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Investigation of crudes of synthesis of neuropeptide Y by high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

Neuropeptide Y (NPY) and its modified form, [Leu^{31},Pro^{34}]NPY, are both thirty six amino acids long and they have relative molecular masses of 4250 and 4220. Solid-phase synthesis of both peptides resulted in complex crudes of reaction, which were investigated by means of combined high-performance liquid chromatography–electrospray mass spectrometry (HPLC–ES-MS). The combination of these two powerful analytical techniques allowed rapid and reliable identification of the target peptides and furnished comprehensive information on other reaction products, which were mainly peptidic chains containing a smaller number of amino acids compared to those present in the intact peptides. The possible origin of such side-products and the eventual purification and unambiguous identification of both peptides are discussed.

1. Introduction

Electrospray ionization (ESI) mass spectrometry (MS) has been extensively used for the characterization of small proteins [1–4] and peptides [5,6].

Neuropeptide Y (NPY) was first sequenced by Tatemoto [7] and is considered a major regulatory peptide both in the central and the peripheral nervous system [8]. In the central system, NPY is believed to be involved in the regulation of food intake, memory processing and circadian rhythm. In the peripheral nervous system, the same peptide functions as transmitter in sympathetic nerves, where it acts together with norepinephrine in the regulation of vascular tone. NPY has also been identified as a therapeutic agent against shocks restoring blood pressure to its normal level [9,10]. In general, substantial and pure quantities are needed for three-dimensional structural characterization of this peptide. Most of present day synthesis of peptides and small proteins is mainly based on the solid-phase method, first introduced by Merrifield [11]. In this procedure of synthesis, a progressively growing peptidic chain is bounded to an insoluble resin by the carboxylic function of the C-terminal amino acid. The synthesis

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proceeds through successive couplings between the activated amino acids and the free amine function of the peptidic chain already linked to the insoluble resin. This synthetic procedure commonly results in unwanted products associated with the absence of one or more amino acids. To reduce the number of such products and to simplify inevitable high-performance liquid chromatography (HPLC) separation, the capping procedure is often adopted [11]. Such a procedure involves the acetylization of the unreacted amine functions following each coupling process. The complexity of the resulting crude of synthesis renders combined HPLC–mass spectrometry an indispensable analytical tool for fast and reliable identification of the target peptide and associated side-products.

2. Experimental

All presented mass spectrometric measurements were obtained using a single-quadrupole instrument (VG Platform, Fisons Instruments, Manchester, UK), which has an upper mass range of 3000. Direct-injection (no HPLC separation) measurements were performed using a Phoenix 20 CU pump (Fisons Instruments); a mobile phase composed of acetonitrile–water (50:50; v/v), 10 μl/min flow-rate, a 10-μl loop and sample concentrations of 20 pmol/μl (artificial mixture) and 85 mg/l (crude of synthesis) were used.

2.1. Analytical HPLC

Initial analytical HPLC of the two crudes of synthesis was performed using the following conditions: Perkin-Elmer 3B HPLC pump (Perkin-Elmer and Applied Biosystem, Foster City, CA, USA) equipped with a Perkin-Elmer LC75 UV spectrophotometer, a Perkin-Elmer 56 recorder and a Perkin-Elmer Sigma 15 data station; Vydac narrow-bore C_{18} RP, 300 Å (150 × 2.1 mm I.D., 5 μm particle size) column (Vydac, Hesperia, CA, USA); 200 μl/min flow-rate, 220 nm wavelength and 1 mg/ml sample concentration. The solvents used in the gradient method were: solvent A, acetonitrile–water (3:1, v/v) containing 0.1% trifluoroacetic acid (TFA); solvent B, water containing 0.1% TFA and sample concentration of 1 mg/ml. The gradient sequence was: time (t) 0–3 min 24% A, which over 64 min reached 66.6% A, remaining constant for 5 min, and going to 100% A in 10 min. The same HPLC conditions were used for the analysis of [Leu^{31},Pro^{34}]NPY crude of synthesis with two variations: 216 nm instead of 220 nm wavelength and gradient sequence from (t) 0–3 min: 40% A, which over 29 min reached 49.5% A, remaining constant for 5 min and going to 100% A in 3 min.

2.2. HPLC–MS

The analytical conditions were: Phoenix 20CU HPLC pump (Fisons Instruments); VG Platform mass spectrometer (Fisons Instruments) equipped with an electrospray ion source, Ultramex 5 C_{18} (250 × 1 mm I.D.) column (Phenomenex, Torrance, CA, USA); 30 μl/min flow-rate, 2-μl loop and sample concentration of 0.25 mg/ml. The gradient solvents used were the same as described above. The gradient sequences were: (a) artificial mixture: from 30% A to 70% A in 30 min, (b) NPY crude of synthesis: from 45% A to 89% A in 50 min, and (c) [Leu^{31},Pro^{34}]NPY crude of synthesis: from 65% A to 75% A in 40 min.

2.3. Preparative HPLC

The analysis conditions were: Shimadzu LC-8A pump, Shimadzu-6A UV detector, Shimadzu C-R6A chromatopack data station (Shimadzu Europe, Duisburg, Germany), Deltapack C_{18} RP (300 × 1.9 mm I.D., 15 μm particle size) column, 20 ml/min flow-rate and 220 nm wavelength. The gradient sequence was: $t = 0$ 100% B, reaching 73.4% B in 10 min, 33.4% B in 150 min, remaining constant for 10 min and reaching 100% A in 2 min.

2.4. Synthesis

The synthesis of NPY and [Leu^{31},Pro^{34}]NPY was based on solid-phase methods using an automated peptide synthesizer (Applied Biosystem 431A). The resin (Bachem Feinchemikalien, Bubendorf, Switzerland) contained Fmoc-4-methoxy-4′-(carboxypropyl)-benzydrylamine linked to alanyl-aminomethyl-polystyrene–1% divinylbenzene (substitution 0.6 mmol/g). For NPY the activation of the Fmoc-amino acid was performed in situ with HBTU–HOBT–DIPEA. A four-fold excess of the activated amino acid was used and acetylation of the unreacted amino functions was performed at the end of each coupling step using 10% acetic anhydride in NMP. For [Leu^{31},Pro^{34}]NPY the activation was performed with HOBT–DCC in NMP for 30 min. A four-fold excess of the active ester was used but no capping was done. In both syntheses every Arg(Mtr) was recoupled. After completion of the sequence the peptide was cleaved from the solid support with simultaneous deprotection of the side chains using a mixture of TFA–thioanisole–phenol–ethanedithiol–water (84:4:6:4:3) at 25°C for 4 h. After filtration and removal of the volatiles, the residual Mtr groups were cleaved with TFA–thioanisole–trimethylsilylbromide (6:1:1) at 0°C for 1 h. The mixture was then concentrated under reduced pressure, water was added and the solution was extracted with diethyl ether. The crude peptides were recovered by lyophilization.

2.5. Materials

Fmoc-L-Pro-OH, Fmoc-L-Ala-OH, Fmoc-L-Glu-(OtBu)-OH, Fmoc-L-Asp(OtBu), Fmoc-L-Leu-OH, Fmoc-L-Arg(Mtr)-OH, Fmoc-L-Tyr(tBu)-OH, Fmoc-L-Ser(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Ile-OH, Fmoc-L-Asn(Trt)-OH, Fmoc-L-Thr(tBu)-OH, Fmoc-L-Gln-(Trt)-OH were purchased from Applied BioSystems; Fmoc-L-Ala-NCA, Fmoc-L-Arg(Mtr)-NCA, Fmoc-L-Ser(tBu)-NCA, Fmoc-L-Leu-NCA, Fmoc-Ile-NCA, Fmoc-L-Asn(Trt)-NCA, Fmoc-Thr(tBu)-NCA, Fmoc-Gln(Trt)-NCA were purchased from Propeptide (Vert-le-Petit, France).

N-Methylpyrrolidone (NMP), dichloromethane, piperidine, 1-hydroxybenzo-triazole, N,N′-dicyclohexylcarbodiimide, acetic anhydride and trifluoroacetic acid were purchased from Applied BioSystems; diisopropylethylamine, ethanedithiol and thioanisole from Fluka Chemie (Buchs, Switzerland); acetonitrile and phenol from Carlo Erba Reagenti (Milan, Italy).

3. Results and discussion

It is commonly argued that under certain ion source conditions, electrospray ionization can yield simple ES mass spectra with negligible fragmentation allowing reasonable identification of the molecular identities within a mixture without the need for chromatographic separation prior to the mass spectrometric analysis. However, the usefulness of this characteristic of ES ionization is highly dependent on the complexity of the investigated medium and the chemical nature of its components. To underline this observation, three mixtures are considered, where the first is artificially made by mixing equal concentrations of [Leu^{31},Pro^{34}]NPY (M_r 4220), [D-Phe^{12}]bombesin (M_r 1628) and alytesin (M_r 1534), while the second and third mixtures are the crudes of synthesis of NPY and [Leu^{31},Pro^{34}]NPY obtained in two different procedures of synthesis. These analysis were performed using 30°C source temperature, 35 V extraction (cone) voltage and 3 kV capillary voltage.

3.1. Artificial mixture of [Leu^{31},Pro^{34}]NPY, [D-Phe^{12}]bombesin and alytesin

Equal concentrations (20 pmol/μl) of the three peptides in acetonitrile–water (50:50, v/v) were directly injected into the ion source. The resulting ES mass spectrum in Fig. 1a contains the multiply charged ions 1056 $[M + 4H]^{4+}$ and 604 $[M + 7H]^{7+}$ of [Leu^{31},Pro^{34}]NPY. The singly and doubly protonated alytesin are observed at A (m/z 1535) and A2 (m/z 768), while the

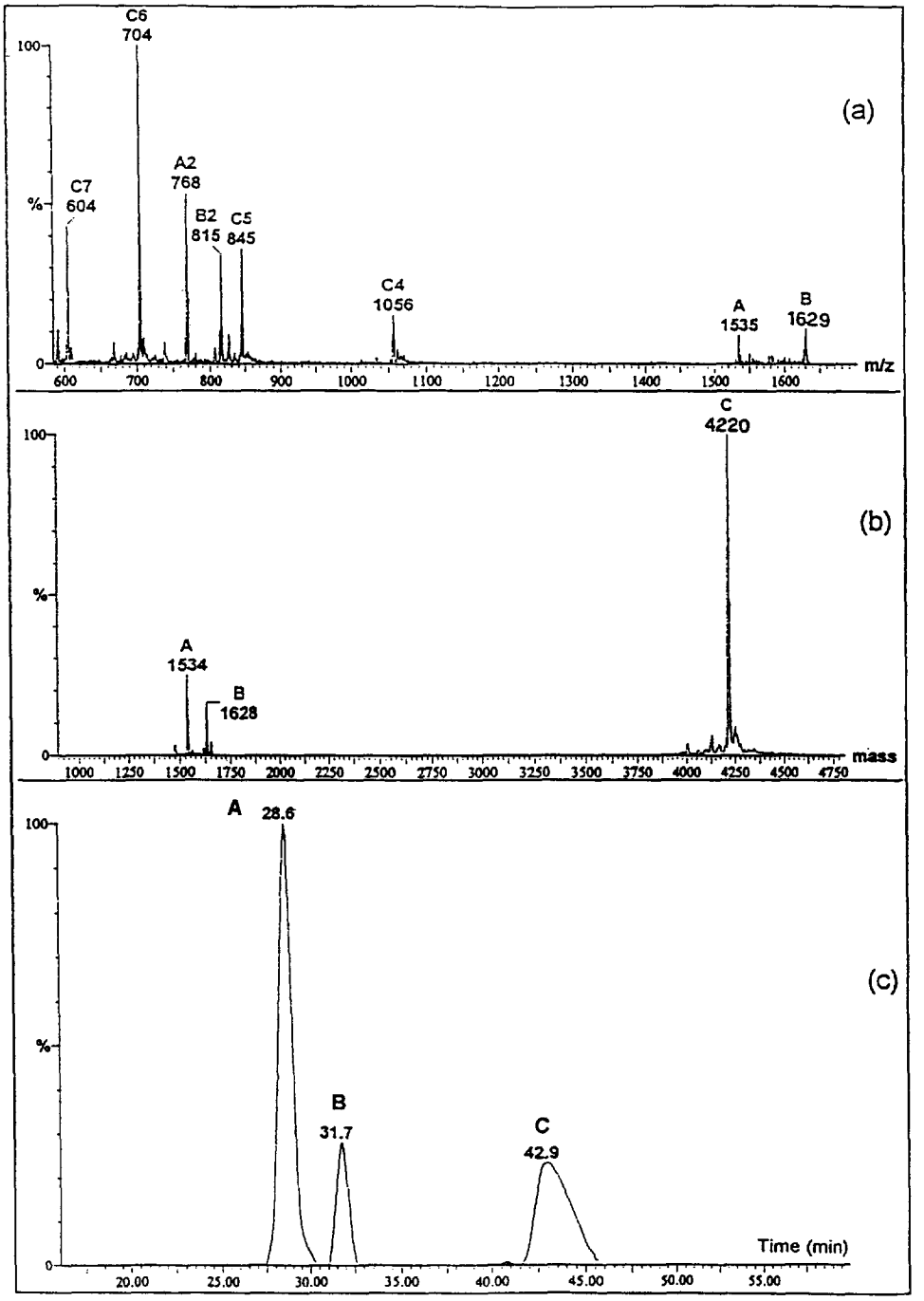

Fig. 1. (a) Charge-state distribution in the ES mass spectrum of an artificial mixture containing alytesin, [Phe^{12}]bombesin and [Leu^{31},Pro^{34}]NPY injected into the ion source without chromatographic separation; (b) measured relative molecular masses of the three peptides obtained by algorithmic transformation of the above charge-state distributions and (c) total-ion current (TIC) chromatogram of HPLC–ES-MS of the same mixture, with peak A (alytesin), peak B ([Phe^{12}]bombesin) and peak C ([Leu^{31},Pro^{34}]NPY).

corresponding ions of [D-Phe^{12}]bombesin are observed at B (m/z 1629) and B2 (m/z 815). Algorithmic transformation of the three charge-state distributions yielded the relative intensities of the three ion species in the mixture as a function of their relative molecular masses (Fig. 1b).

The investigation of the same mixture by HPLC–ES-MS resulted in the total-ion current (TIC) chromatogram in Fig. 1c. The three peaks with the retention times (t_R) 28.6, 31.7 and 42.9 min are attributed to alytesin, [D-Phe^{12}]bombesin and [Leu^{31},Pro^{34}]NPY, respectively.

The ES mass spectra under these peaks (Figs. 2a,b,c) contain charge-state distributions which are not substantially different from those reported in Fig. 1a. This agreement between the two sets of ES data obtained with and without HPLC separation was not evident in the data of the crudes of synthesis, which are discussed below. Apart from this deduction, the data pertaining to the artificial mixture served two purposes. First, optimization of the ion source conditions prior to the investigation of completely unknown crudes of synthesis and second, the same data furnished valuable information on the charge-states distribution of [Leu^{31},Pro^{34}]NPY in the presence of other components in the electrosprayed solution.

3.2. HPLC–ES-MS of the NPY crude of synthesis

Solid-phase synthesis [10] of the peptide NPY yielded a crude mixture which upon investigation by gradient HPLC resulted in the UV chromatogram in Fig. 3a. As well as the target NPY ($t_R = 40.4$ min), the chromatogram contains other, unidentified peaks with the indicated retention times. The complexity of the crude was further confirmed by its ES mass spectrum (Fig. 3b) obtained by direct injection into the ion source of a solution containing $8.5 \cdot 10^{-2}$ mg/ml dissolved in acetonitrile–water (50:50, v/v) and using a 10 μl/min flow-rate. The multiply charged ions marked A4–A7 are attributed to $[M + 4H]^{4+}$ and $[M + 7H]^{7+}$ of the NPY, while other significant charge-state distributions marked B3–B6 and C3–C4 are assigned to two fragments with relative molecular masses 3492 and 2271, respectively.

The identification of other significant peaks in the same spectrum required further HPLC–ES-MS measurements. To gain further information on the various components observed in the UV chromatogram, HPLC–ES-MS analysis was performed. However, before performing such analysis, the analytical HPLC parameters were modified to allow micro-bore column analysis. Such modification had two advantages: improved concentration detection limits and a lower flow-rate (30 μl/min) more adequate for electrospray ionization.

The HPLC–ES-MS of the crude of synthesis resulted in the total-ion current chromatogram in Fig. 3c, in which the target NPY is observed at the retention time of 36.8 min. The ES mass spectrum under this peak (Fig. 4a) exhibits the multiply charged ions (A3–A6) of the NPY.

The ES mass spectra under the TIC peaks with the retention times 38.5 and 45.0 min are given in Fig. 4b,c. The first spectrum contains a single charge-state distribution (A2–A4) yielding a relative molecular mass of 2270, while the spectrum in Fig. 4c exhibits two charge-state distributions, A and B, which yielded the relative molecular masses 3490 and 2852. Based on the mass spectra in Fig. 4 and other mass spectra which are not presented here, the relative molecular masses and most likely amino acid composition of the various peptidic chains within the crude of synthesis are summarized in Table 1. Close inspection of this table reveals that most of the acetylated peptidic chains contain 8–16 amino acids [Ac(28–36), Ac(20–36)]. These and similar incomplete (difficult) sequences are commonly observed in solid-phase peptide synthesis.

The chemistry related to repeated sequential coupling of amino acid residues to a growing terminal section of a peptide attached to a lightly cross-linked solid resin has been extensively investigated [12]. Couplings considered difficult and/or of low yield tend to occur with particular sequences. These difficulties have been variously attributed to association complexes, swelling

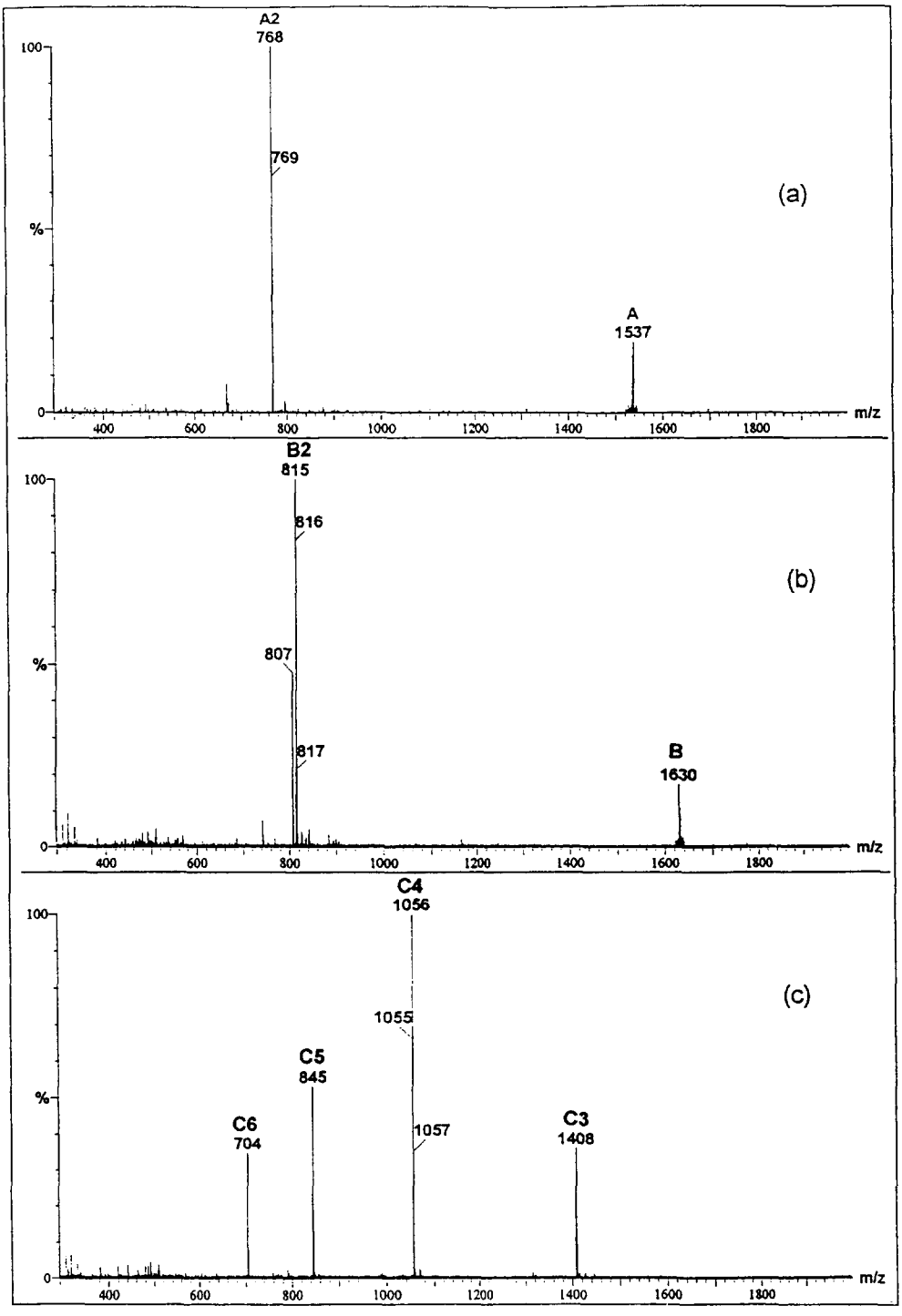

Fig. 2. Charge-state distributions under the TIC peaks in Fig. 1c; (a) $t_R = 28.6$ min, (b) 31.7 min, and (c) 42.9 min.

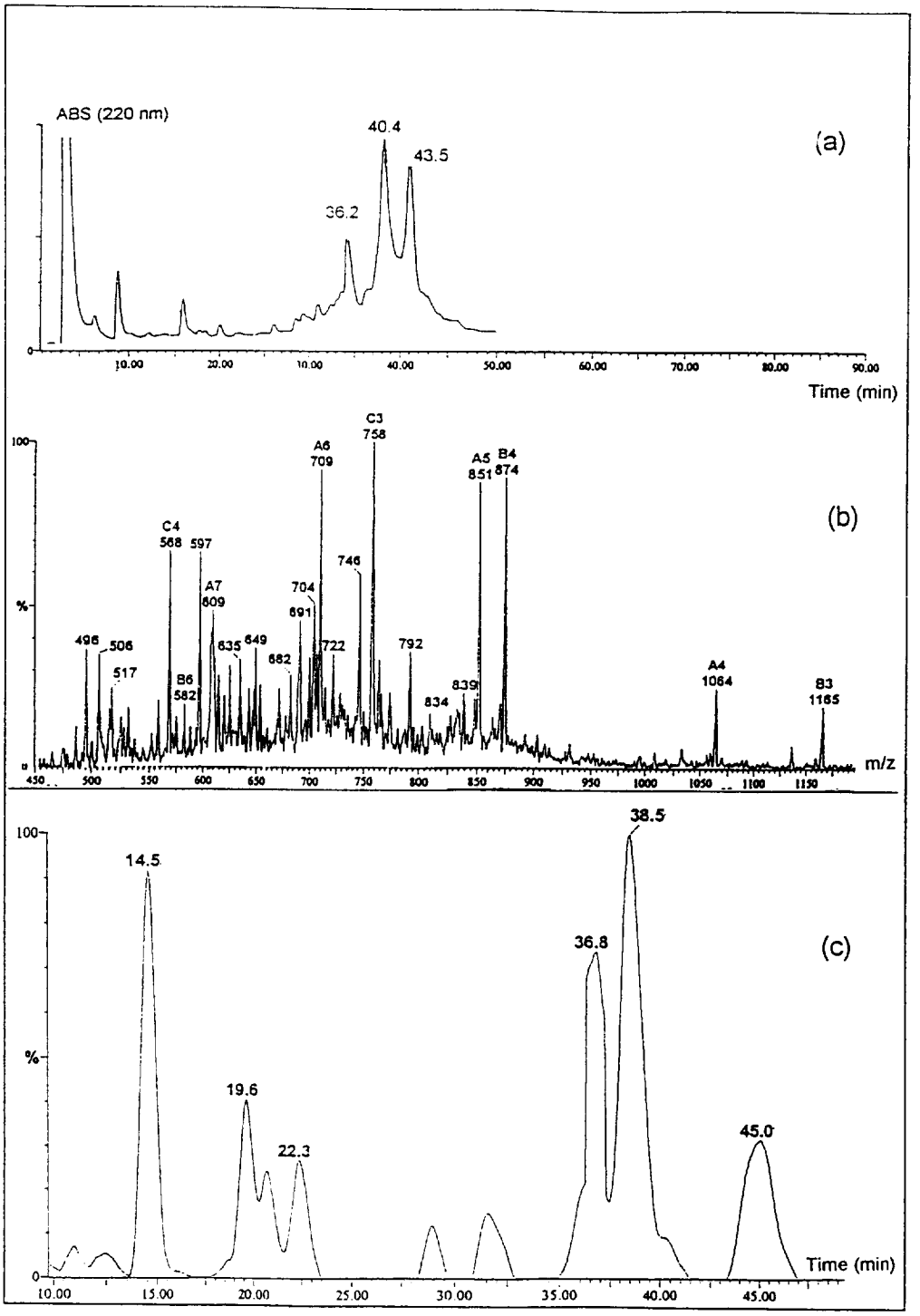

Fig. 3. (a) UV (220 nm) chromatogram of the NPY crude of synthesis, (b) charge-state distributions in the ES mass spectrum of the mixture injected into the ion source without chromatographic separation and (c) TIC chromatogram obtained by HPLC–ES-MS of the same mixture.

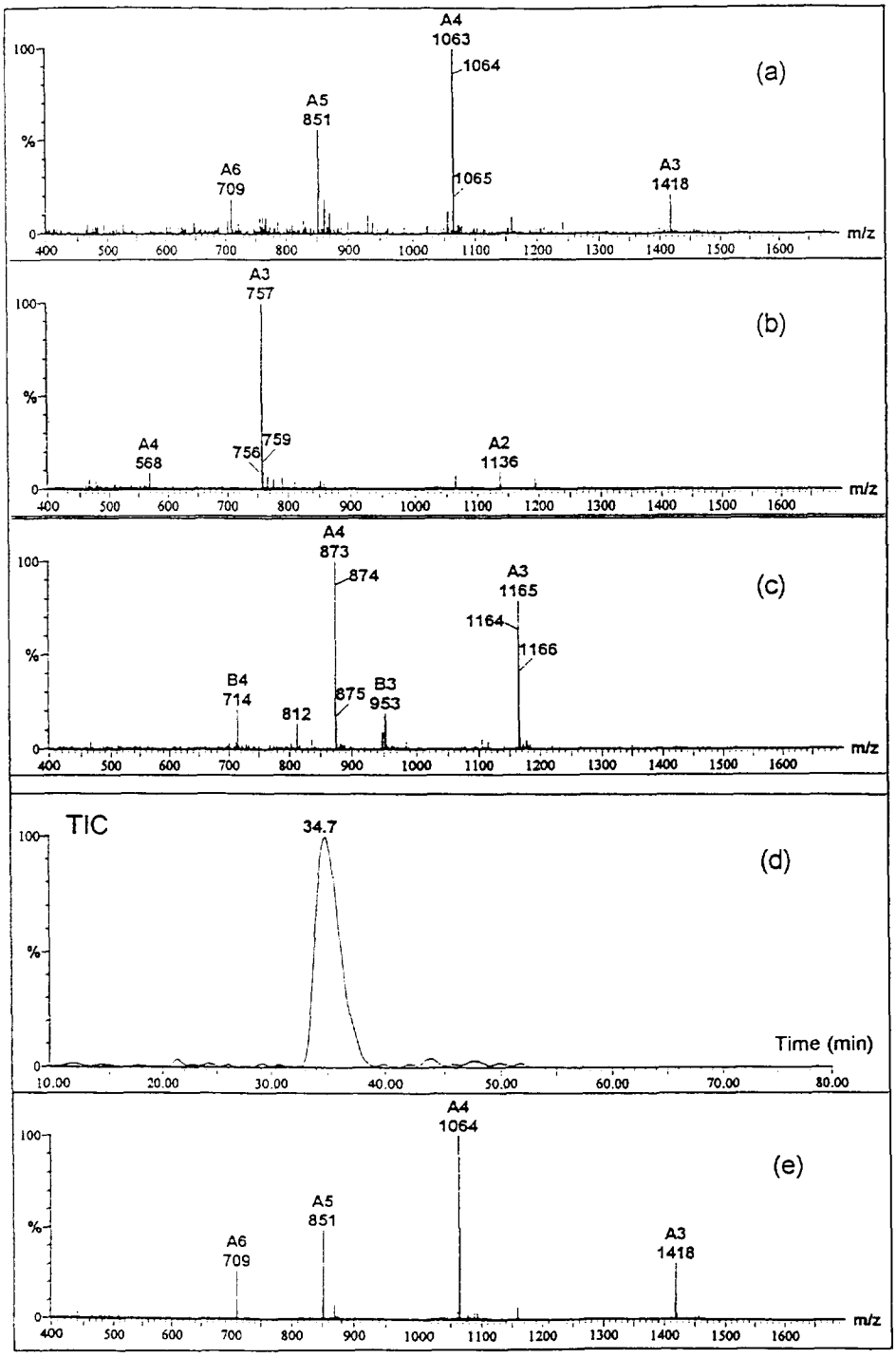

Fig. 4. Charge-state distributions in the ES mass spectra under the TIC peaks in Fig. 3c at the retention times (t_R) of: (a) 36.8 min, (b) 38.5 min and (c) 45.0 min; (d) TIC chromatogram of the purified NPY, and (e) charge-state distribution in the ES mass spectrum under the NPY peak (d).

Table 1
Relative molecular masses (M_r) of acetylated chains and the numeric position (x) of the final amino acid attached

x	M_r = Ac(x − 36)		x	M_r = Ac(x − 36)	
36	222		18	2496	
35	378		17	2609	
34	506		16	2724	
33	662		15	2853	*
32	763		14	2924	
31	876		13	3021	
30	989	*	12	3092	
29	1103		11	3207	
28	1216	*	10	3336	
27	1379	*	9	3393	
26	1516	*	8	3490	*
25	1672	*	7	3604	
24	1785	*	6	3719	
23	1856	*	5	3816	
22	1943	*	4	3944	
21	2106	*	3	4031	
20	2269	*	2	4128	
19	2425			–	

The symbol * refers to the peptidic chains which have been identified in the present work.

problems and hydrogen-bond aggregation of the growing peptide chains [13,14]. Although it has been always assumed that the solid support could isolate the growing peptide chains, creating chemically dilute environments [15], it has now been shown that site–site reactions between different chains do occur, resulting in incomplete sequences.

Based on the data presented, it is evident that the use of HPLC–ES-MS allowed reliable identification of the target NPY and it underlined the need for further purification. This was done using preparative HPLC followed by HPLC–ES-MS analysis, which resulted in a single TIC peak centered at t_R = 34.7 min (Fig. 4d), under which the ES mass spectrum in Fig. 4e was obtained. The observation of a single charge-state distribution (A3–A6) centered at m/z 1063 infers the presence of a single identity with a relative molecular mass of 4250 corresponding to the NPY peptide.

3.3. HPLC–ES-MS of [Leu^{31},Pro^{34}]NPY crude of synthesis

The method of synthesis of this peptide did not involve capping. In other words, acetylization was not applied to the sites where coupling was not successful. In theory, such procedure is expected to result in a more complicated crude of synthesis compared to that obtained in the synthesis of NPY. Such expected complexity and in particular the lack of resolution is clearly evident in the UV chromatogram in Fig. 5a. The total-ion current (TIC) chromatogram of the same crude of synthesis obtained using 30 μl/min flow-rate, Ultramex column and positive ES ionization is given in Fig. 5b.

The qualitative differences between the two chromatograms in Fig. 5 can be attributed to two reasons. First, the two chromatograms were generated under different experimental conditions including different columns, different sample concentrations and different flow-rates. Second, the top chromatogram refers to UV absorption by various components present in solution, while the bottom chromatogram reflects the protonation efficiency of gas-phase molecules.

The ES mass spectrum (Fig. 6a) under the twin peak with retention times 21.7 and 22.9 min contains three charge-state distributions, marked A, B and C.

The first distribution (A3–A6) is attributed to the target compound [Leu^{31},Pro^{34}]NPY, while the distributions (B3–B5) and (C3–C5) are due to incomplete peptidic chains with relative molecular masses of 4110 and 3816, respectively.

The dominant TIC peak centered at 13.0 min is due to a relatively pure component with relative molecular mass of 1486, while the peak at t_R = 27.9 min contains two molecular identities with relative molecular masses of 3978 (A), and 747 (B), respectively.

The data in Figs. 5 and 6 underlined the need for further purification of the target compound. Such purification was performed in two stages, involving preparative HPLC followed by a relatively slow gradient HPLC, which resulted in the UV chromatogram in Fig. 7a. The observation of

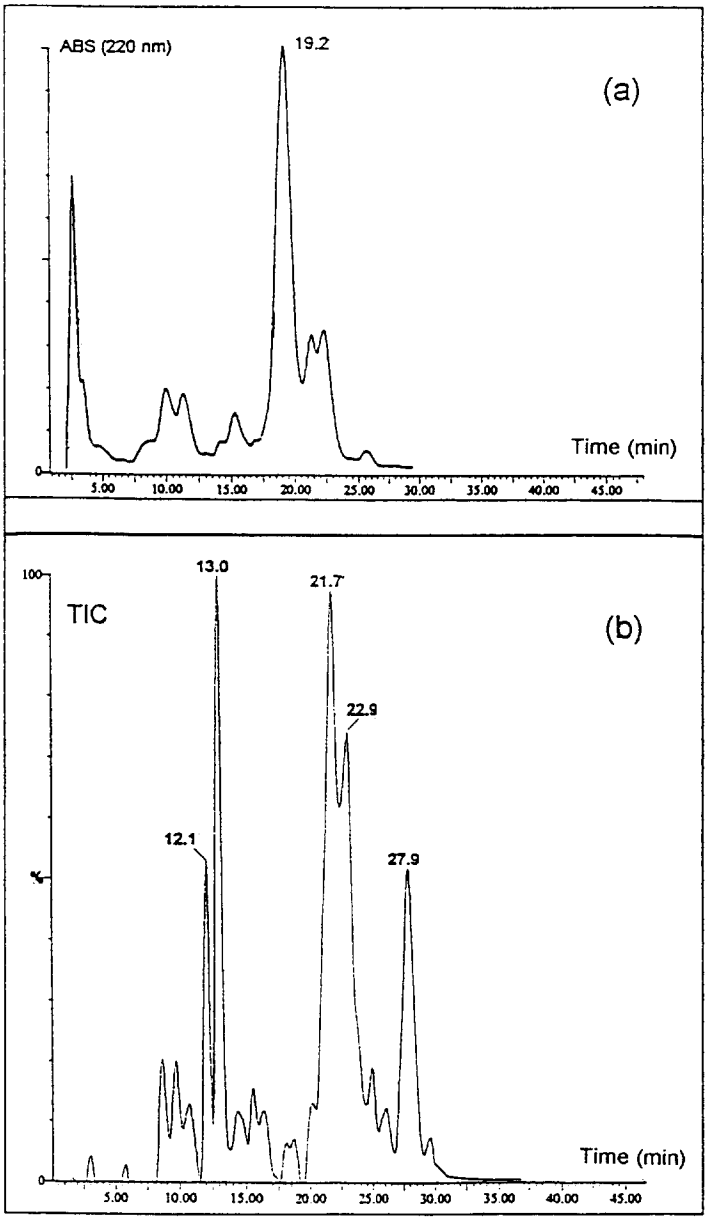

Fig. 5. UV (220 nm) chromatogram of [Leu^{31},Pro^{34}]NPY crude of synthesis and (b) TIC chromatogram of the same mixture obtained by HPLC–ES-MS.

a single Gaussian peak and the absence of substantial baseline noise gives the impression of a pure product. This apparent purity is not confirmed by the ES mass spectrum under this peak (Fig. 7b), which contains two charge-state distributions, (A3–A5) and (B3–B5), which upon transformation (Fig. 7c) gave two molecular masses, 4220 [Leu^{31},Pro^{34}]NPY, and 4106.

The observation of the latter molecular identity can be simply attributed to collision-induced dissociation of the intact peptide in the intermediate region of the ion source, which is held at

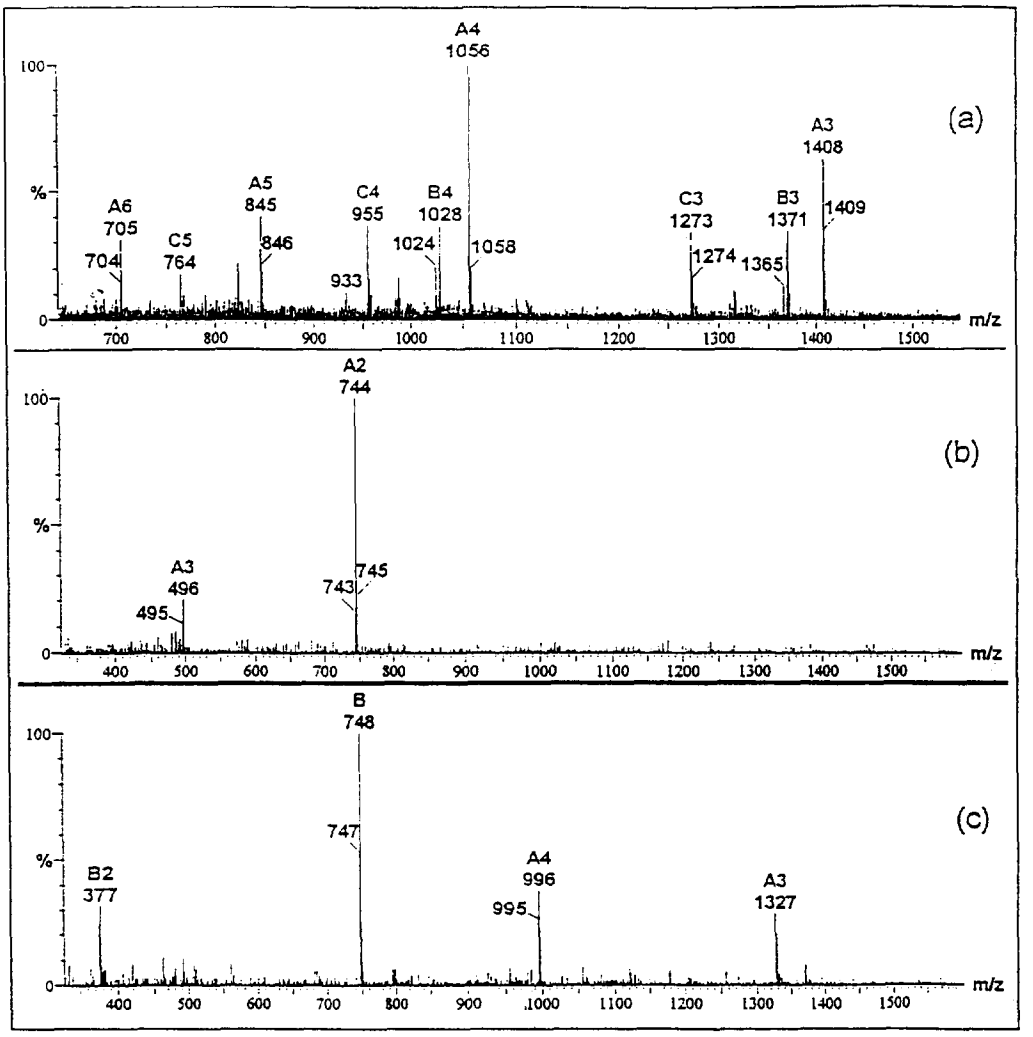

Fig. 6. Charge-state distributions in the ES mass spectra under the TIC peaks in Fig. 5b at the retention times: (a) 21.7–22.9 min, (b) 13.0 min, and (c) 27.9 min.

a pressure of ca. 0.1 mbar. This interpretation, however, cannot be fully sustained for a number of reasons. First, both C- and N-terminals contain tyrosine (M_r 181), which excludes the loss of 114 as the first step of dissociation; second, peak slicing by means of preparative HPLC resulted in three fractions pertaining to the leading part of the peak, the center and the trailing part, which were isolated and examined by ES mass spectrometry.

The transformed mass spectra in Fig. 8a,b,c indicate that the leading part of the purified HPLC peak contains exclusively [Leu^{31},Pro^{34}]NPY, while the central and trailing portions of the same peak contain the same peptide as well as a second component with a molecular mass of 4106. The absence of the latter molecular identity from the leading portion of the HPLC peak is a clear indication that this component is not the result of fragmentation within the ion source.

Considering the HPLC–ES-MS data and the procedure of synthesis, the molecular mass 4106 is attributed to the absence of one of the as-

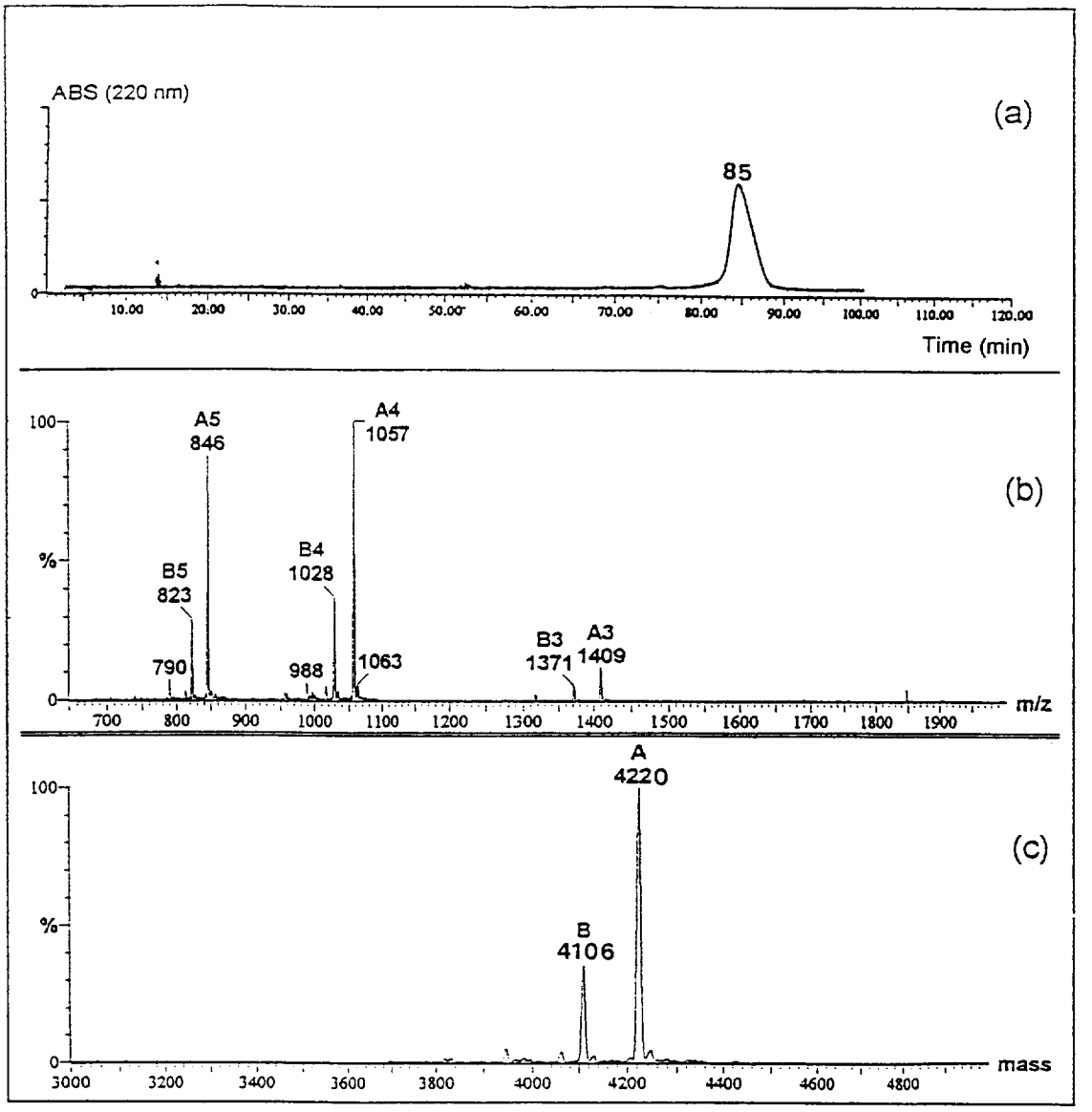

Fig. 7. (a) UV (220 nm) chromatogram obtained by preparative HPLC of [Leu^{31},Pro^{34}]NPY crude of synthesis, (b) charge-state distributions in the ES mass spectrum under the above peak and (c) measured relative molecular masses obtained by algorithmic transformation of the charge-state distributions.

paragine residues (M_r 114) from the amino acids sequence of [Leu^{31},Pro^{34}]NPY.

4. Conclusions

The combination of HPLC and electrospray ionization mass spectrometry provides an efficient analytical tool for fast separation and reliable identification of the major components present in a fairly complex medium, which are commonly encountered in the crudes of synthesis of large peptides and small proteins. The mildness and mass specific capabilities of ES mass spectrometry permitted the following deductions. First, unambiguous identification of the

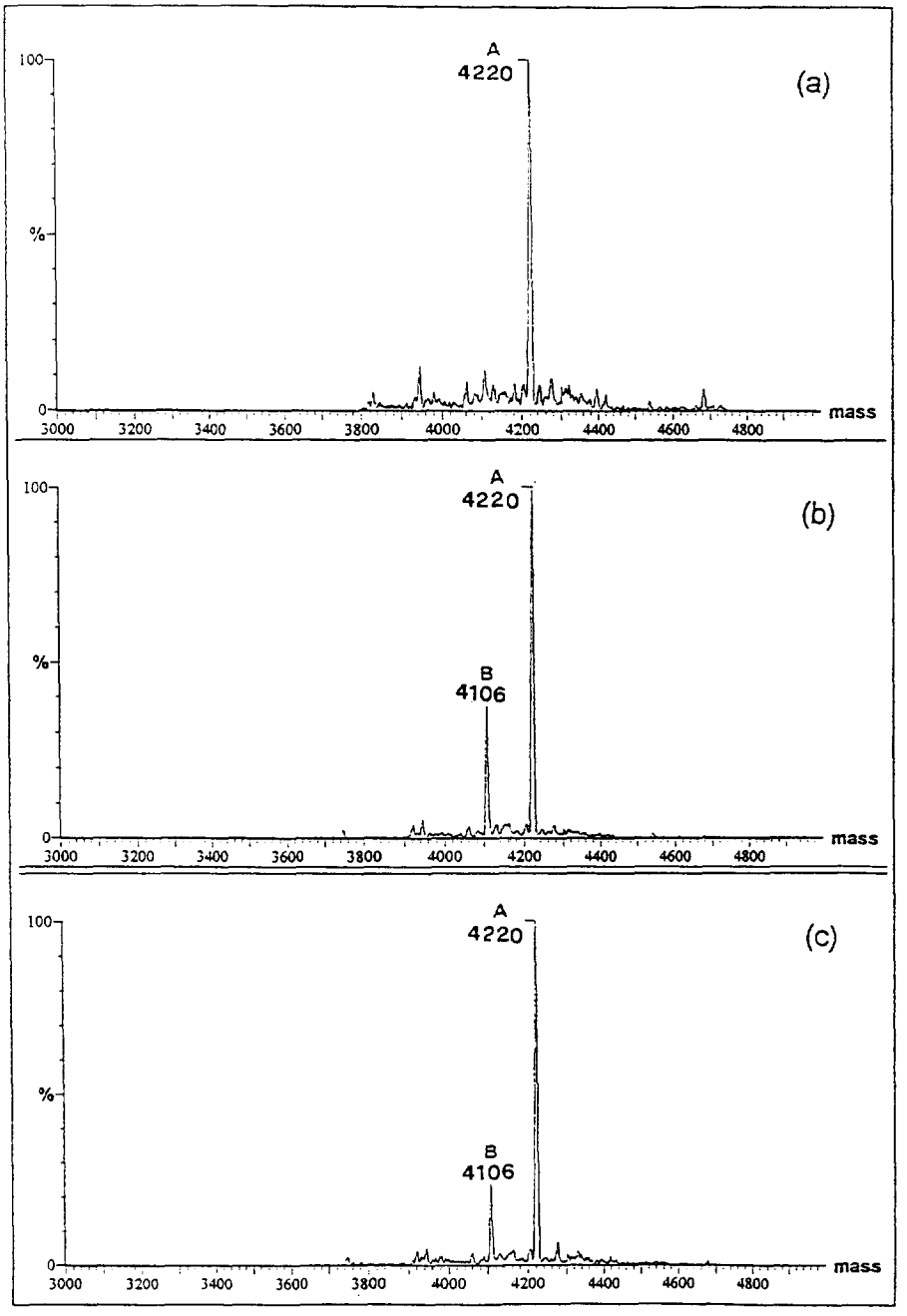

Fig. 8. Measured relative molecular masses obtained by algorithmic transformation of the charge-state distributions of the three fractions obtained by preparative HPLC slicing of the peak in Fig. 7a: (a) leading fraction, (b) central fraction, and (c) trailing fraction.

target peptides in the various stages of chromatographic separation and purification. Second, reliable molecular mass identification of various incomplete peptidic chains within the resulting crudes of synthesis and third, differentiation between two peptidic components within a single chromatographic peak which, because of their closely related amino acid sequence, could not be distinguished by HPLC alone.

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