Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 93 (2005) 65–72
Food Chemistry
www.elsevier.com/locate/foodchem

Identification of rennet-whey solids in "traditional butter" by means of HPLC/ESI-MS of non-glycosylated caseinomacropeptide A

Ivano De Noni *, Pierpaolo Resmini

Department of Food Science and Technology, State University of Milan, via G. Celoria 2, Milan 20133, Italy

Received 15 January 2004; received in revised form 5 September 2004; accepted 6 September 2004

Abstract

"Traditional butter" (TB) is directly obtained from milk cream according to Reg. EC No. 2991/1994. Ascertainment of TB authenticity implies the development of an analytical method for detecting illegal addition of cream from rennet-whey cream (RWC) to milk cream. The reference HPLC method adopted for detecting the presence of rennet-whey solids in skim milk powder (EC Reg. No. 213/2001) is based on the determination of non-glycosylated caseinomacropeptide A (CMP A, i.e. k-CN_A f 106–169). In this paper, the same method, coupled to ESI-MS, has been applied to the water phase of butter in order to detect CMP A deriving from usage of RWC for butter manufacturing. The reliability of this approach has been evaluated by studying the effect of both natural creaming and cream ripening in originating CMP A or peptides with CMP-like chromatographic behaviour. Results demonstrated that peptides other than CMP A, and interfering in the HPLC profile, can form in cream after prolonged ripening with commercial starters of lactic acid bacteria. Furthermore, proteolysis caused by psychrotrophic bacteria was studied by inoculating milk cream with *Pseudomonas fragi* ATCC 4973. In this case, different peptides, including CMP A, are cleaved from k-casein when bacterial count is $>10^6$ cfu/g. For these reasons, only LC/MS can unequivocally show the presence of non-glycosylated CMP A and hence the usage of RWC in manufacturing of TB. In this regard, monitoring of multicharged ions at m/z 1697.5 and 2263.2 was adopted for recognition of monophosphorylated CMP A. Control of commercial samples by means of HPLC/ESI-MS revealed that usage of RWC is sometimes performed in countries where butter consumption is not widespread and a large volume of rennet whey is available.

Keywords: Caseinomacropeptide; Traditional butter; Rennet-whey cream; ESI-MS; HPLC

1. Introduction

Butter is traditionally prepared from cream obtained by milk centrifugation (milk cream, MC). Churning and working are successive steps of butter-making, during which the oil-in-water emulsion is broken, leading to aqueous phase separation and formation of water-in-oil emulsion. According to the Council Regulation No. 2991/94 (1994), the term "traditional" may be used, together with the name "butter", provided the cream is obtained directly from milk by either centrifugation or natural creaming.

Raw materials other than MC are characteristic of countries where cheese-making is widespread and, in this regard, centrifugation of rennet whey represents the main source. Normally, the lower quality of the rennet-whey cream (RWC) implies blending with MC before churning in order to produce butter with satisfactory microbiological and sensory properties. Nevertheless, the derived butter can not be labelled as "traditional" and assessment of its genuineness implies the identification of the type of used raw material.

* Corresponding author. Tel.: +39 25 031 6670; fax: +39 25 031 6672.
E-mail address: ivano.denoni@unimi.it (I.D. Noni).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.09.011

Analytical methods for ascertaining presence of rennet whey solids (RWS) in dairy products are mainly based on either direct or indirect detection of caseinomacropeptides (CMPs) released from *k*-casein during milk renneting. A colorimetric method for the determination of sialic acid, present in CMPs, was reported for detecting RWS in skim milk powder (SMP) (De Koning, Eisses, & De Vries, 1966). Also, Polak and Bradley (1994) proposed a colorimetric method for detecting whey cream in sweet cream and butter. Nevertheless, these methods prove to be not specific because proteolytic phenomena in milk can split peptides other than CMPs from *k*-casein but still containing sialic acid (Recio, Lopez Fandino, Olano, Olieman, & Ramos, 1996). For this reason, more selective analytical methods have been studied, in order to identify true CMPs in SMP or in buttermilk powder (Olieman & van Riel, 1989; Van Riel & Olieman, 1995). In particular, the HPLC method proposed by Olieman and van Riel (1989) has been adopted in EC Regulations (No. 2799/99, 1999; No. 213/01, 2001) for the control of SMP intended for animal feed. This method ascertains the presence of non-glycosylated caseinomacropeptide (CMP A) released from *k*-casein A (k-CN_A) during renneting of milk. The objective of this work was to investigate the potential of the cited HPLC method in ascertaining the usage of RWC in manufacturing of TB. To this end, the influence of the individual steps of the buttermaking process on reliability of the method was also investigated.

2. Materials and methods

2.1. Materials

Experimental butter samples were prepared near an Italian manufacturer, using an industrial continuous buttermaker (Contimab-Simon). MC and RWC samples were also prepared by centrifugation of raw bulk milk and rennet whey from industrial cheese-makings, respectively.

Bulk samples ($n = 2$) of cream from natural creaming of milk were provided by manufacturers of Grana Padano and Parmigiano Reggiano cheeses.

One sample of pasteurized MC (35% fat) was purchased at the market and inoculated with three different starters from Sacco (Cadorago, Italy). Starters were defined strain cultures of *Lactococcus lactis* subsp. lactis and *Lactococcus lactis* subsp. cremoris (MO 0.30), *Lactococcus lactis* subsp. lactis, *Lactococcus lactis* subsp. cremoris and *Lactococcus lactis* subsp. lactis biovar. diacetylactis (M 0.31R) or *Lactococcus lactis* subsp. lactis, *Lactococcus* subsp. cremoris, subsp. *Lactococcus* lactis biovar. diacetylactis and Leuconostoc (M 0.32T). Each starter contained an amount of viable cells that allowed the addition of at least one million cells per gramme of cream. Cultured samples were then ripened at 22 °C for 24 h.

Fresh culture of a selected strain of *Pseudomonas fragi* ATCC 4973, grown at 30 °C for 16 h in Nutrient Broth (Sharlau, Barcelona, Spain), was inoculated into commercial pasteurized MC. In particular, cells were centrifuged at 3000*g* for 10 min, collected and suspended in 4 ml distilled water. Appropriate aliquots of the bacterial suspension were inoculated into 200 ml MC in order to obtain an initial concentration of 3×10^6 cfu/ml. Aliquots of cultured MC were then kept either at 4 °C for 72 h or at 30 °C for 24 h. Counts were determined by Gelisate Agar (incubation at 30 °C for 72 h).

Commercial butter samples were collected at the retail market in Italy ($n = 26$), France ($n = 6$), Germany ($n = 2$), United Kingdom ($n = 2$) and Denmark ($n = 2$). Samples were analysed within seven days from purchase or frozen before analysis.

2.2. Methods

2.2.1. Sample preparation

About 400 g butter were melted in a water bath (40 °C) and centrifuged at 8000*g* for 30 min at 40 °C. The supernatant (oil phase) was discharged and the water phase collected. Ten ml of the water phase were treated with 5 ml 24% (w/w) trichloroacetic acid under stirring over a period of 2 min. Finally, after standing at 25 °C for 1 h, the sample was filtered through a 0.45 μm pore membrane (Millipore, Billerica, MA) and 50 μl injected into the HPLC. When cream was analysed, 40 g cream were centrifuged (3000*g* for 20 min, 40 °C) and 20 ml water phase were treated with 10 ml 24% (w/w) trichloroacetic acid under stirring. Subsequent preparation steps were the same as for butter analysis.

2.2.2. HPLC/electrospray ionisation-mass spectrometry conditions

An Alliance 2695 HPLC quaternary gradient pump system was used (Waters, Milford, MA). Chromatographic conditions of the reverse phase HPLC method reported in *Annex XIX* to the EC Reg. No. 213/01 (2001) were adopted. For this purpose, SMP with 5% RWS was purchased at NIZO (Ede, The Netherlands) and used as reference material for identifying the peak of CMP A. A post-column flow splitter was used to introduce 1:15 of the HPLC flow stream (1.0 ml/min) into the ESI source.

Electrospray ionisation-mass spectrometry (ESI-MS) data were acquired in positive mode using a LCQ Deca XP (Thermo Finnigan, San Jose, CA) ion trap equipment. Nitrogen was used as nebulisation gas (relative flow value = 12). Capillary temperature was set at 275 °C, the potential applied was 5 kV and the cone voltage was 32 V. Full scan mass spectra were acquired, scanning the ranges *m/z* 1500–2500 or *m/z* 500–2000,

and detection of monophosphorylated CMP A was based on recognition of ions at *m/z* 1697.5 and 2263.2 or by selected ion monitoring (SIM) of the latter ion. CMP A was quantified by evaluating the intensity of ion at *m/z* 2263.2 during SIM. Deconvoluted mass spectra were determined on the measured protonated ($[M + nH^+]n^+$) molecules with the Bioworks software (Thermo Finnigan, San Jose, CA). Mass accuracy in the range *m/z* 1500–2500 was ensured by calibration with PPG2700/PPG2000 mixture (about 100 ng/μl in 10 mM sodium acetate in methanol:water 1:1), infused separately. Alternatively, a mixture of caffeine, reserpine and PFK (in methanol:water 1:1, 0.1% acetic acid) was adopted when scanning the range *m/z* 500–2000. The average molecular weight (M_r) of peptides was calculated by summing the corresponding masses of amino acid residues present in the primary amino acid sequence of *k*-casein according to Mercier, Ribadeau-Dumas, and Grosclaude (1973). All reagents and solvents were of analytical or HPLC grade and purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland).

3. Results and discussion

3.1. Feasibility and reliability of the HPLC/ESI-MS approach

Rennet coagulation of milk involves a specific cleavage of *k*-casein to yield *para-k*-casein and soluble CMPs, which remain in the rennet whey. Presence (>1% w/w) of RWS in SMP is detectable by determining the presence of non-glycosylated CMP A (f 106–169) from *k*-CN_A according to the HPLC method reported in Reg. EC No. 213/01 (2001). The method requires the elution gradient to be adjusted in order to elute CMP A at 26 ± 2 min retention time (RT). Under this condition the peak of CMP A can be identified as it shows a RT which differs no more than ± 0.2 min with respect to that of the CMP A peak of the reference sample, i.e. SMP treated with 5% RWS.

In principle, the same analytical approach could be applied to ascertain the usage of RWC during butter manufacturing. Indeed, caseinomacropeptides from rennet coagulation remain in the whey and possibly in the water phase of butter after whey cream churning. The feasibility of this approach was verified by preparing two industrial samples of butter produced from either MC or RWC, obtained from the same milk and submitted to pasteurization (85 °C for 30 s) and churning. The HPLC patterns of the water phase of these butter samples are shown in Fig. 1, along with that of the reference sample prepared according to Reg. No. 213/01. The peak at 27 min RT, in butter from RWC, could be ascribed to CMP A, on the basis of both RT and absence of absorbance at 280 nm (not shown). This feature is related to the absence of aromatic amino acids in the primary structure of CMP A (Mercier et al., 1973). In order to allow univocal identification of CMP A, the HPLC was coupled to ESI-MS. As shown in Fig. 2(a), the base peaks observed in ESI-MS spectrum are ions at *m/z* 1697.5 and 2263.2. These peaks correspond to the $[M + 4H^+]^{4+}$ and $[M + 3H^+]^{3+}$ multicharged ions for non-glycosylated monophosphorylated CMP A, as demonstrated by the deconvoluted spectrum (Fig. 2(b)), which shows a M_r of 6786.3 Da ($n = 6$, mean: 6786.3, std dev: 0.40). The level of protonation is consistent with the basic sites (3 Lys and 0 Arg residues) on this peptide (Mercier et al., 1973) and the M_r fits the expected average mass for CMP A (i.e. 6787.45 Da). The difference of about 1 Da little accounts for the MS resolution but much more for ionization of the phosphoserine residue ($pK_a = 1.6$) at position 149 of CMP A. Indeed, trifluoroacetic acid in the HPLC eluents lowers the pH value of the mobile phase to about 1–2, so promoting dissociation of the phosphoric group.

At least three other unresolved peaks are eluted as front or tail to CMP A in the HPLC pattern of reference SMP (Fig. 1). Nevertheless, ESI-MS spectra (not shown)

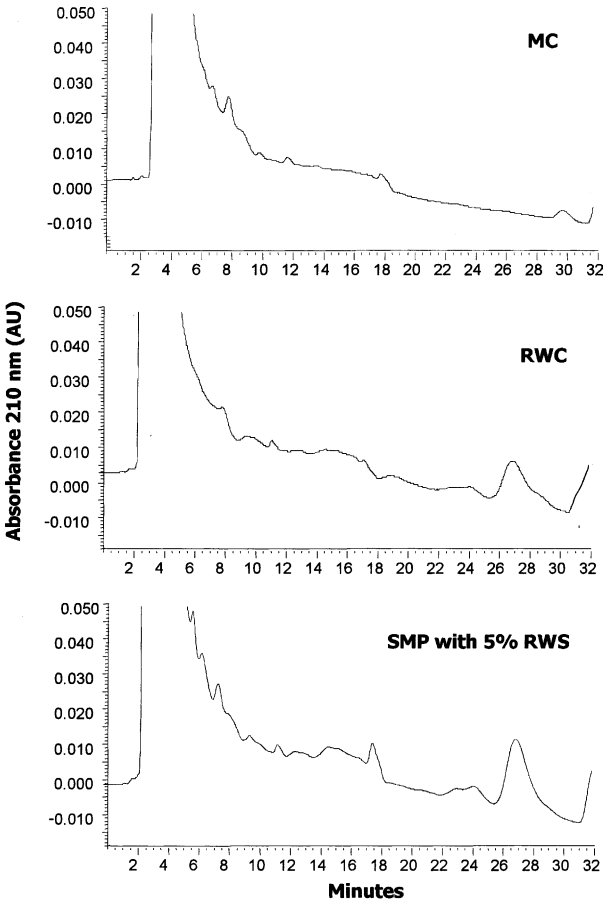

Fig. 1. HPLC profiles of water phase of butter samples from milk cream (MC), rennet whey cream (RWC) and skim milk powder (SMP) treated with 5% (w/w) rennet whey solids.

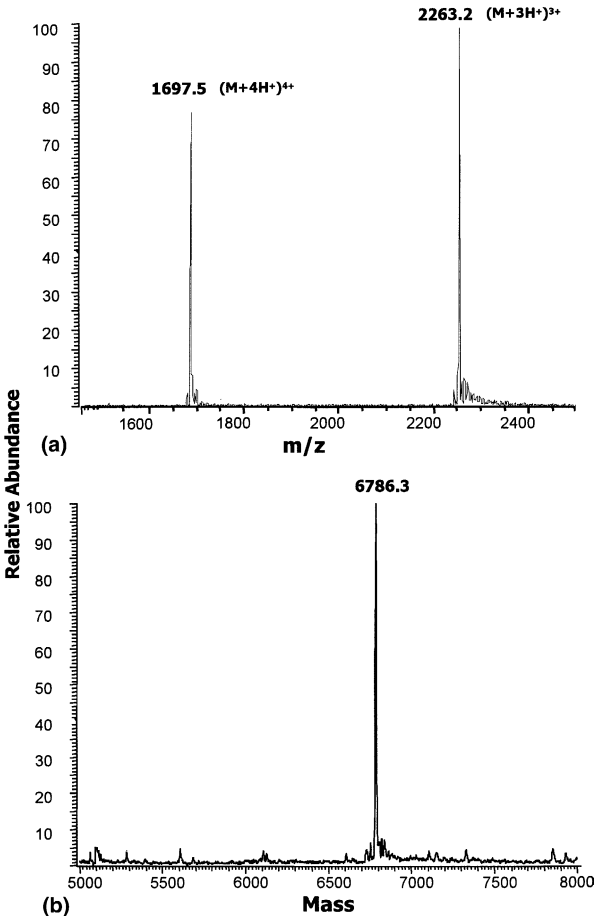

Fig. 2. (a) Normalised full scan ESI-MS spectrum obtained from the peak eluting at 27 min in the HPLC profile of water phase of butter from RWC and (b) related deconvoluted spectrum.

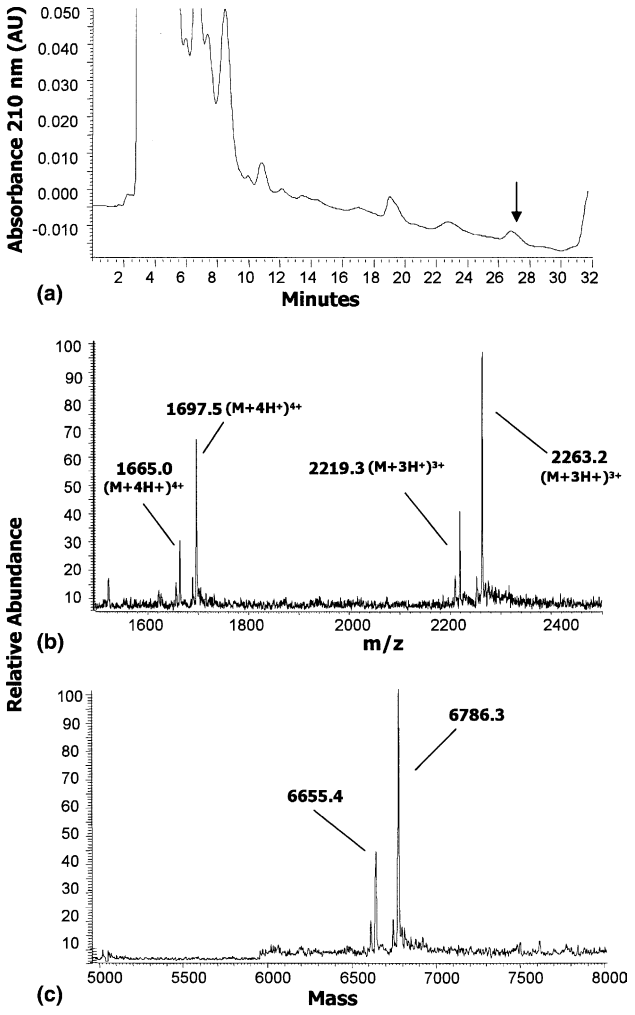

Fig. 3. (a) HPLC profile of the water phase of a commercial butter sample. (b) Full scan mass ESI-MS spectrum obtained at 26.8–27.0 min in the HPLC profile and (c) related deconvoluted spectrum.

of this peak cluster show the presence of five peptides with M_r of 6–7 kDa and including fragments 106–169 (1P) from k-CN_B (M_r = 6754.9 Da) and non-glycosylated diphosphorylated CMP A (M_r = 6865.5 Da).

The reliability of HPLC/ESI-MS is demonstrated in Fig. 3(a), which refers to a commercial sample of butter. According to the RT and absence of absorbance at 280 nm, the peak eluted at 26.8 min could be ascribed to CMP A. Nevertheless, the mass spectrum presented base peaks at *m/z* 1665.0 and 2219.3 (Fig. 3(b)), which correspond, respectively, to $(M + 4H^+)^{4+}$and $(M + 3H^+)^{3+}$of the deconvoluted mass spectrum at 6655.4 Da (Fig. 3(c)). This M_r suggests that additional proteolysis of k-CN_A has occurred between positions 106 and 107, the methionine residue located at position 106 in the primary structure of k-CN_A accounting for the mass difference. Proteases from *Pseudomonas* spp. are mainly reported to be responsible for a splitting of k-CN_A, which leads to formation of "pseudo-CMP" (*i.e.* k-CN_A f 107–169) (Adams, Barach, & Speck, 1976; Driessen, 1983; Law, 1979). The same splitting was reported to occasionally occur in acid buttermilk, due to proteolytic activity of used starters (Olieman & van Riel, 1989; van Riel et al., 1995). Presence of this component clearly introduces a source of uncertainty in TB authentication but, in principle, also in the ascertainment of SMP genuineness. Indeed, the need for an accurate optimization of the chromatographic separation is related to the possible presence of interfering peaks originating from the chymosin-like action of some proteolytic enzymes (Olieman et al., 1989). These enzymes are naturally present in milk (e.g. plasmin) or mainly produced by psycrotrophic bacteria. Moreover, these enzymes seem to be responsible for the presence of a small peak having the same retention time as that of CMP A in the HPLC pattern of some genuine SMP samples.

3.2. Effects of cream storage at low temperature

For better understanding the effect of proteolysis, pasteurised MC was inoculated with *Pseudomonas fragi*

ATCC 4973 at 3×10^6 cfu/g and kept either at 30 °C for 24 h or 4 °C for 72 h. Indeed, proteolytic activity by psycrothrophic bacteria is reported to occur in refrigerated milk when more than $>10^6$ cfu/ml are present (Olieman & van Riel, 1989; Paquet, Driou, Bracquart, & Linden, 1987). At the end of ripening, the viable counts were 7×10^7 and 1.1×10^8 cfu/g in samples kept at 30 or 4 °C, respectively. Both cream samples were coagulated while no drop of the initial pH value (6.75) occurred.

No peaks were observed at 26–28 min RT in the chromatographic patterns of cream samples (Fig. 4(a)). Despite this, base peaks of both CMP A (not shown) and "pseudo CMP A" (Fig. 4(b)) have been recognised by ESI-MS. The HPLC profile of the cream sample kept at 4 °C for 72 h also shows ions at *m/z* 1647.0 and 2195.9. The derived deconvoluted spectrum presents a M_r of 6584.3 (Fig. 4(c)), i.e. a mass difference of 202 Da with respect to M_r of CMP A. A Met-Ala dipeptide may accounts for this difference which, therefore, was related to splitting of *k*-CN_A at the 107–108 position. These results demonstrate that proteases from *Pseudomonas fragi* can split *k*-CN_A at positions 105, 106 or 107, producing peptides with similar chromatographic behaviour. The same type of splitting was observed for *in vitro* digestion of *k*-CN with proteases from *Pseudomonas fluorescens* B52 (Recio, Garcia Risco, Ramos, & Lopez Fandino, 2000). Peptides originating from such a splitting were also observed in UHT milk (Recio et al., 1996). On this basis, it can be supposed that other *Pseudomonas* spp. strains can split *k*-CN_A at the same positions. Despite the deep proteolysis induced by the exceptionally high bacterial count, only ESI-MS allowed the presence of CMP A to be detected, the related chromatographic peak not being detectable at 210 nm. From this point of view, presence of both true and "pseudo" CMP A in the water phase of the commercial butter of Fig. 3 can be related to partial usage of RWC with poor microbiological properties.

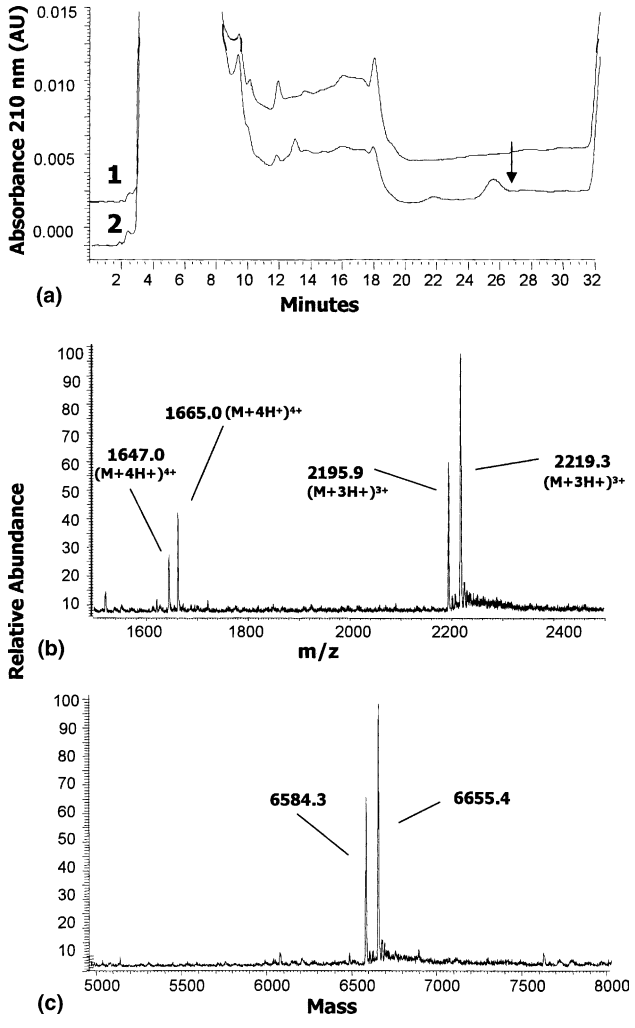

Fig. 4. (a) HPLC profiles of pasteurized cream inoculated with *Pseudomonas fragi* ATCC 4973 and kept at either (1) 30 °C for 24 h or (2) 4 °C for 72 h. (b) Full scan mass ESI-MS spectrum obtained at 26–27 min RT in the HPLC profile (2) and (c) related deconvoluted spectra.

It must also be taken into account that the initial bacterial count of the inoculated cream highly exceeded the maximum count (10^5 cfu/ml) provided by EC Directive 92/46 (1992). Moreover, both cream samples showed unacceptable soapy and rancid flavours at the end of ripening so that usage for butter-manufacturing is unlikely.

3.3. Effects of cream ripening and natural creaming

Reliability of the studied analytical approach could also be affected by phenomena occurring during cream ripening. In principle, proteases from culture microrganisms can originate peptides with CMP-like chromatographic behaviour which could hardly be distinguished from true CMP A by means of HPLC separation. Batches of pasteurized samples of MC and RWC were ripened (20 h/12 °C) with a commercial starter culture, the microbial composition of which was not disclosed by the manufacturer. Only in the case of butter produced from RWC, did the HPLC profile (not shown) present a peak at 27 min RT which in turn showed a MS spectrum with ions at *m/z* 1697.5 and 2263.2.

Other samples of pasteurized MC (35% fat) were laboratory-ripened (25 °C for 24 h) by inoculating 3 commercial cultures (MO 0.30, M 0.31R and M 0.32T) containing different lactic acid strains (see materials). The pH value of all cream samples dropped to 4.3 and HPLC analysis gave similar patterns. In particular, the chromatogram related to samples inoculated with culture M 0.32T (Fig. 5(a)) showed a major peak at 29.5 min RT with M_r of 1718.9 $(M + H^+)^+$Da (Fig. 5(b)). This peak has also been recognised in some genuine butter samples collected within the European market; nevertheless, its nature and origin were not further

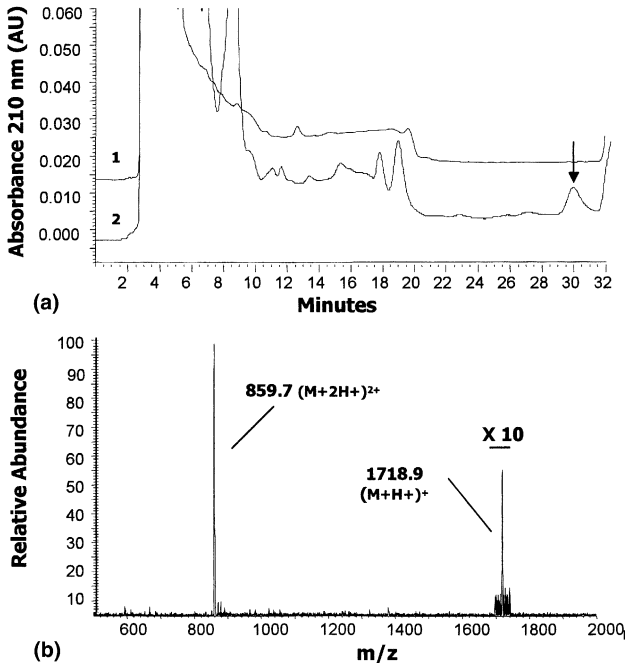

Fig. 5. (a) HPLC profiles of the water phase of pasteurized cream (1) before and (2) after ripening with starter M 0.32T. (b) Full scan mass ESI-MS spectrum of the peak eluting at 29.5 min in the HPLC profile.

investigated. Other small peaks have been detected at 24–27 min RT in the three samples of ripened cream but none showed significant mass spectra. Moreover, SIM of the ion at *m/z* 2263.2 did not show the presence of CMP A. Extreme conditions of ripening were also tested by analysing butter from MC cultured with MO 030 and ripened for 8 h at 25 °C plus seven days at 4 °C. Absence of both CMP A and related interfering peaks confirmed the previous findings. These experiments demonstrate that the microbial activities of added starter cultures have no chymosin-like activity and do not originate peptides which could interfere in the ESI-MS spectrum. These results are in agreement with those of Olieman and van Riel (1989) for selected microflora used in butter production. Release of fragment 107–169 of *k*-$CN_A$was not observed despite this splitting being reported to occur in some cultured buttermilk (Van Riel et al., 1995).

The effect of natural creaming of milk on reliability of the HPLC/ESI-MS method was also studied. In particular, raw bulk milk was naturally creamed for 10 h at 12 °C and the cream directly churned. Derived butter was analysed but no peak at 27 min RT or with a CMP A-like MS-spectrum was detected. Further experiments were addressed to show the effect of natural microflora present in raw milk submitted to natural creaming. Indeed, specific bacterial strains could promote different types and degrees of proteolysis. For this purpose, cream samples from raw milk intended for manufacturing of Grana Padano or Parmigiano Reggiano cheeses were churned after creaming under different conditions (6–12 h at 10–16 °C). Again, neither the chromatographic profile nor the ESI-MS spectrum of the two samples showed presence of CMP A.

3.4. Effect of butter washing

After draining of buttermilk in the buttermaking process, butter grains are washed so that the water phase of butter is progressively diluted. Experiments were conducted in order to ascertain if butter washing could originate false negative responses, i.e. not due to RWC usage in butter manufacturing. To this end, RWC was churned and the butter submitted to either one or six washing steps. The intensity (full scan) of ion at *m/z* 2263.2 was three times lower (1.1×10^6 vs 3×10^6) in the six-times washed butter, indicating that, at least for butter from RWC, extensive washing can not completely eliminate CMP A in the water phase.

Due to different washing conditions adopted by industrial manufacturers, quantitative determination of RWC used for butter-making is not possible. Despite this, the response of the HPLC/ESI-MS technique was studied. For this purpose, different amounts of RWC (2.5%, 5%, 10%, 25% and 50%, w/w) were mixed with MC and the derived blends were churned and washed twice, according to a typical industrial butter-making process. The calibration curve, based on intensities for SIM at *m/z* 2263.2, is shown in Fig. 6. A linear dynamic response covered the entire tested range and the minimum signal detected for 2.5% RWC sample was 1.7×10^4 (signal-to-noise ratio = 10). Presence of CMP A was still detectable when this sample was submitted to six washings.

3.5. Control of commercial samples of butter

The developed method was applied to control butter samples collected at the retail market both in Italy ($n = 26$) and in other the European countries ($n = 12$). Among the Italian samples, three were labelled as TB; the other samples were sold as "butter" and nine of them reported on the package: "butter produced from milk cream". None of the butter samples from other European countries was labelled as "traditional". The results obtained are listed in Table 1 where + or − signs indicate presence or absence in the full scan mass range of ions at *m/z* 1697.5 and 2263.2. As previously discussed, intensities of these ions can not be used for quantitation of RWC. As expected, the Italian samples labelled as "butter" were also produced with RWC, with two exceptions. These last were not correlated with extensive washing because the content of milk-solids non-fat of both butter samples fulfilled the limit (>2%, w/w) provided by Reg. No. 2991/94 (1994). These samples were produced in Trentino, a mountain region of

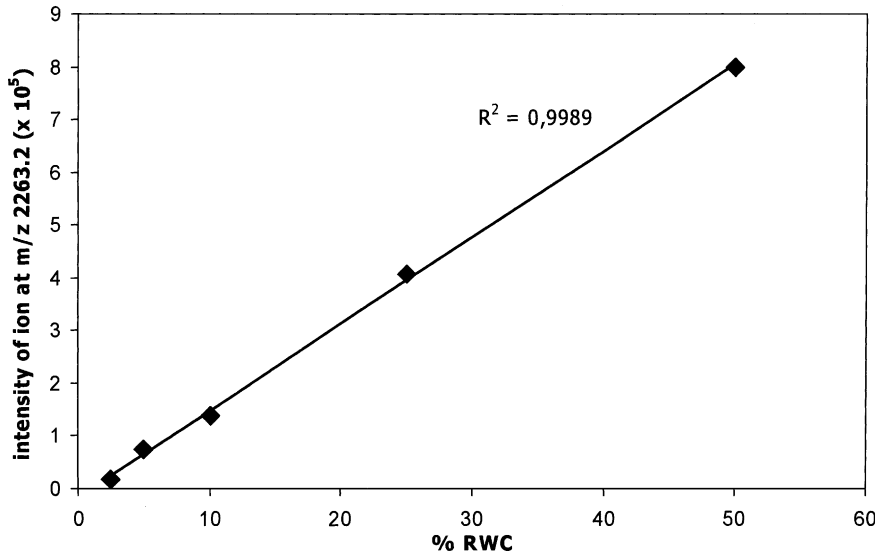

Fig. 6. ESI-MS response of SIM at *m/z* 2263.2 as function of percentage of RWC used in butter manufacturing.

Table 1
HPLC/ESI-MS of commercial butter samples of different origins

Origin	Number of samples			RWC[a]	
	Total	Traditional butter	Butter	+	–
Italy	26		23	21	2
		3		2	1
France	6		6	2	4
United Kingdom	2		2		2
Denmark	2		2		2
Germany	2		2		2

[a] Usage (+) of RWC based on presence of ion at *m/z* 2263.2.

Italy where butter consumption is widespread and high quality products are requested by the consumer. Two samples of TB gave positive response to HPLC/ESI-MS analysis and therefore they have to be considered illegal. The *scenario* for countries other than Italy showed presence of RWC only in two butter samples from France. Among the considered countries, France is the most important cheese producer, along with Italy.

4. Conclusion

The proposed HPLC/ESI-MS method proves to be reliable and not affected by proteolytic phenomena which can occur during natural creaming or cream ripening steps in butter-making. Only unacceptable contamination of cream by psychrotrophic bacteria can originate CMP A and therefore give potentially false-positive response.

A further advantage of coupling HPLC to ESI-MS is to avoid the need to optimise the chromatographic separation through an accurate set-up of elution gradient. Indeed, unambiguous identification of CMP A is achieved by SIM of the ion at *m/z* 2263.2, even when this peptide is eluted before or after 26 ± 2 min RT.

Despite washing lowering the quantity of residual CMP A in the water phase, the sensitivity of HPLC/ESI-MS allows usage of very low levels of RWC in butter manufacturing to be detected. Exhaustive washing of butter in order to completely remove CMP A is not economically feasible and will result in excessive dilution of the milk-solids non-fat content.

Control of commercial samples has highlighted how usage of RWC in butter (also traditional) manufacturing is performed in countries where cheesemaking is widespread and therefore, a large volume of rennet-whey is available. Although HPLC/ESI-MS is an expensive technique, the developed method appears to be a reliable analytical approach for detecting usage of RWC in TB manufacturing.

References

Adams, D. M., Barach, J. T., & Speck, M. L. (1976). Effect of psycrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultra high temperature treatment. *Journal of Dairy Research, 59*, 823–827.

Commission Regulation (EC) No 2799/1999 (1999). *Official Journal L 340*, 31/12/1999, pp. 3–27.

Commission Regulation (EC) No 213/2001 (2001). *Official Journal L 037*, 07/02/2001, pp.1–99.

Council Directive 92/46/EEC (1992). *Official Journal L 268*, 14/09/1992, pp. 1–32.

Council Regulation (EC) No 2991/94 (1994). *Official Journal L 316*, 09/12/1994, pp. 2–7.

De Koning, P. J., Eisses, J., & De Vries, H. (1966). A method for the detection of small percentages of whey powder in milk powder. *Netherlands Milk and Dairy Journal, 20*, 204–212.

Driessen, F. M. (1983). Lipases and proteinases in milk. Occurrence, heat inactivation and their importance for the keeping quality of milk products. *Netherlands Milk and Dairy Journal, 37*, 193–196.

Law, B. A. (1979). Reviews of the progress of dairy science. Enzymes of psycrotrophic bacteria and their effect on milk and milk products. *Journal of Dairy Research, 46*, 573–588.

Mercier, J. C., Ribadeau-Dumas, B., & Grosclaude, F. (1973). Amino-acid composition and sequence of bovine kappa-casein. *Netherlands Milk and Diary Journal, 27*(2/3), 313–322.

Olieman, C., & van Riel, J. A. M. (1989). Detection of rennet whey solids in skim milk powder and buttermilk with reverse-phase HPLC. *Netherlands Milk and Dairy Journal, 43*, 171–184.

Paquet, D., Driou, A., Bracquart, P., & Linden, G. (1987). Effect of refrigerated storage of milk on proteolysis. Relationship to the proteose-peptone content. *Netherlands Milk and Dairy Journal, 41*, 81–92.

Polak, P., & Bradley, R. L. (1994). Quantitative detection of whey cream in sweet cream and butter. *Journal of AOAC International, 77*, 488–490.

Recio, I., Lopez Fandino, R., Olano, A., Olieman, C., & Ramos, M. (1996). Study of the formation of caseinomacropeptides in stored ultra-high-temperature-treated milk by capillary electrophoresis. *Journal of Agricultural and Food Chemistry, 44*, 3845–3848.

Recio, I., Garcia Risco, M. R., Ramos, M., & Lopez Fandino, R. (2000). Characterization of peptides produced by the action of psychrotrophic proteinases on kappa-casein. *Journal of Dairy Research, 67*, 625–630.

Van Riel, J., & Olieman, C. (1995). Determination of caseinomacropeptide with capillary zone electrophoresis and its application to the detection and estimation of rennet whey solids in milk and buttermilk powder. *Electrophoresis, 16*, 529–533.