be coupled directly to the HPLC system should allow online identification of peptides.

Table 2 Principal component analysis

	Principal components (PC)			
Variance	1	2	3	4
Explained by PC	78%	6%	5%	2%
Cumulated	78%	84%	89%	91%

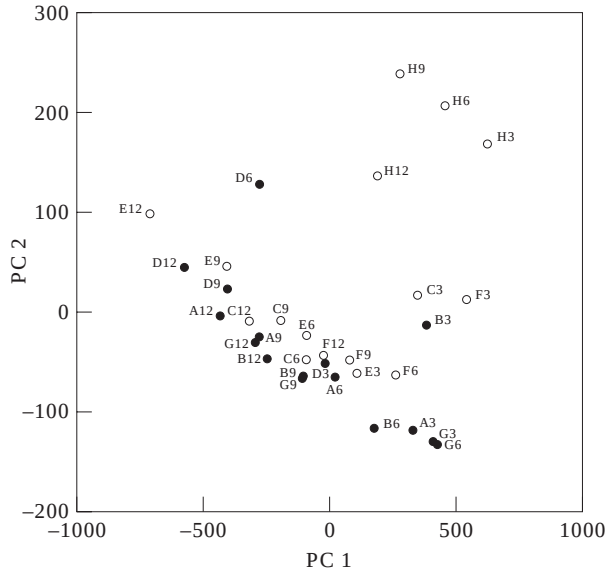

Fig. 2 Plotting of first and second principal components (● with, ○ without, *Lactobacillus casei* subsp. *casei*) A–H = cheese manufacturer, 3–12 = cheese age (months)

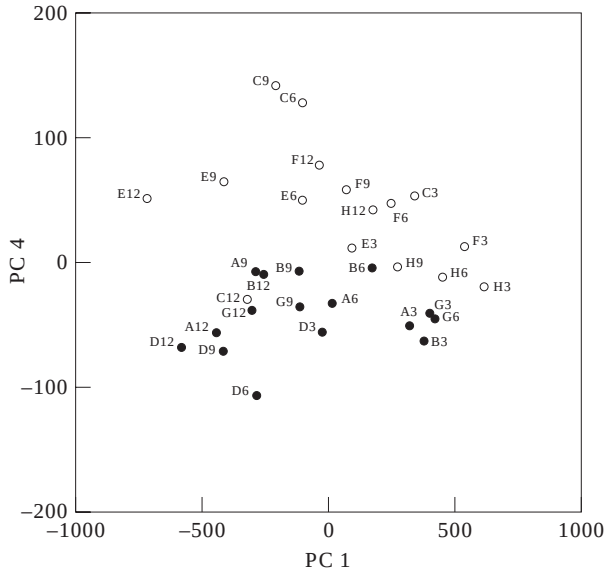

Fig. 3 Plotting of first and fourth principal components (● with, ○ without *Lactobacillus casei* subsp. *casei*). A–H = cheese manufacturer, 3–12 = cheese (months)

References

1 Cliffe, A. J., Revell, D. and Law, B. A. A method for the reversed phase high performance liquid chromatography of peptides from Cheddar cheese. *Food Chemistry*, **34**, 147–160 (1989)

2 Christensen, T. M.I.E., Kristiansen, K. R. and Madsen, J. S. Proteolysis in cheese investigated by high performance liquid chromatography. *Journal of Dairy Research*, **56**, 823–828 (1989)

3 Ardö, Y. and Gripon, J. C. Chromatographic methods used to measure proteolysis in cheese. *IDF Bulletin*, **261**, 29–33 (1991)

4 Bican, P. and Spahni, A. Proteolysis in Swiss-type cheeses: a high-performance liquid chromatography study. *International Dairy Journal*, **3**, 73–84 (1993)

5 Belitz, H.-D. and Kaiser, K.-P. Monitoring Cheddar cheese ripening by chemical indices of proteolysis. 3. Identification of several high-molecular mass peptides. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung*, **197**, 118–122 (1993)

6 Singh, T. K., Fox, P. F., Hojrup, P. and Healy, A. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. *International Dairy Journal*, **4**, 111–122 (1994)

7 Gonzales De Llano, D., Polo, M. C. and Ramos, M. Study of proteolysis in artisanal cheeses: high perform-

ance liquid chromatography of peptides. *Journal of Dairy Science*, **78**, 1018–1024 (1995)
8 Turin, L., Mutti, B. and Bonomi, F. Isolation and characterization of large-size water soluble peptides produced in the ripening of Taleggio and Caciotta cheese. *Milchwissenschaft*, **50**, 371–375 (1995)
9 McSweeney, P. L.H. and Fox, P. F. Chemical methods for the characterization of proteolysis in cheese during ripening. *Lait*, **77**, 41–76 (1997)
10 McSweeney, P. L.H., Pochet, S., Fox, P. F. and Healy, A. Partial identification of peptides from the water-insoluble fraction of Cheddar cheese. *Journal of Dairy Research*, **61**, 587–590 (1994)
11 Champion, H. M. and Stanley, D. W. HPLC separation of bitter peptides from Cheddar cheese. *Canadian Institute of Food Science and Technology Journal*, **15**, 283–288 (1982)
12 Tobler, M., Windemann, H. and Baumgartner, E. HPLC-Auftrennung von Peptiden aus Casein von Kuh- und Ziegenkäse. *Mitteilungen auf dem Gebiete der Lebensmitteluntersuchungen und Hygiene*, **74**, 132–139 (1983)
13 Pham, A.-M. and Nakai, S. Application of stepwise discrimant analysis to high pressure liquid chromatography profiles of water extract for judging ripening of Cheddar cheese. *Journal of Dairy Science*, **67**, 1390–1396 (1984)
14 Addeo, F., Chianese, L., Sacchi, R., Musso, S. S., Ferranti, P. and Malorni, A. Characterization of the oligopeptides of Parmigiano-Reggiano cheese soluble in 120 g trichloroacetic acid/I. *Journal of Dairy Research*, **61**, 365–374 (1994)
15 Gonzalez De Llano, D., Polo, M. C. and Ramos, M. Production, isolation and identification of low molecular mass peptides from blue cheese by high performance liquid chromatography. *Journal of Dairy Research*, **58**, 363–372 (1991)
16 Bican, P., Spahni, A. and Schaller, J. Partial characterization of peptides from emmentaler cheeses. *Food Biotechnology*, **8**, 229–241 (1994)
17 Singh, T. K., Fox, P. F. and Healy, A. Water-soluble peptides in Cheddar cheese: isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. *Journal of Dairy Research*, **62**, 629–640 (1995)
18 Singh, T. K., Fox, P. F. and Healy, A. Isolation and identification of further peptides in the diafiltration retentate of the water-soluble fraction of Cheddar cheese. *Journal of Dairy Research*, **64**, 433–443 (1997)
19 Gouldsworthy, A. M., Leaver, J. and Banks, J. M. Application of a mass spectrometry sequencing technique for identifying peptides present in Cheddar cheese. *International Dairy Journal*, **6**, 781–790 (1996)
20 Muehlenkamp, M. R. and Warthesen, J. J. β-Casomorphins: analysis in cheese and susceptibility to proteolytic enzymes from *Lactococcus lactis* ssp. *cremoris*. *Journal of Dairy Science*, **79**, 20–26 (1996)
21 Bachmann, H. P., Bütikofer, U., Badertscher, R., Dalla Torre, M., Lavanchy, P., Bühler-Moor, U., Nick, B., Jimeno, J., Warmke, R., Grosch, W., Sieber, R. and Bosset, J. O. Reifungsverlauf von in Folien verpacktem Emmentaler Käse mit und ohne Zusatz von *Lactobacillus casei* subsp. *casei*. I. Mikrobiologische, chemische, rheologische und sensorische Untersuchungen. *Lebensmittel-Wissenschaft und -Technologie*, **30**, 417–428 (1997)
22 Bosset, J. O., Bütikofer, U., Gauch, R. and Sieber, R. Reifungsverlauf von in Folien verpacktem Emmentaler Käse mit und ohne Zusatz von *Lactobacillus casei* subsp. *casei*. II. Untersuchung einiger flüchtiger, neutraler Verbindungen mit Hilfe einer GC-dynamischen Dampfraumanalyse. *Lebensmittel-Wissenschaft und - Technologie*, **30**, 464–470 (1997)
23 Rychlick, M., Warmke, R. and Grosch, W. Ripening of Emmental cheese wrapped in foil with and without addition of *Lactobacillus casei*. spp. *casei*. III. Analysis of character impact flavour compounds. *Lebensmittel-Wissenschaft und -Technologie*, **30**, 471–478 (1997)
24 SYSTAT: Systat for Windows: Statistics. Version 5.0 Edition. Evanston, IL: SYSTAT Inc.(1992)
25 Larmann, J. P., Lemmo, A. V., Moore, A. W. and Jorgenson, J. W. Two-dimensional separations of peptides and proteins by comprehensive liquid chromatography–capillary electrophoresis. *Electrophoresis*, **14**, 439–447 (1993)
26 Willis, B. Analytical chemistry instrumentation and systems: a view to the future. *International Laboratory*, May 1995, 11–17 (1995)
27 Steffen, C., Bühlmann, C., Schnider, J., Schär, H. and Rentsch, F. Vergleichende Untersuchungen in Emmentalerkäsen mit und ohne Nachgärung. I. Probenerhebung, statistische Auswertung und Fabrikationsdaten. *Schweizerische Milchwirtschaftliche Forschung*, **8**, 3–8 (1979)