Study of the Efficiency of a Mobile Phase Used in Size-Exclusion HPLC for the Separation of Peptides from a Casein Hydrolysate According to their Hydrodynamic Volume

L. Lemieux[1] / J.-M. Piot[2] / D. Guillochon[2] / J. Amiot[3]*

[1]Centre de Recherche STELA, Département de Sciences et Technologie des Aliments, Université Laval, Sainte-Foy, Québec G1K 7P4, Canada.

[2]Laboratoire de Technologie des Substances Naturelles, IUT A-Lille 1, BP 179, 59653 Villeneuve d'Ascq Cedex, France.

[3]Département de Sciences et Technologie des Aliments, Université Laval, Sainte-Foy, Québec G1K 7P4, Canada.

Key Words

Column liquid chromatography
Size-exclusion
Peptides
Casein hydrolysate
FAB-MS

Summary

A casein hydrolysate was eluted on a TSK G-2 000 SW size-exclusion high-performance liquid chromatography (SE-HPLC) column using a mobile phase which gives a correlation coefficient of 0.98 for the separation of 20 pure and synthetic peptides and proteins of molecular weight (M.W.) ranging from 243.28 (Ala-Pro-Gly) to 43 000 (Ovalbumine) fractions. Further isolation and purification of peptides present in each of the 8 different fractions collected from the SE-HPLC were done by reversed-phase HPLC (RP-HPLC). Peptide experimental M.W. determined from the calibration curve obtained for synthetic or purified peptides was compared to their precise M.W. obtained by fast atom bombardment-mass spectrometry analysis (FAB-MS). The results do not show a separation of this complex hydrolysate by SE-HPLC according to the hydrodynamic volume of its constituents.

Introduction

Caseins are the most abundant among the major classes of milk proteins which are synthesised in the mammary gland as larger precursors. Beside a nutritional role, casein is also a source of biologically active peptides playing important physiological roles such as opiate-like activator, platelet inhibitor, immunostimulator, chymosin inhibitor [1]. Hydrolysis of caseins with enzymes such as trypsin and chymotrypsin is made to produce small peptides and amino acids for use in dietetic foods and to liberate active peptidic fragments. Maubois and Brulé [2] for instance developed a continuous-flow membrane enzymatic reactor for the production of casein hydrolysates. Such hydrolysates were shown to contain more than 200 peptides of different sizes [3]. Therefore, a rapid high-resolution method for separating peptides by size and thus providing estimates of their molecular weights would be a valuable tool for the characterization of such hydrolysate. Some attempts have been done in this way using size-exclusion HPLC [4–9] but were not successful in separating a complex mixture of peptides by size. It is limited, indeed, by several factors such as the adsorption of peptides to the stationary phase by ionic and/or hydrophobic interactions, the use and the difficulty of removal of high concentration of salts or denaturing agents in the mobile phase buffers, and the inefficiency to separate small peptides [8].

In a recent publication [3] we have shown that the peptides from a casein hydrolysate phosphorylated were not eluted on a TSK G-2 000 SW column according to their hydrodynamic volume. A further extensive study of the different interactions present in the chromatographic system was made possible by isolation and identification of each peptide and by comparison with retention times obtained on SE-HPLC. From this study, we proposed a new mobile phase susceptible to allow a reliable correlation between the M.W. and elution volume of peptides from casein hydrolysates on a TSK G-2 000 SW column [10].

The present study was conducted to verify the efficiency of this new mobile phase for the separation of peptides contained in a casein hydrolysate phosphorylated in accordance with their hydrodynamic volume. A first separation was done by high-performance liquid chromatography on a size-exclusion column and was followed by the isolation and purification of peptides by RP-HPLC. FAB-mass spectrometry was performed on peptides randomly selected to measure directly their true molecular weight.

0009-5893/91/12 0499-06 $ 03.00/0 © 1991 Friedr. Vieweg & Sohn Verlagsgesellschaft mbH

Materials and Methods

Materials

Commercial casein hydrolysate phosphorylated (CHPS) was provided by Laboratoire Sopharga (France). It was prepared according to the procedure developed by Maubois and Brulé [2], which involves a controlled hydrolysis of casein by proteases, chymotrypsin and trypsin, in a continuous-flow membrane enzymic reactor. It was provided with the following specifications for peptides molecular weight distribution: > 5 000 daltons, 3%; 1 000–5 000 daltons, 28%; < 1 000 daltons, 69%, including 8% of free amino acids [11]. According to the manufacturers, CHPS was shown to contain caseinophosphopeptides, but it might also be contaminated by dephosphorylated peptides [11].

HPLC-grade water and methanol, and monobasic and dibasic sodium phosphate were obtained from Fisher Scientific (Quebec, Canada). Trifluoroacetic acid (TFA) was supplied by Pierce (USA). Analytical ammonium acetate and acetic acid (R.P. Normapur) and acetonitrile (HPLC-grade) were supplied by Prolabo (Paris, France). 1-thioglycerol and glycerol were provided by Interchim (Paris, France). Distilled water purified by passing it through a Culligan water purification system (Lille, France) was used for the reversed-phase LC and FAB-MS. RP-LC samples were filtered on Sartorius (Palaiseau, France) 0.20 µm filters. Eluents were degassed with helium (Air Liquide, Bois d'Arcy, France) during analysis.

Size-Exclusion HPLC

Primary separation of CHPS was performed by SE-HPLC on a TSK G-2 000 SW column (600 x 7.5 mm I.D.) with a guard column (60 x 7.5 mm I.D.) (Toyo Soda, Tokyo, Japan). An LKB (Bromma, Sweden) HPLC system equipped with a Model 2150 pump, a Model 2152 controller, an autosampler Model 2157 having a Rheodyne M7010 sample injection valve with a 20-µL loop, and a Model 1040A diode-array detector (Hewlett-Packard) set at 214 nm and connected to a Model HP 9133H disc drive and to a Model HP 9000 serie-300 computer were used. The mobile phase consisted of 0.1% TFA, 0.12M phosphate buffer (pH 5) and 10% methanol. The system was run isocratically at a flow-rate of 0.75 mL/min and at constant temperature (22 °C). The mobile phase was filtered through a 0.45 µm filter (Millipore) and sonicated before use. Hydrolysate solutions of 20 mg/mL were prepared in the mobile phase and filtered through 0.45 µm filters (Millipore) before being applied to the column. Fractions eluted from 21 runs were manually collected and evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY, USA).

Reversed-Phase LC

Fractions obtained by HPSEC were rechromatographed for a further separation of the peptides on an LKB Pep – S 5 µm (C_2 – C_{18} Superpac) reversed-phase column (250 x 4 mm I.D.). After equilibration of the column with 10 mM ammonium acetate buffer pH 6.0 (solvent A) at a flow-rate of 1 mL/min, peptides were eluted by linearly increasing the concentration of solvent B [50% (v/v) acetonitrile in 50% (v/v) solvent A] as follows: 0–30 min, 0–30% B; 30–50 min, 30–100% B; 50–55 min, 100–0% B. The time required for one complete run, including the equilibration time, was 65 min. Degassing of the mobile phases with helium was continuously done during the runs. Samples were dissolved in 1 mL of eluent A and 150 µL were injected. Peptides were monitored at 226 nm with an absorbance scale of 0.05. Peaks eluted from the column were manually collected and freeze-dried.

Fast Atom Bombardment – Mass Spectrometry

A Concept II HH (Kratos Analytical Ltd, Urmston, Manchester UK) four sector high-resolution tandem mass spectrometer, which consists of two double-focusing forward-geometry instruments joined back to back (E-B-E-B) was used to acquire spectra. It was equipped with a commercial Kratos FAB source, an Ion Tech B 11 NF saddle-field fast atom gun (Ion Tech Ltd, Teddington, UK) energised with the B-50 current-regulated power supply and a Kratos DS-90 (DGDG/30) data system to collect and process the spectra.

A beam of Xe atoms of approximately 7-keV impact energy and equivalent to 0.8 mA emission current was employed to ionise peptides dissolved in matrix (glycerol/water/trifluoroacetic acid in ratio 10:88:2 (v/v) or thioglycerol/trifluoroacetic acid in ratio 98:2 (v/v)). The positive FAB-produced ions were then accelerated through a potential of 8KV and mass-selected by using MS-I (ESA-I and the first magnetic sector) at a resolution of about 2 000 (full width at 5% height).

Peptides randomly selected from each SE-HPLC fraction were dissolved in purified distilled water (50 µg in 10 µL) and 1 µL of the solution were loaded on the stainless steel tip of the probe with 2 µL of the matrix. The mass range was scanned at 10 s/decade and caesium iodide was the standard used for mass calibration. The peptide accurate relative molecular mass was deduced from the m/z value of $(M + H)^+$ by substraction of one mass unit for the attached proton.

Results and Discussion

Size-Exclusion LC

According to the SE-HPLC elution conditions described above, 480 µg of CHPS hydrolysate could be injected per run and the chromatographic pattern so obtained is shown in Figure 1. Twenty-one injections were performed, giving the same elution pattern and 8 fractions (F_1 to F_8), as indicated in the Figure 1 were manually collected, pooled and freeze-dried. Since sample components were not well resolved, selected collection times were the same as those previously used [9] in order to ease comparison between both mobile phases. The molecular weight ranges of the different SE-HPLC-fractions are given in Table I. They were

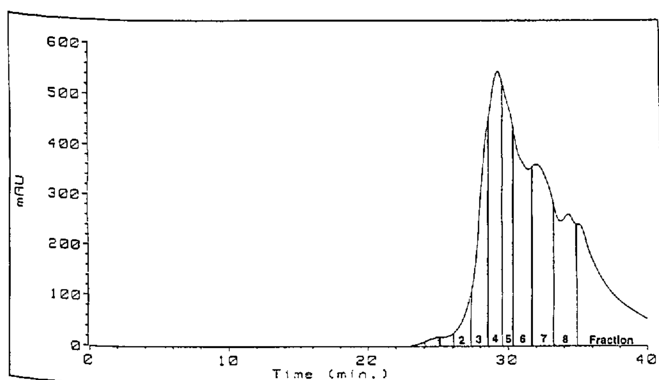

Figure 1

Elution profile of a casein hydrolysate phosphorylated on a TSK G-2 000 SW size-exclusion column.

assigned from the relationship between log (molecular weight) and elution time established for 20 pure or synthetic peptides and proteins of M.W. varying from 243.28 (Ala-Pro-Gly) to 43 000 (Ovalbumine) daltons and which gave a correlation coefficient of 0.98. The estimated quantity of peptides present in each SE-HPLC-fraction is also given in % and in mg for a better valuation of the amount available for the FAB-MS analysis. As previously shown [3], over 50% of peptides are located in fractions 4 to 6.

Reversed-Phase LC

Freeze-dried fractions obtained from SE-HPLC were solubilised in solvent A and analysed by HPLC on a C_2 – C_{18} Superpac reversed-phase column. The elution profiles for fractions 3 to 8 are shown in Figure 2. Fractions 1 and 2 did not give any peak under these chromatographic conditions. Six injections of the same product were performed and individual fractions were collected in separate test tubes and evaluated for purity on the basis of symmetry and narrowness of the peak. To verify their purity, some individual fractions were freeze-dried, dissolved in eluent A, injected once more under the same reversed-phase chromatographic conditions and collected again for further analysis by FAB-MS. When this step was not done more than one M.W. could be attributed to the selected peaks as it was the case for peaks 5_6, 6_7, 7_{10}, 8_4, and 8_7. As seen in Figure 2, peaks obtained with this RP-HPLC system were well resolved. The volatile mobile phase used and the linear gradient were efficient in separating this complex mixture of peptides having largely different hydrophobicities. Indeed, a good demarcation is observed between hydrophilic peptides (eluted till about 22 min) and hydrophobic peptides (eluted from about 27 min).

Mass Spectrometry

A total of 24 peptides randomly selected (Figure 2) for FAB-MS analysis are listed in Table II with their accurate relative molecular mass. Representative FAB-MS spectra of peptides 5_5, 6_5, 7_9, 8_6 and 8_7 are shown in Figure 3. Since sequences of peptides from CHPS have been previously identified and located in the known casein structures from their amino acid content and their N- and C-terminal amino acid analysis [3], it was possible to ascribe an estimated sequence to most of the selected peptides based on their molecular mass determined by FAB-MS. Peaks 5_{11} and 7_{10} were not identified since two peptides with different sequences have an identical M.W. of 688 daltons. Tandem MS should have been performed to determine the right sequence attributable to each of these two peptides. Moreover, no casein sequence has been assigned to peptides 3_2, 4_4, 4_8, 4_{10}, 6_{10} and 7_3 since none of the previously identified peptides corresponded to their M.W. These peptides might be among the missing links previously identified [3] in the casein sequences.

From the Table II it can be seen that even though peptides were randomly selected, identical peptides were eluted in different fractions (for example: peptides 3_6 and 4_9; 7_9 and 8_3). This can be explained by the low power of resolution of the TSK G-2 000 SW column as previously shown by results obtained with another mobile phase [3]. Peaks obtained with the present system were still neither sharp nor well-separated. Mass determination for peptides 7_9 and 8_3 (α_{s2}: 126–137; 1378 daltons) indicated that this sequence was not

Table I Comparison between experimental molecular weight distribution of peptides from CHPS separated by HPSEC and random selected peptide molecular weight range determined by positive FAB-MS; quantities of peptides recovered in each HPSEC fraction and estimated from the integration results are also given.

	Fractions							
	1	2	3	4	5	6	7	8
Experimental M.W. from the calibration curve (daltons)	10700–3667	3667–1929	1929–1070	1070–594	594–415	415–183	183–86	86–
M.W. range of random peptides identified by positive FAB-MS	–	–	1194–2105	686–1759	688–2556	747–1382	688–1089	666–2104
Estimated quantity of peptides of each fraction collected from HPSEC (% and mg)	1.4 % 0.2 mg	2.7 % 0.3 mg	10.8 % 1.1 mg	20.0 % 2.0 mg	13.2 % 1.3 mg	18.8 % 1.9 mg	16.9 % 1.7 mg	16.2 % 1.6 mg

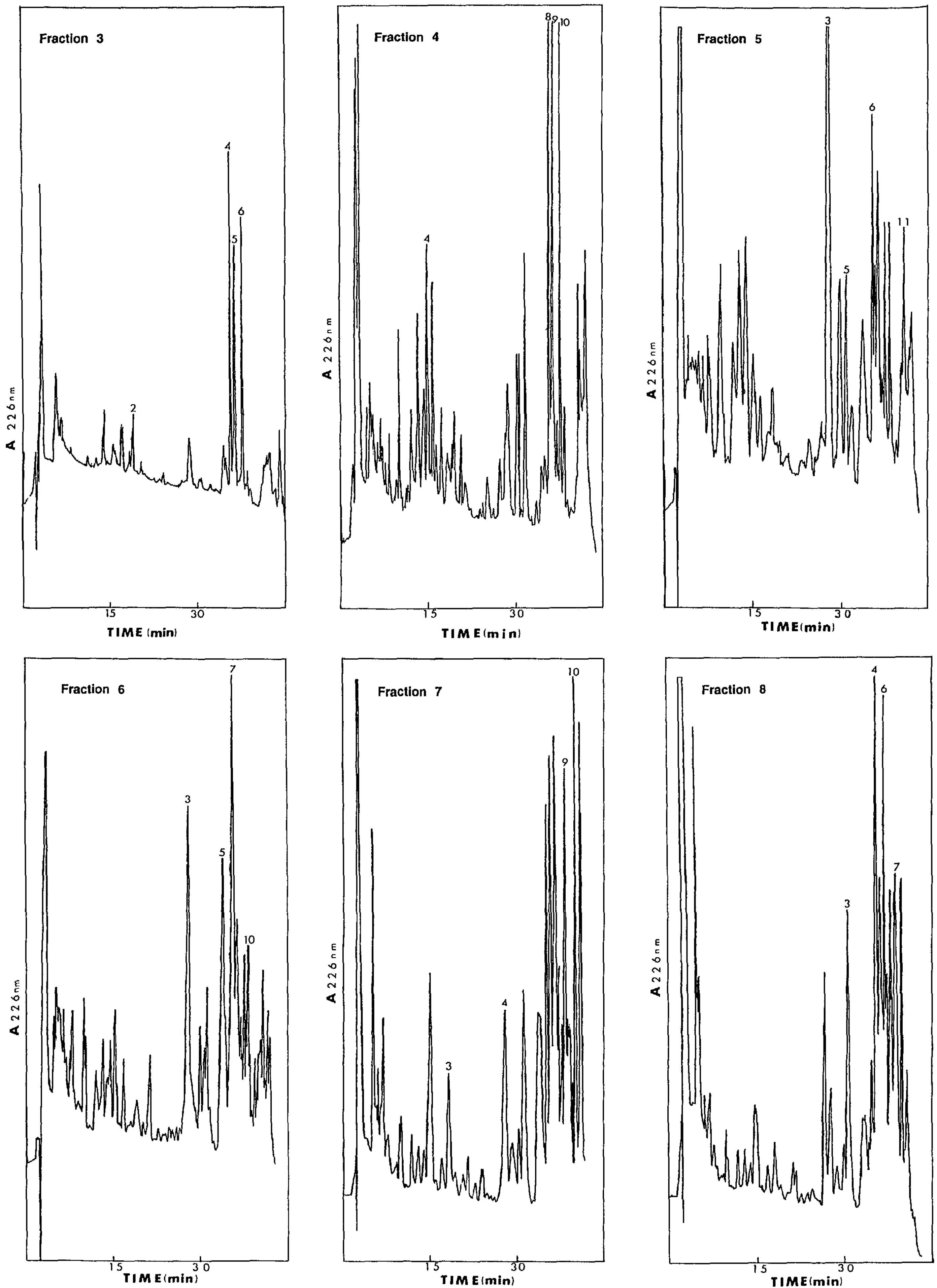

Figure 2
RP-HPLC of hydrolysate fractions (3–8) obtained from SE-HPLC and performed on a C_2 – C_{18} Superpac column.

Table II List of the randomly selected peptides for FAB-MS analysis with their accurate molecular weight and their estimated sequences*.

Selected peptides for FAB-MS analysis	Molecular Weight daltons	Possible Sequence
3_2	1348	–
3_4	2105	α_{s1}: 174–193
3_5	1194	α_{s2}: 115–125
3_6	1760	α_{s1}: 8–22
4_4	779	–
4_8	686	–
4_9	1759	α_{s1}: 8–22
4_{10}	764	–
5_3	829	β: 177–183
5_5	2556	α_{s1}: 170–193
5_6	1195	α_{s2}: 115–125
	2103	α_{s1}: 174–193
5_{11}	688	α_{s1}: 37–42 }
		β: 134–139 }
6_3	829	β: 177–183
6_5	747	β: 108–113
6_7	939	α_{s1}: 166–173
	1269	α_{s1}: 192–202
6_{10}	1382	–
7_3	756	–
7_4	902	α_{s1}: 25–32
7_9	1382	α_{s2}: 126–137
7_{10}	688	β: 134–139 }
		α_{s1}: 37–42 }
	1089	α_{s1}: 25–34
8_3	1378	α_{s2}: 126–137
8_4	939	α_{s1}: 166–173
	1269	β: 192–202
	2104	α_{s1}: 174–193
8_6	666	α_{s2}: 92–96
8_7	814	α_{s1}: 145–150
	904	α_{s1}: 25–32

* From [3]

phosphorylated as should have been expected from a pure CHPS hydrolysate, thus indicating a contamination with CHDS (a commercial casein hydrolysate dephosphorylated) as previously mentioned [11].

Table I shows that, except for fractions 3 and 4, the M.W. of the peptides selected for identification by FAB-MS are not corresponding to the expected values determined experimentally by SE-HPLC. Indeed, the M.W. of the peptides are rather scattered among the different fractions instead of being well distributed. As the number of selected peptides is representative for each fraction, these results can thus be extrapolated to the other peptides remaining in the different fractions. A separation of the CHPS-peptides according to their hydrodynamic volume should not then be expected under these chromatographic conditions.

The mobile phase used in this experiment with a TSK G-2 000 SW column was developed to minimise possible interactions between peptides, the mobile phase and/or the stationary phase as previously described [3, 11]. From the results obtained, we may therefore conclude that TSK G-2 000 SW column is not appropriate to characterise complex mixtures of peptides such as casein hydrolysates on the basis of peptides individual molecular weights. However, such a chromatographic system has proved to be efficient for other uses such as the differentiation between the bitter and astringent soluble fractions extracted from Cheddar cheese [13] or to follow cheese maturation with different

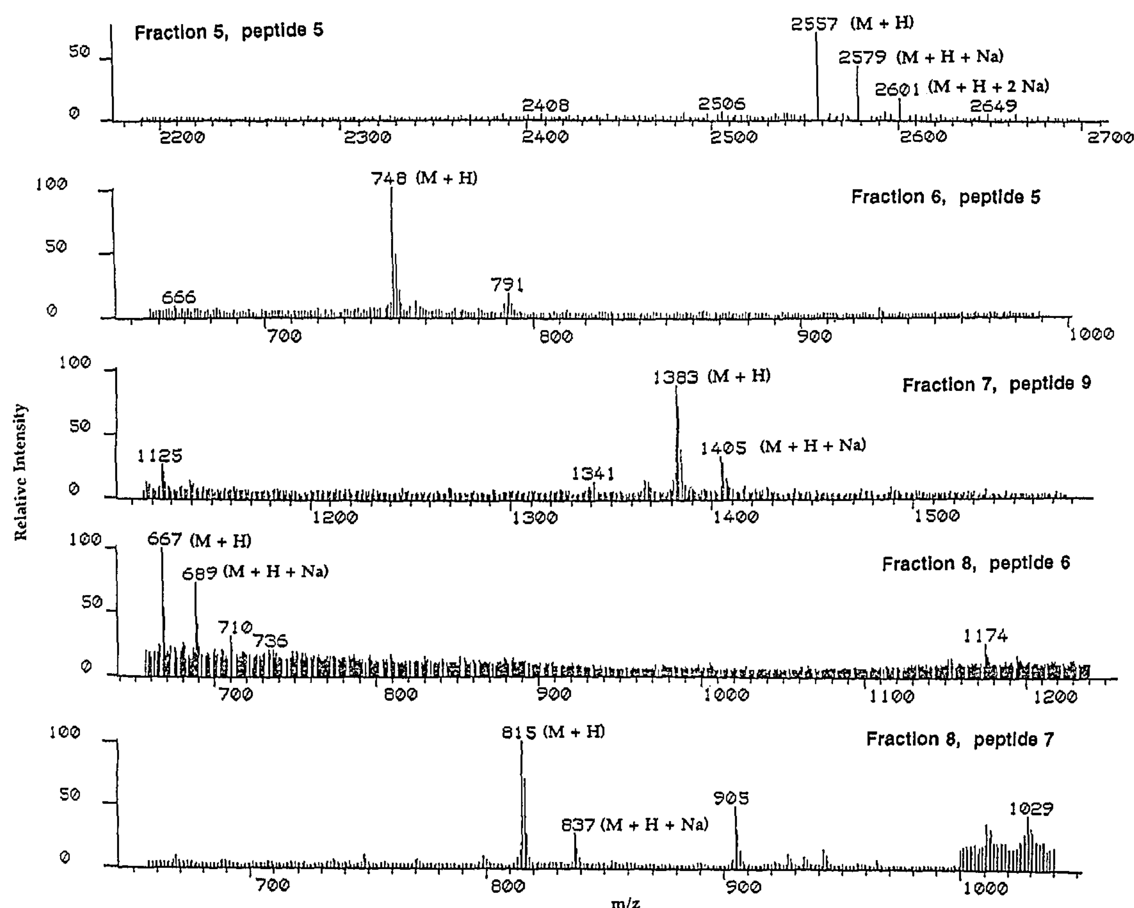

Figure 3
Positive FAB-MS spectra of peptides 5_5, 6_5, 7_9, 8_6 and 8_7.

bacterial strains [13]. As previously indicated, RP-HPLC has proved to be efficient to separate individual peptides from casein hydrolysates and to give information on their hydrophilic and hydrophobic properties. FAB-MS, however, appears to be a precise and reliable method for the determination of peptides molecular weights, including those isolated by RP-HPLC from complex mixtures such as casein enzymatic hydrolysates. Indeed, this methodology has already been used for rapid analysis of polar and charged molecules such as oligopeptides [14, 15] or more complex structures such as cyclic peptides or aminoblocked and other posttranslationally modified peptides [16].

Acknowledgements

Ministère des Affaires internationales du Québec (Programme d'échanges France-Québec sur les biotechnologies).

References

[1] *A.-M. Fiat, P. Jollès,* Molecular and Cellular Biochem. **85**, 5 (1989).
[2] *J. L. Maubois, G. Brulé,* Lait **62**, 484 (1982).
[3] *L. Lemieux, J. Amiot,* J. Chromatogr. **519**, 229 (1990).
[4] *N. Ui,* Analyt. Bioc. **97**, 65 (1979).
[5] *M. E. Himmel, P. G. Squire,* Int. J. Peptide Protein Res. **17**, 365 (1981).
[6] *G. B. Irvine, C. Shaw,* Anal. Biochem. **155**, 141 (1986).
[7] *R. Wahl, H. J. Maasch, W. Geissler,* J. Chromatogr. **351**, 39 (1986).
[8] *G. D. Swergold, C. S. Rubin,* Anal. Biochem. **131**, 295 (1983).
[9] *M. A. Vijayalakshmi, L. Lemieux, J. Amiot,* J. Liq. Chromatogr. **9**, 3559 (1986).
[10] *L. Lemieux, J. Amiot,* J. Chromatogr., submitted for publication.
[11] *L. Lemieux, J. Amiot,* J. Chromatogr. **473**, 189 (1989).
[12] Reabilan, *Scientific Dossier,* Société Sopharga, Roussel UCLAF, DS/FT, France (1984).
[13] *L. Lemieux, R. Puchades, R. E. Simard,* J. Food Sci. **54**, 1234 (1989).
[14] *R. M. Caprioli,* Biochemistry **27**, 513 (1988).
[15] *J. M. Piot, Q. Zhao, D. Guillochon, P. Dhulster, G. Ricart, D. Thomas,* Chromatographia **30**, 205 (1990).
[16] *E. R. Grant, R. G. Cooks,* Science **250**, 61 (1990).

Received: Aug. 8, 1991
Revised manuscript
received: Sep. 9, 1991
Accepted: Sep. 30, 1991