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ScienceDirect
Food Chemistry 101 (2007) 964–972
Food Chemistry
www.elsevier.com/locate/foodchem

Use of mass spectrometry to characterize proteolysis in cheese

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Received 14 March 2005; received in revised form 23 February 2006; accepted 23 February 2006

Abstract

A rapid method for characterizing proteolysis in different cheese varieties was developed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS). pH 4.6-soluble extracts of different cheese varieties were sub-fractionated using ethanol and 70% ethanol-soluble extracts were analyzed using MALDI-ToF-MS. Sample analysis time was ~3 min compared to ~100 min for reversed-phase HPLC; moreover, the method yielded accurate molecular masses of peptides originating as a result of proteolysis. Small peptides (<3500 *m/z*) were monitored and data for relative intensities of peptides were analyzed using multidimensional scaling (MDS) to demonstrate the similarity between profiles obtained from different cheese varieties. The similarities between the profiles of MALDI-ToF mass spectra were demonstrated in a two-dimensional space by performing MDS on the similarity matrix. Use of MALDI-ToF-MS is thus a fast and effective method for monitoring small peptides produced in cheese as a result of proteolysis.

Keywords: MALDI-ToF; Small peptides; Multidimensional scaling; Proteolysis; Cheese

1. Introduction

Cheese maturation involves complex biochemical reactions, of which proteolysis is regarded as being the most important for many varieties. To gain an insight into the complex process of proteolysis in cheese during ripening, numerous non-specific and specific techniques have been developed over the years (see Fox, McSweeney, & Singh, 1995; IDF, 1991, 1999; McSweeney & Fox, 1993, 1997; Upadhyay, McSweeney, Magboul, & Fox, 2004; Wallace & Fox, 1998). Non-specific methods (e.g., determination of soluble nitrogen by Kjeldahl) provide information about the extent of proteolysis and the proteolytic agents involved, but do not characterize the evolution of peptides during ripening, which is very important in understanding the complexity of proteolysis. Use of more advanced analytical techniques (i.e., electrophoresis and chromatography), in combination with multivariate statistical analysis (e.g., principal component analysis, discriminant analysis, principal component similarity analysis) has emerged as a powerful tool to improve the understanding of proteolysis in cheese (Pripp, Shakeel-Ur-Rehman, McSweeney, & Fox, 1999; Pripp, Shakeel-Ur-Rehman, McSweeney, Sørhaug, & Fox, 2000a; Pripp, Stepaniak, & Sørhaug, 2000b; Pripp, Tobiassen, Stepaniak, & Sørhaug, 1998; Shakeel-Ur-Rehman, Pripp, McSweeney, & Fox, 1999).

Although chromatographic and electrophoretic techniques provide valuable information about peptides produced in cheese during ripening, the use of such techniques involves considerable analysis time. During the early 1980s, mass spectrometric techniques, such as electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS), emerged for the analysis of large biomolecules (Alomirah, Alii, & Konishi, 2000). These methods are characterized by ionization without fragmentation, accurate mass determination, picomole to femtomole sensitivity and broad applicability (Abell & Sporns, 1996). Unlike other mass spectrometric techniques,

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0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.02.048

MALDI-ToF-MS can also obtain accurate results for peptide masses in the presence of salts and other buffer components (Alomirah et al., 2000).

MALDI-ToF-MS has been widely used in analytical and structural characterization of food proteins (Alomirah et al., 2000). Numerous studies have been performed on milk proteins using MALDI-ToF-MS, including identification of milk protein variants, analysis of structural modifications in milk proteins and studies of glycoforms and degree of glycosylation (de Frutos, Cifuentes, Diez-Masa, Camafeita, & Mendez, 1998; Fanton et al., 1998; Ferranti et al., 1995; Ferranti et al., 1997; Kim, Leszyk, & Taub, 1997; Siciliano, Rega, Amoresano, & Pucci, 2000; Zappacosta, Di Luccia, Ledda, & Addeo, 1998). Recently, MALDI-ToF-MS has been employed as a technique for characterizing water buffalo milk and to detect adulteration, with bovine milk, of buffalo milk used for the manufacture of Mozzarella cheese (Angeletti, Gioacchini, Seraglia, Piro, & Traldi, 1998; Cozzolino, Passalacqua, Salemi, & Garozzo, 2002). The technique has also been used to detect the presence of cows' milk in ewes' or buffaloes' milk and powdered milk in fresh raw milk (Cozzolino et al., 2001; Fanton et al., 1998) and to determine proteolysis in yoghurt caused by strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Fedele, Seraglia, Battistotti, Pinelli, & Traldi, 1999). MALDI-ToF-MS has been employed to identify peptides from Cheddar cheese separated by RP-HPLC (Broadbent, Strickland, Weimer, Johnson, & Steele, 1998; Gouldsworthy, Leaver, & Banks, 1996) and to quantify the bitter peptide β-CN (f193–209) in Queso Fresco cheese (Soeryapranata et al., 2002a), to correlate the concentration of β-CN (f193–209) with bitterness in Cheddar cheese (Soeryapranata et al., 2002b) and to differentiate peptidase activities of starter and adjunct cultures on β-CN (f193–209) under stimulated cheese-like conditions (Soeryapranata, Powers, Weller, Hill, & Siems, 2004).

To our knowledge, however, MALDI-ToF-MS has not previously been used to characterize directly proteolysis in cheese. The aim of this study was to demonstrate the suitability of MALDI-ToF-MS as a tool for evaluating proteolysis in different cheese varieties by monitoring short peptides and to explore the use of multivariate statistical analysis to treat data from mass spectra.

2. Materials and methods

2.1. Samples

Samples of Camembert, Stilton, Edam, Cooleeney [an Irish Camembert-type farmhouse cheese], Swiss, Ricotta, Parmigiano–Reggiano and Port du Salut were purchased from a local supermarket in Cork, Ireland; Goats' milk cheese, Pecorino, Ricotta and Parmigiano–Reggiano were purchased from a local supermarket in Potenza, Italy; Cheddar cheese was also manufactured in the pilot plant in University College Cork and ripened for 1 d (young) or 4 mo (ripened); smear-ripened Cheddar cheese was manufactured in the Dairy Products Research Centre, Moorepark and a Gruyere-type cheese was obtained from a commercial source; core and surface samples (2 mm) were collected for smear-ripened Cheddar and Gruyere-type cheese.

2.2. Preparation of cheese extracts

The pH 4.6-soluble extracts were prepared from the cheeses by a modification of the procedure of Kuchroo and Fox (1982); grated cheese was homogenized with twice the amount of water for 5 min in a Colworth Stomacher 400 (Seward Laboratory, UK). This homogenate was adjusted to pH 4.6 using 1.0 M HCl, left to stand at room temperature for 30 min and pH was readjusted to pH 4.6, if necessary. After pH adjustment, the homogenate was held at 40 °C for 1 h and centrifuged at 3000*g* for 30 min at 4 °C. The supernatant was filtered through glass wool and Whatman No. 113 filter paper and freeze-dried.

2.3. MALD1-ToF analysis

Freeze-dried samples of the pH 4.6-soluble extracts (10 mg) were dissolved in 1 ml of 70% ethanol, held at room temperature for 30 min and centrifuged at 13,000*g* for 10 min. Duplicate aliquots of supernatants containing peptides soluble in 70% ethanol, were mixed (1:1) with matrix solution (α-cyano-4-hydroxy-cinnamic acid in 50% v/v acetonitrile containing 0.5% v/v trifluoroacetic acid). This mixture (0.3 μl) was applied in duplicate (two different spots) on a sample slide tray and allowed to dry in air. Mass spectra were acquired in reflectrion mode, using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences, Uppsala, Sweden). Calibration of the time-to-mass scale was performed using two external standard peptides (ile7AngIII, M+H 897.531, monoisotopic, and hACTH 18-39, M+H 2465.191, monoisotopic). MALDI-ToF analysis was carried out in duplicate on two different days; a total of 8 spectra for each sample were thus collected.

2.4. Data processing and statistical analysis

Spectra were processed using the software integrated with the MALDI-ToF-MS (version Pro 2.0 SP2). For each spectrum, the mass list and corresponding relative intensity (%) were exported to a data sheet. For each cheese, replicate spectra were processed by discarding peaks with relative intensities below 10% of the highest peak and peaks that were not present in all replicates. The remaining peaks constituted the set of variables that was used for statistical analysis.

The matrix of Spearman rank-order correlation coefficients was built for this set of variables. This measure is a nonparametric version of the Pearson correlation coefficient, and is based on the ranks of the data rather than the actual values, which makes it appropriate for data from MALDI-ToF mass spectra because of the high variability observed in peak intensity. The matrix of Spearman was

submitted to multidimensional scaling (MDS), and scaled in two dimensions by using the Guttman loss function. For the representation of the relative positions of each profile, the bivariate 99% confidence ellipse, enclosing the replicate profiles, was plotted for each cheese extract. MDS is a technique that computes coordinates for a set of points in a space, such that the distances between pairs of these points fit as closely as possible to measured dissimilarities between a corresponding set of objects (Everitt & Dunn, 2001); i.e., objects that are close in the bi-dimensional space are similar.

3. Results and discussion

The mass spectra of 70% ethanol-soluble fractions of different cheese varieties are shown in Fig. 1. The ratios of molecular mass to charge (m/z) of various peptides and the corresponding relative intensities of different cheese varieties are shown. Generally charge of ion (z) was +1 and hence m/z can be compared to molecular mass (MW) of the peptide. As the 70% ethanol-soluble fraction contains the small peptides produced during ripening, only peptides with $m/z < 3500$ were monitored in this study. In order to understand the origin of the peptides, their m/z values were compared to the reported molecular masses of peptides identified from different cheeses (Addeo et al., 1992, 1994; Broadbent et al., 1998; Gagnaire, Mollé, Herrouin, & Léonil, 2001; Gouldsworthy et al., 1996; Lund & Ardö, 2004; Soeryapranata et al., 2002a).

In the peptide profiles, the peak detected at m/z 1881, most likely corresponding to the peptide β-CN (f193–209) (MW 1881 Da) or α_{s2}-CN (f5–18)4P, was the most common peptide in most of the cheese varieties, particularly in Cheddar cheese at 4 months, Cooleeney, the surface and core regions of smeared Cheddar, Port du Salut and ewes' and goats' milk cheeses. This peptide is produced due to the action of chymosin on β-casein at $Leu_{192} - Tyr_{193}$ (Visser & Slangen, 1977) and its accumulation in cheese is frequently associated with bitterness (Smit, Kruyswijk, Weerkamp, de Jong, & Neeter, 1996).

Chymosin acts on α_{s1}-casein at Phe_{23}-Phe_{24} (Hill, Lahav, & Givol, 1974; McSweeney, Olson, Fox, Healy, & Højrup, 1993), yielding the peptides α_{s1}-CN (f1–23) and α_{s1}-CN (f24–199). Peptide α_{s1}-CN (f24–199) is insoluble at pH 4.6, while α_{s1}-CN (f1–23) (MW 2764 Da) is soluble at pH 4.6. A peak at m/z 2763.5, which has the same mass as α_{s1}-CN (f1–23), was detected in Edam, Cooleeney, Camembert, Swiss, Parmigiano–Reggiano (purchased in Italy), Port du Salut, Cheddar (young), smeared Cheddar and Gruyere-type (surface and core). The peak at m/z 2764, however, was not detected in ripened Cheddar cheese, suggesting further hydrolysis of α_{s1}-CN (f1–23) by proteolytic agents in that cheese during ripening.

Cell envelope-associated proteinases (CEP) and endopeptidases of starter and non-starter bacteria are able to hydrolyse α_{s1}-CN (f1–23) rapidly at the bonds Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, Glu_{14}-Val_{15} and Leu_{16}-Asn_{17} (Exterkate & Alting, 1993). Products of such hydrolysis include α_{s1}-CN (f17–23) (MW 905 Da), α_{s1}-CN (f1–9) (MW 1141 Da), α_{s1}-CN (f14–23) (MW 1246 Da), α_{s1}-CN (f1–13) (MW 1535 Da), α_{s1}-CN (f1–14) (MW 1664 Da), α_{s1}-CN (f1–16) (MW 1874 Da) and α_{s1}-CN (f1–17) (MW 1991 Da), respectively, which probably correspond to peaks detected at m/z of 905.44, 1140.72, 1246.72, 1535.89, 1664.92, 1877.20 and 1991 (Table 1). Some of these peptides were found in Camembert, Cooleeney, Edam, Port du Salut, Parmigiano–Reggiano, Swiss, Gruyere-type and Cheddar cheeses (Table 1). Kaminogawa, Yan, Azuma, and Yamauchi (1986) indicated accumulation of some of these peptides in Gouda cheese. The presence of these peptides has been previously detected in Cheddar (Broadbent et al., 1998; Gouldsworthy et al., 1996) and Swiss cheese (Gagnaire et al., 2001; Combes, Paterson, & Amado, 2002). Differences in production of these peptides in different cheese varieties can be explained by the specificity of CEPs and endopeptidases of the specific starter cultures used.

Signals were detected at m/z 1555.85 and 1782.08 in mass spectra of some cheese varieties, which possibly correspond to the peptides β-CN (f193–206) (MW 1555.8 Da) and β-CN (f193–208) (MW 1782.1 Da), respectively. The peptide β-CN (f193–209) (m/z 1881), which is formed by hydrolysis of bond Leu_{192}-Tyr_{193} in β-casein, may be cleaved by a carboxypeptidase at Ile_{208}-Val_{209} to form β-CN (f193–208), while β-CN (f193–206) may be produced by hydrolysis of the Ile_{208}-Val_{209} bond by CEPs, followed by carboxypeptidase-catalysed hydrolysis; however, no carboxypeptidase activity has been detected in lactococci although some activity toward N-terminal-blocked peptides has been reported in strains of lactobacilli (Abo-Elnaga & Plapp, 1987).

Stilton cheese showed the presence of the β-CN (f193–206), β-CN (f193–208) and β-CN (f193–209), along with a strong signal at m/z 1668.95, which probably corresponds to β-CN (f192–206) (MW 1669 Da). The profile of Camembert cheese indicated a strong signal at m/z 1589.94, which probably corresponds to β-CN (f195–209). Profiles of Parmigiano–Reggiano cheeses (Fig. 1a, b) were complex but showed a strong signal at m/z 1198.69. Detection of a peak in Fig. 1a at m/z 1278.80, which probably corresponds to phosphopeptide β-CN (f15–24)3P (MW 1279 Da), has been previously reported in Grana Padano cheese by Ferranti et al. (1997) using fast atom bombardment mass spectrometry.

Compared to other cheeses, cheeses manufactured from ewes' milk (Pecorino) and goats' milk had a large number of small peptides with low molecular masses (m/z 800–2000; Fig. 1m, n; Table 1). This difference may be due to different caseins of ewes' and goats' milk, compared to cows' milk, and the microflora of the cheeses during ripening.

Ricotta cheese showed signals only at m/z 851.39 and 995.46 (Fig. 1q); this cheese is manufactured from cheese whey, which is heated at high temperature. The whey used for the manufacture of Ricotta may contain peptides

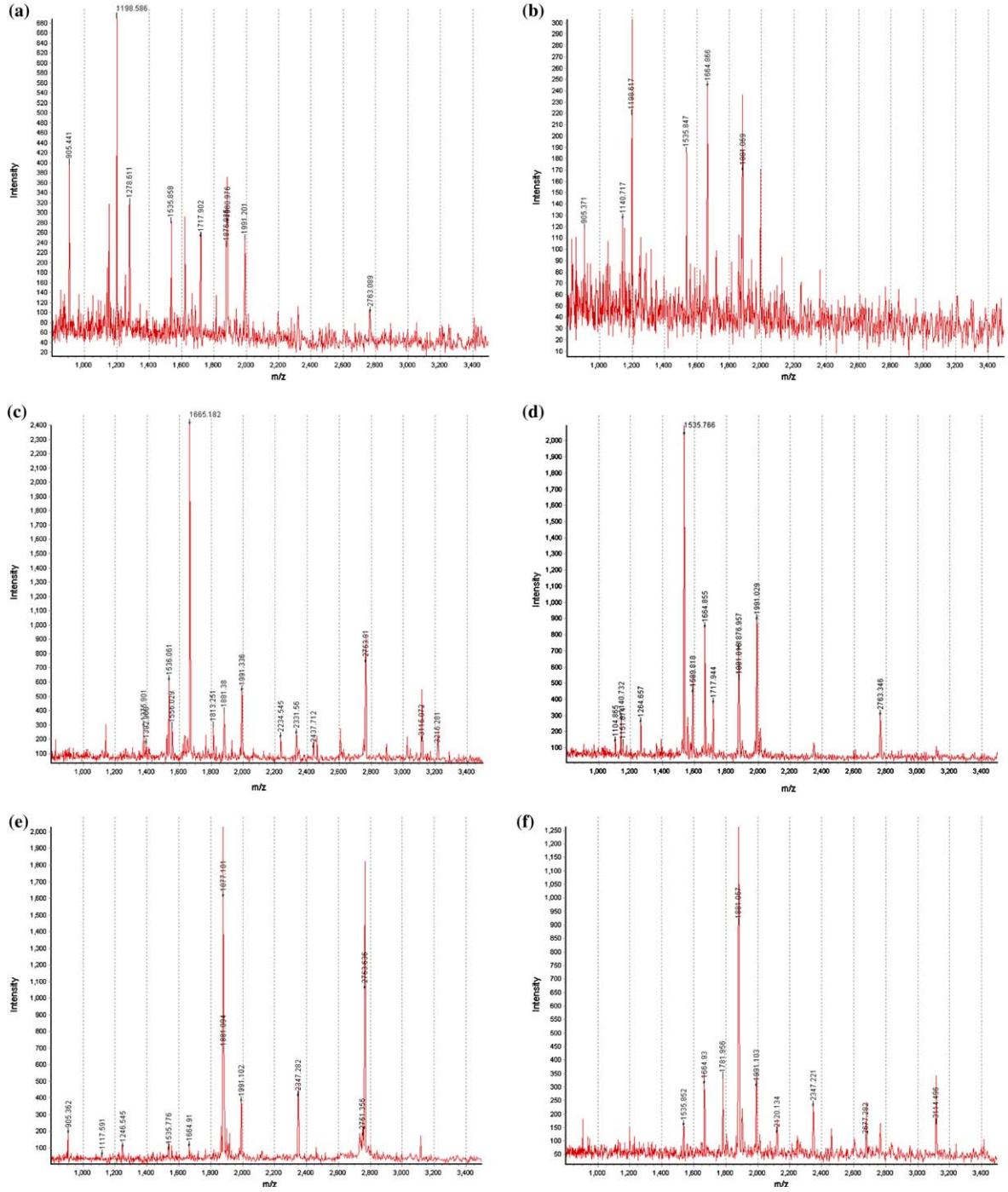

Fig. 1. MALDI-ToF mass spectra of 70% ethanol-soluble fractions of (a) Parmigiano–Reggiano purchased in Italy, (b) Parmigiano–Reggiano-purchased in Ireland, (c) Swiss, (d) Edam, (e) Young Cheddar (1 d), (f) ripened Cheddar (4 mo), (g) core sample of smeared Cheddar cheese, (h) surface sample of smeared Cheddar cheese, (i) Camembert, (j) Cooleeney Camembert, (k) Stilton, (l) Port du Salut, (m) Pecorino, (n) Goat's milk cheese, (o) core sample of Gruyere-type cheese, (p) surface sample of Gruyere-type cheese and (q) Ricotta.

derived from casein hydrolysis during cheesemaking or some casein fines. However, the peptide profile of Ricotta cheese did not show the presence of such peptides.

Smeared Gruyere-type cheese, showed the presence of a number of peptides in the m/z region 2000–3500 (Fig. 1o, p), which were not apparent in other cheeses. Samples from the surface region of Gruyere-type had peaks at m/z 1052.61 and 2331.39, which probably correspond to α_{s1}-CN (f24–32) (MW 1052 Da) and α_{s1}-CN (f106–125) (MW 2331 Da), respectively. Some of these peptides were also identified in Pecorino, goat's milk and Swiss cheese in this study. Addeo et al. (1992) identified these peptides in Parmigiano–Reggiano cheese by using fast-atom-bombardment mass spectrometry.

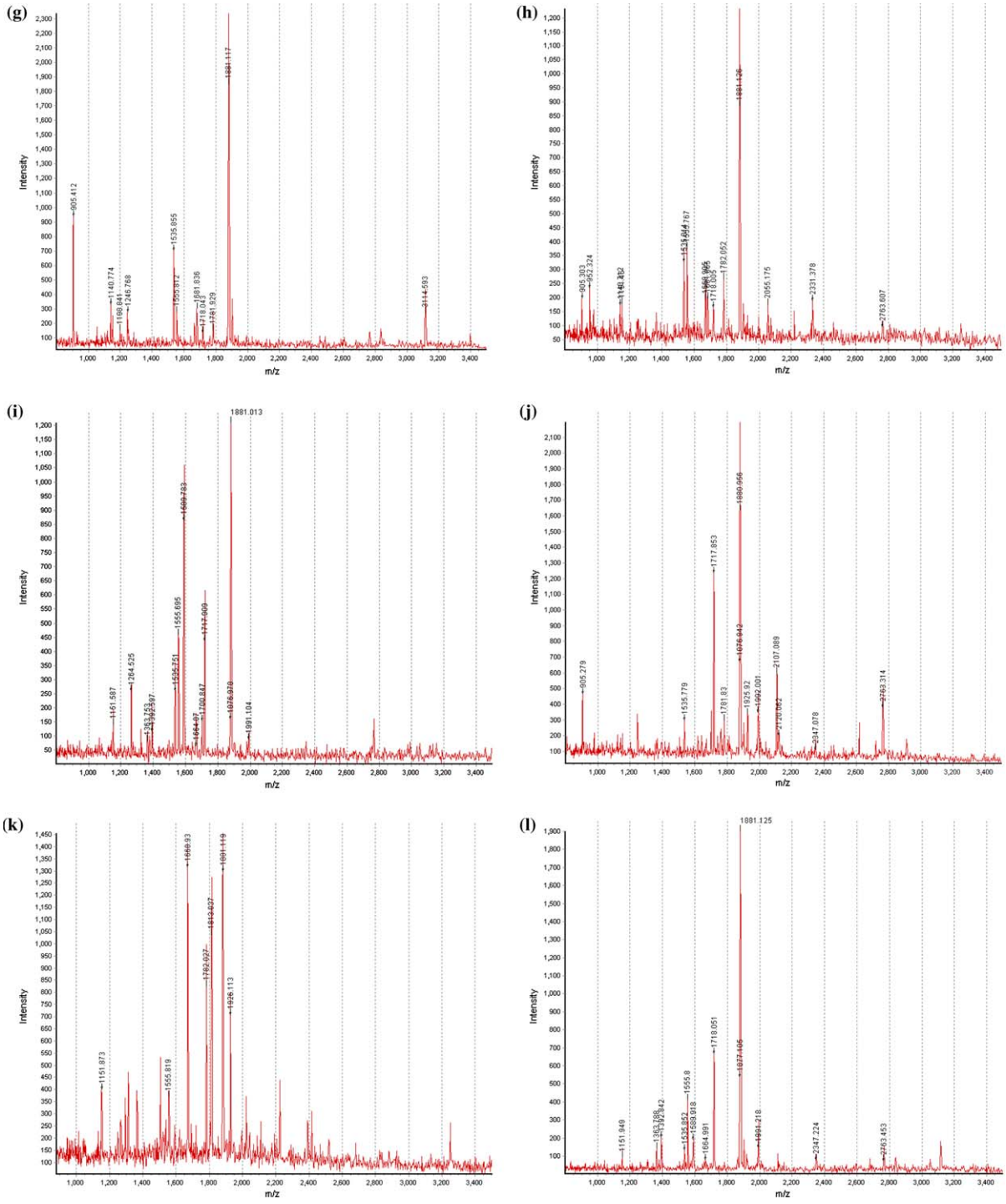

Fig. 1 (*continued*)

The similarities between the profiles of MALDI-ToF mass spectra were demonstrated in a two-dimensional space by performing MDS on the similarity matrix, which was obtained by Spearman rank-order correlation (Fig. 2). The proportion of variance explained was 86.4%. Each cheese was represented as a confidence ellipse (computed at the 99% level) enclosing the positions of the 8 replicates. Since peak matching was performed during data processing, the variability between the replicates in Fig. 2 derives from the variability associated with the peak intensity, which is typical of spectra obtained by MALDI-ToF analysis. However, in most of the cases, the variability observed between the peptide profiles of different cheese types was greater than that observed among replicates and, hence, it was possible to discriminate between cheese types (Fig. 2). Due to its unique peptide profile, Ricotta cheese, which was made from whey, was not included in MDS.

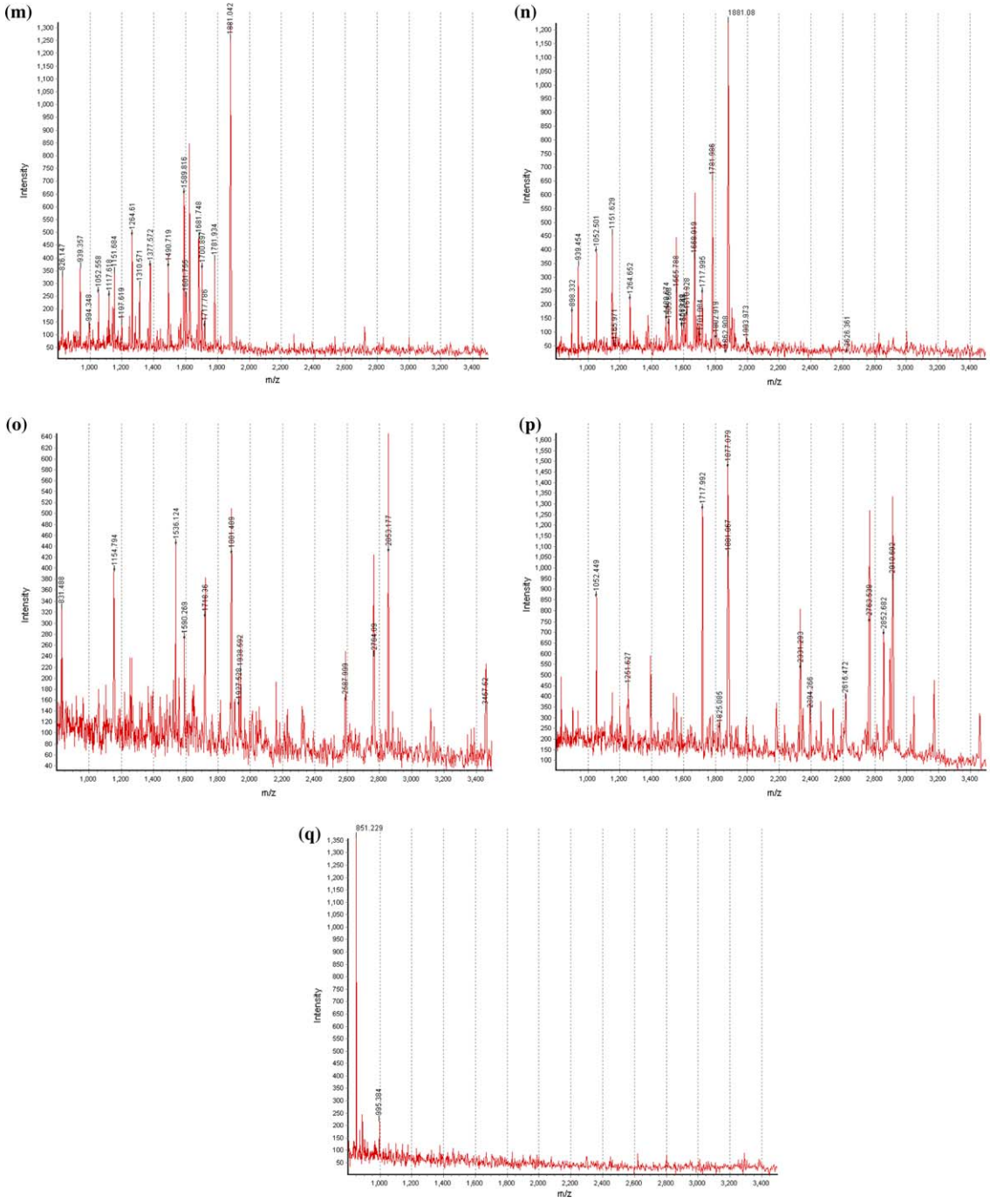

Fig. 1 (*continued*)

Based on these considerations, cheeses made from ewes' (Pecorino) or goats' milk were dissimilar from all other cheeses, and had low values on dimension 2 (Fig. 2). Gruyere-type cheeses (core and surface) were also distinguished from all the other cheeses, with high values on dimension 1. The dissimilarity observed between these cheeses (Pecorino, goats' milk cheese, surface and core of Gruyere-type cheese) was mainly due to the high number of specific peptides that characterized those cheeses (Table 1). Core and surface samples of Gruyere-type cheese were separated over the first dimension. The peptide at m/z 1535.89 (possibly α_{s1}-CN (f1–13); MW 1535.8 Da) was most abundant at the core, and it was absent from the surface, while the peptides with m/z 2331.39 (possibly α_{s1}-CN (f106–125)) and m/z 1052.61 (possibly α_{s1}-CN (f24–32)) were present at surface but not at core (Table 1).

Stilton cheese, which is an internal mould-ripened cheese, was differentiated from the rest of the samples because of the high relative intensities of unique peptides. These peptides possibly originated in cheese due to action of chymosin retained in cheese curd or enzymes from moulds on caseins. A group of cheeses showed similar

Table 1
List of peptides (m/z ± standard deviation, SD) detected in MALDI-ToF mass spectra of 70% ethanol-soluble fractions of cheeses and corresponding possible fragment identities

m/z (SD)	Possible casein fragment[a]	ST	ED	SW	GC	EC	CO	CM	PS	RI	PR-I	PR-E	CH-1	CH-4	CH-SC	CH-SS	LD-C	LD-S
905.44 (0.1)	α_{s1}-CN (f17–23)	–	–	–	–	–	30 (12)	–	–	–	50 (14)	38 (11)	39 (18)	–	37 (17)	18 (5)	–	–
939.51 (0.12)		–	–	–	48 (20)	25 (5)	–	–	–	–	–	–	–	–	–	–	–	–
1052.61 (0.12)	α_{s1}-CN (f24–32)	–	–	–	39 (21)	20 (8)	–	–	–	–	–	–	–	–	–	–	–	57 (16)
1117.67 (0.13)	α_{s1}-CN (f15–23)	–	–	–	–	13 (4)	–	–	–	–	–	–	8 (2)	–	–	–	–	–
1140.73 (0.13)	α_{s1}-CN (f1–9)	–	13 (4)	–	–	–	–	–	–	–	–	42 (16)	–	–	14 (5)	13 (4)	–	–
1151.75 (0.15)	β-CN (f199–209)	40 (10)	12 (2)	–	61 (21)	22 (9)	–	20 (6)	10 (6)	–	–	–	–	–	–	14 (4)	–	–
1198.69 (0.15)		–	–	–	–	–	–	–	–	–	100 (0)	100 (0)	–	–	10 (4)	–	–	–
1246.72 (0.12)	α_{s1}-CN (f14–23)	–	–	–	–	–	–	–	–	–	–	–	12 (2)	–	14 (3)	–	–	–
1264.72 (0.12)	α_{s1}-CN (f115–124)	–	22 (5)	–	26 (6)	22 (10)	–	42 (14)	–	–	–	–	–	–	–	–	–	–
1363.77 (0.17)		–	–	–	–	–	–	14 (5)	8 (2)	–	–	–	–	–	–	–	–	–
1392.58 (0.37)		–	–	9 (4)	14 (5)	–	–	28 (11)	–	–	–	–	–	–	–	–	–	–
1490.83 (0.14)		–	–	–	–	35 (15)	–	–	–	–	–	–	–	–	–	–	–	–
1535.89 (0.16)	α_{s1}-CN (f1–13)	–	100 (0)	25 (11)	–	–	20 (11)	100 (0)	22 (12)	–	48 (13)	65 (14)	10 (3)	10 (5)	27 (11)	22 (9)	97 (7)	–
1555.85 (0.17)	β-CN (f193–206)	38 (12)	–	31 (21)	19 (5)	–	–	59 (11)	22 (4)	–	–	–	–	–	10 (3)	27 (7)	–	–
1589.95 (0.13)	β-CN (f195–209)	–	42 (21)	–	12 (2)	43 (18)	–	79 (9)	13 (3)	–	–	–	–	–	–	–	36 (15)	–
1601.93 (0.15)		–	–	–	11 (3)	15 (6)	–	–	–	–	–	–	–	–	–	–	–	–
1664.92 (0.19)	α_{s1}-CN (f1–14)	–	52 (11)	95 (11)	–	–	–	24 (12)	8 (4)	–	–	79 (11)	7 (2)	22 (9)	–	–	–	–
1668.95 (0.13)	β-CN (f192–206)	100 (1)	–	–	36 (9)	–	–	–	–	–	–	–	–	–	–	17 (7)	–	–
1681.92 (0.15)		–	–	–	–	21 (9)	–	–	–	–	–	–	–	–	14 (3)	11 (6)	–	–
1700.95 (0.23)	β-CN (f12–24)	–	–	–	11 (3)	20 (9)	–	18 (12)	–	–	–	–	–	–	–	–	–	–
1718.08 (0.17)	β-CN (f194–209)	–	19 (11)	–	29 (8)	14 (4)	69 (16)	53 (18)	42 (10)	–	47 (17)	–	–	–	7 (3)	17 (3)	44 (19)	93 (12)
1782.08 (0.18)	β-CN (f193–208)	49 (11)	–	–	56 (9)	24 (8)	20 (7)	–	–	–	–	–	–	28 (4)	11 (4)	22 (5)	–	–
1812.95 (0.11)		80 (10)	–	22 (7)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1877.2 (0.23)	α_{s1}-CN (f1–16)	–	41 (6)	–	–	–	68 (14)	49 (22)	62 (27)	–	49 (11)	–	100 (0)	–	–	–	–	83 (20)
1881.13 (0.23)	β-CN (f193–209)/ α_{s2}-CN(f5–18)4P	72 (18)	56 (29)	55 (36)	100 (0)	100 (0)	100 (0)	57 (18)	99 (3)	–	51 (14)	64 (14)	52 (10)	100 (0)	100 (0)	100 (0)	58 (21)	65 (13)
1926.03 (0.15)		45 (10)	–	–	–	–	23 (6)	–	–	–	–	–	–	–	–	–	–	–
1991.15 (0.21)	α_{s1}-CN (f1–17)	–	88 (22)	21 (10)	–	–	33 (13)	18 (9)	18 (10)	–	39 (15)	–	18 (5)	16 (9)	–	–	–	–
2120.19 (0.19)		–	–	–	–	–	20 (7)	–	–	–	–	–	–	11 (5)	–	–	–	–
2331.39 (0.21)	α_{s1}-CN (f106–125)	–	–	20 (14)	–	–	–	–	–	–	–	–	–	–	–	12 (5)	–	51 (17)
2347.38 (0.19)	α_{s1}-CN (f1–20)	–	–	–	–	–	23 (9)	–	10 (3)	–	–	–	14 (7)	12 (7)	–	–	–	–
2763.54 (0.25)	α_{s1}-CN (f1–23)	–	17 (11)	31 (8)	–	–	18 (7)	–	7 (3)	–	18 (11)	–	46 (13)	–	–	9 (5)	30 (13)	53 (9)
2852.75 (0.23)		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	50 (25)	46 (11)
3114.71 (0.22)		–	–	22 (13)	–	–	–	–	–	–	–	–	–	10 (4)	10 (4)	–	–	–
Specific peptides			1104.7	1375.6	898.5	826.2	2107.1			851.3	1278.8		2751.4	2677.5		952.4	831.4	1251.7
				2233.9	1166.0	994.4				995.4						2055.4	1154.6	825.2
				2437.5	1488.9	1197.6											1937.9	2394.4
					1505.9	1310.6											2587.5	2616.5
					1619.0	1377.6											3457.6	2910.7
					1994.3													
					2627.1													

The distribution of % relative intensities and its SD among cheeses is also reported. Specific peptides in the Table indicate peptides particular to a cheese variety. Labels for cheeses are given in Fig. 2.

[a] The peptide fragment is based on comparison between the m/z of peptides in the mass spectra and the molecular mass and its corresponding peptide fragment, previously reported in cheese.

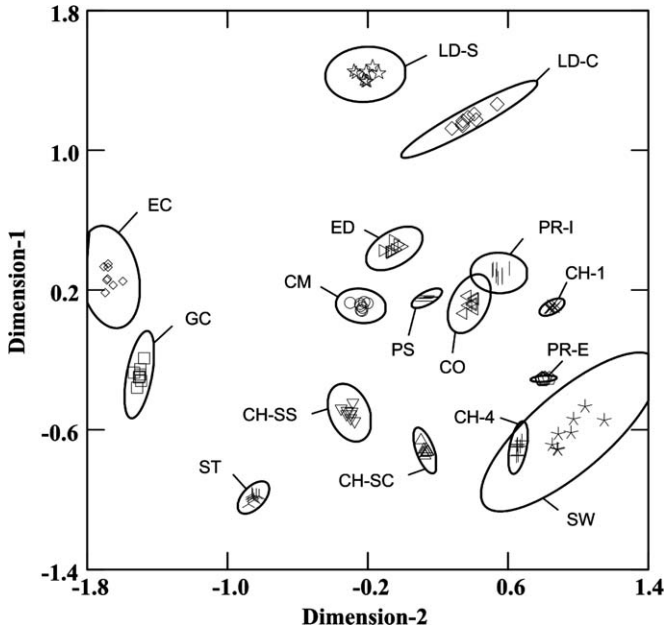

Fig. 2. Similarities between the MALDI-ToF mass spectra represented by multidimensional scaling of the matrix of Spearman correlations. Confidence ellipses (99%) enclose the 8 replicate profiles for each cheese. Cheeses were labelled according to their name: Cheddar aged 1 d and 4 months (CH-1 and CH-4); smeared Cheddar sampled at core and surface (CH-SC and CH-SS); Gruyere-type sampled at core and surface (LD-C and LD-S); Parmigiano–Reggiano cheese purchased in Italy and in Ireland (PR-I and PR-E); Stilton (ST); Swiss cheese (SW); Edam (ED); Cooleeney Camembert (CO); Port du Salut (PS); Camembert (CM); goats's milk cheese (GC); ewes' milk cheese (EC).

coordinates on dimension 2 (0.2), thus indicating a degree of similarity in their peptide profiles; this group included surface-ripened cheeses (Port du Salut, Cooleeney, Camembert), Edam, and Parmigiano–Reggiano cheeses. Young (1 d) and ripened Cheddar (4 mo), Parmigiano–Reggiano purchased in Ireland and Swiss cheeses had high scores on dimension 1. The Swiss cheese sample showed a high variability between replicates. The similarity observed between Parmigiano–Reggiano cheese purchased in Ireland and Swiss cheese was mainly due to the relatively high intensity observed for a common peptide at m/z 1664.92 (possibly α_{s1}-CN (f1–14)), which was absent from Parmigiano–Reggiano purchased in Italy. Although both Parmigiano–Reggiano cheeses were distinguished, a strong signal (100% relative intensity) at m/z 1198.69 (unknown peptide) was observed in both their peptide profiles. Both core and surface samples of smeared Cheddar cheeses were placed between Stilton and surface-ripened cheeses.

In conclusion, small peptides extracted from different cheese varieties, were distinguished using MALDI-ToF-MS. Use of MALDI-ToF-MS had the advantage of rapid analysis of samples (analysis time was less than 3 min) compared to reversed-phase HPLC (sample analysis time generally ~100 min) for monitoring peptide profiles in the study of proteolysis. Moreover, it was possible to obtain very accurate molecular masses of peptides, hence facilitating better understanding of their origins, based on the known specificities of chymosin, plasmin and enzymes from starter, non-starter or secondary microflora in the cheese. The use of multivariate statistical analysis for data handling helped to describe the variance associated with MALDI-ToF mass spectra, due to the shot-to-shot variation between the replicates, and was useful for discriminating proteolysis of different cheese varieties, and, potentially, in traceability studies.

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