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Journal of Chromatography A, 849 (1999) 161–173

JOURNAL OF CHROMATOGRAPHY A

Evaluation of volatile eluents and electrolytes for high-performance liquid chromatography–electrospray ionization mass spectrometry and capillary electrophoresis–electrospray ionization mass spectrometry of proteins
I. Liquid chromatography

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Received 19 February 1999; accepted 19 April 1999

Abstract

Proteins ranging in molecular mass from 14 000 to 80 000 were analyzed by reversed-phase high-performance liquid chromatography–electrospray mass spectrometry (RP-HPLC–ESI-MS) using 60×1.0 mm I.D. microbore-columns packed with 2.3 μm highly crosslinked, octadecylated poly(styrene–divinylbenzene) particles. Proteins were eluted at temperatures of 80–90°C with gradients of acetonitrile in 0.10–0.50% aqueous solutions of trifluoroacetic acid, formic acid or acetic acid. Substitution of trifluoroacetic acid, the most commonly used mobile phase additive for RP-HPLC, by formic acid resulted in a 35–160-fold improvement in analyte detectability at the cost of an only 32–104% increase in peak width at half height of eluting chromatographic peaks. The lower limits of detection for carbonic anhydrase (M_r 29 022.7) in full scan and selected ion monitoring mode were 37 and 2.3 fmol, respectively. Measurement of protein masses by RP-HPLC–ESI-MS was accurate and highly reproducible with maximum mass deviations of 0.025% and relative standard deviations of less than 0.011%. Calibration plots of peak area versus concentration allowed the reliable quantitation of proteins in a concentration range of 0.010–1.0 mg/ml. Finally, the optimized method was applied to the separation, identification and quantification of proteins in real samples such as commercial protein preparations, monoclonal antibody fragments, allergen extracts and whey drinks.

Keywords: Liquid chromatography–mass spectrometry; Electrospray ionization; Mobile phase composition; Poly(styrene–divinylbenzene) stationary phases; Proteins; Formic acid

1. Introduction

Separation, purification and characterization of proteins are of utmost importance for numerous applications in biological sciences. Reversed-phase high-performance liquid chromatography (RP-HPLC) has become the predominant separation technique for proteins largely because of its versatility, high resolving power, and preparative purification capability [1,2]. Stationary phases of the micropellicular configuration offering favorable mass transfer properties due to the absence of internal pore struc-

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00532-4

ture have been found to be particularly suitable for the high-speed analysis of proteins [3–6]. Nevertheless, chromatographic separation together with UV detection of proteins alone are not always sufficient to solve the problems posed by modern analytical biotechnology [7]. By measuring the molecular mass, however, many of the questions in protein identification and structural characterization can be simply answered [8]. During the past decade, mass spectrometry [9,10], especially electrospray ionization mass spectrometry (ESI-MS) [11,12], has emerged as one of the preferred methods to detect proteins and to determine accurately their molecular mass. Hence, the on-line conjugation of RP-HPLC and ESI-MS is a logical objective to improve the productivity of both techniques in collecting information about proteins contained in complex mixtures of biological origin [13,14].

The on-line coupling of HPLC to ESI-MS is relatively straightforward with modern chromatographic and mass spectrometric instrumentation [12]. Nevertheless, it is still important to consider solution chemistry to achieve the best separation together with optimal analyte detectability. This usually implies that HPLC separation conditions have to be adjusted to be suitable for ESI-MS analysis. The biggest problem that must be overcome for HPLC–ESI-MS is the incompatibility with ESI-MS of eluents commonly used for RP-HPLC with conventional UV detection. Typical mobile phase additives such as phosphoric acid [15], trifluoroacetic acid [16], detergents [15], and chaotropes [15] are not suitable for ESI-MS and must be replaced by alternative mobile phase additives. Salts and nonvolatile organic compounds get deposited in the ion source resulting in decreased ion transmission and eventual blockage of bores that conduct the ions to the mass analyzer. High concentrations of strong electrolytes such as trifluoroacetic acid inhibit the analyte MS-signal because of competitive ionization [17,18]. Moreover, trifluoroacetic acid is known to efficiently inhibit ionization of proteins because of ion pair formation [18,19]. The intensity and stability of the analyte signal in ESI-MS is a direct reflection of the stability of charged droplet formation, the efficiency and consistency of the droplet drying processes, and the efficiency of ion evaporation [19]. Because analyte properties relevant for the efficiency of ESI such as pK_a, diffusivity, and molecular size are determined by its chemical structure that cannot be chosen independently, optimization of ESI detection has to concentrate on the appropriate choice of operational parameters including solvent composition, type and concentration of additives, liquid flow-rate, solution conductivity, solution surface tension, solution pH, electrospray voltage, and sheath gas flow-rate. The current trend in RP-HPLC–ESI-MS of proteins is to use 0.1–1 mm I.D. columns packed with C_{18}-silica stationary phases in combination with hydro–organic eluents containing 0.1–1% volatile organic acids as mobile phase additives [13,14,20]. Although it has been shown that better ESI sensitivity is attainable with acetic acid [13], most applications employ trifluoroacetic acid as additive for RP-HPLC–ESI-MS despite its signal suppressing effects [13,20–22].

We recently investigated the applicability of micropellicular, octadecylated poly(styrene–divinylbenzene) particles (PS–DVB–C_{18}) for rapid chromatographic separation and mass spectrometric detection of proteins [23]. Trifluoroacetic acid as mobile phase additive for RP-HPLC enabled high-resolution separation of proteins with detection limits in the low picomole range. In this communication we report on the use of formic acid and acetic acid as alternative volatile mobile phase additives for HPLC–ESI-MS analysis of proteins. The influence of these additives on separation efficiency and detectability of proteins is studied and various real samples are used to evaluate the performance of HPLC–ESI-MS with micropellicular PS–DVB–C_{18} for the characterization of proteins.

2. Experimental

2.1. Chemicals and standards

Acetonitrile (HPLC gradient grade), and methanol (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Acetic acid (AcOH, analytical-reagent grade), formic acid (HCOOH, analytical-reagent grade), and trifluoroacetic acid (TFA, for UV spectroscopy) were from Fluka (Buchs, Switzerland). For preparation of the eluents, high-purity water (Epure, Barnstead, Newton, MA, USA) was

Table 1
Standard proteins used in this study

Protein	Abbreviation	Source
Carbonic anhydrase	CAH	Bovine erythrocytes
Cytochrome *c*	CYT	Horse heart
α-Lactalbumin	LALB	Bovine milk
β-Lactoglobulin A	LAC A	Bovine milk
β-Lactoglobulin B	LAC B	Bovine milk
Lysozyme	LYS	Chicken egg white
Myoglobin	MYO	Horse heart
Ribonuclease A	RIB	Bovine pancreas
Transferrin	TRA	Human
Trypsin	TRY	Bovine pancreas

used. All concentrations of mobile phase additives and organic modifiers are calculated as % (v/v). All standard proteins (Table 1) were obtained from Sigma (St. Louis, MO, USA). The monoclonal mouse anti-TNP antibody fragment was kindly provided by J. Varga (University of Innsbruck, Innsbruck, Austria). The mite allergen extract (*Dermatophagoides pteronyssinus*) was from Allergopharma (Reinbek, Germany).

2.2. High-performance liquid chromatography

The HPLC system consisted of a low-pressure gradient micropump (Model Rheos 4000, Flux, Karlskoga, Sweden), a degasser (Knauer, Berlin, Germany), a column oven (Model S4110, Sykam, Gilching, Germany), a microinjector (Model 7520, Rheodyne) with a 200-nl internal loop, a variable-wavelength detector (Model Linear UV–Vis 200, Linear Instruments, Fremont, CA, USA) with a 1.2-μl micro detector cell (used for the UV chromatogram in Fig. 3) or a 100-nl capillary detector cell (Grom, Herrenberg, Germany, all other UV chromatograms), and a personal computer-based data system (Chromeleon, vers. 4.12, Gynkotek, Germering, Germany). A flow of 50 μl/min was achieved through splitting a primary flow of 350 μl/min by means of a tee-piece and a 50 μm I.D. fused-silica restriction capillary. The separations were performed using 60×1 mm I.D. stainless steel columns (Grom) packed with octadecylated PS–DVB particles which have been synthesized according to the protocol described elsewhere [24]. Eluents and separation conditions are given in the figure captions.

2.3. Electrospray mass spectrometry and coupling to liquid chromatography

ESI-MS was performed on a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with the electrospray ion source. For LC–MS analysis with pneumatically-assisted ESI a spray voltage of 4–5 kV and a sheath gas pressure of 270 kPa were employed. The temperature of the heated transfer capillary was set to 200–210°C. Protein mass spectra were recorded by scanning the third quadrupole Q3, scan range and scan time are given in the figure captions. The mass spectrometer was calibrated using a solution of L-methionyl–arginyl–phenylalanyl–alanine (20 pmol/μl, Finnigan) and apomyoglobin from horse skeletal muscle (5.0 pmol/μl, Sigma) in methanol–water (50:50, v/v) containing 1.0% acetic acid to give the average molecular masses of the observed signals. The mass spectrometer was tuned for chromatographic conditions using a 2.0 μg/μl solution of cytochrome *c* added at a flow-rate of 1.0 μl/min to the column effluent (50 μl/min, 30% acetonitrile in 0.50% aqueous formic acid) by means of a tee-piece. Mass chromatograms and mass spectra were recorded on a DEC-Alpha workstation with the ICIS software, version 7.01 (Finnigan).

2.4. Quantitative analysis of proteins

Calibration plots of peak area versus concentration were obtained by linear regression analysis of the average of at least three area data points per concentration in a concentration range of 0.010–1.0 mg/ml. The peak areas of α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A were determined by selected ion monitoring of the four or five most abundant multiply charged signals of the proteins (m/z 1577.2, 1774.1, 2026.8, 2364.1 for α-lactalbumin, m/z 1306.9, 1407.6, 1525.1, 1663.1, 1829.3 for β-lactoglobulin B, and m/z 1313.5, 1413.4, 1531.7, 1671.2, 1838.0 for β-lactoglobulin A). A 1.5-ml aliquot of a whey drink (Lattella Molkegetränk Natur, Tirol Milch, Innsbruck, Austria) was centrifuged at 13 000 rpm for 5 min. The supernatant was diluted 1:5 with bidistilled water and a 200-nl sample was injected directly onto the

column. The concentration of the proteins in the whey drink was determined from the average peak areas of three injections.

3. Results and discussion

3.1. Influence of mobile phase additive on analyte detectability

There are three major reasons for the usefulness of acids as mobile phase additives in RP-HPLC of proteins. (1) When silica-based stationary phases are employed, protonation of residual silanol groups on the support surface at low pH diminishes unwanted ionic interactions with the basic amino acid residues of the proteins. (2) Small organic acids form ion pairs with these basic sites and consequently increase the hydrophobic character of a protein. (3) Acidic solvents assist in the process of unfolding and denaturing the three-dimensional structure of a protein resulting in more homogenous species that elute as sharper and more symmetrical peaks [25]. Trifluoroacetic acid is the most commonly used acid because it is volatile (unlike phosphoric acid), it does not corrode stainless steel parts of the HPLC apparatus (unlike hydrochloric acid), and it is UV transparent (unlike acetic or formic acid). A chromatogram of eight standard proteins separated on a 60×1.0 mm I.D. column packed with 2.3 μm PS–DVB–C_{18} particles using 0.20% trifluoroacetic acid as additive is depicted in Fig. 1. Applying a gradient of 24.0–53.3% acetonitrile in 10 min all proteins were separated to baseline within 8 min with peak widths at half height between 4.6 s (ribonuclease A) and 12.6 s (α-lactalbumin). Injection of 170–405 fmol proteins permitted their UV detection at 215 nm with signal-to-noise ratios between 7:1 (lysozyme) and 3:1 (trypsin). However, with 0.20% trifluoroacetic acid the concentration of eluted proteins was too low to yield signals in the reconstructed ion-chromatogram or to enable the acquisition of ESI mass spectra because of the strong signal suppressing effect of trifluoroacetic acid.

To improve the detectability of proteins by on-line HPLC–ESI-MS, trifluoroacetic acid has to be substituted by more polar and less conductive additives such as formic acid or acetic acid. Banks et al.

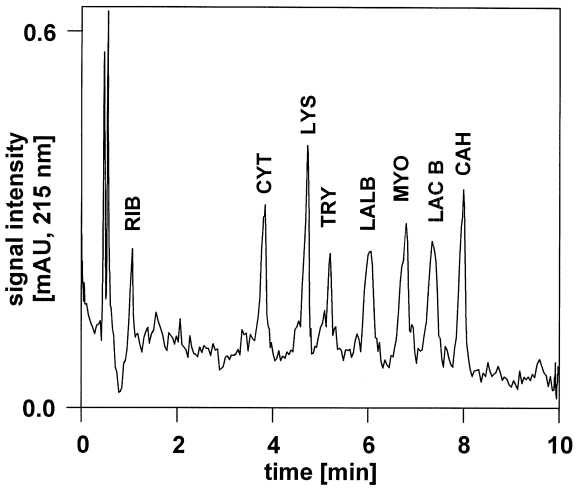

Fig. 1. Separation and UV detection of eight proteins with 0.20% trifluoroacetic acid as mobile phase additive. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.20% trifluoroacetic acid, acetonitrile–water (15:85), (B) 0.20% trifluoroacetic acid, acetonitrile–water (60:40); linear gradient, 20–85% B in 10 min; flow-rate, 50 μl/min; temperature, 90°C; detection, UV, 215 nm; sample, 360 fmol RIB, 405 fmol CYT, 350 fmol LYS, 215 fmol TRY, 350 fmol LALB, 300 fmol MYO, 260 fmol LAC B, 170 fmol CAH.

reported a more than 20-fold increase in ESI-MS signal intensity of a cytochrome *c* sample upon replacing 0.10% trifluoroacetic acid in the spray solvent with 1.0% acetic acid [13]. We observed a total ion current of $2.3 \cdot 10^7$ counts in the ESI-MS spectrum of lysozyme acquired by direct infusion of a 0.20 mg/ml solution in acetonitrile–water (50:50) containing 0.10% (v/v) formic acid compared to $3.5 \cdot 10^6$ counts with a solution of the same concentration acetonitrile–water (50:50) containing 0.10% (v/v) trifluoroacetic acid representing a more than 6.5-fold increase in signal intensity with formic acid. The charge states with 0.10% trifluoroacetic ranged from 8+ (m/z 1789.3) to 6+ (m/z 2385.4) with a maximum at 7+ (m/z 2044.7). With 0.10% formic acid charge states from 12+ (m/z 1193.2) to 6+ (m/z 2385.4) with a maximum at 9+ (m/z 1590.6) were observed. The shift in charge state distribution to higher charge states, i.e., lower m/z values, with 0.10% formic acid is advantageous particularly for HPLC–ESI-MS of proteins because the m/z of even large proteins falls into the mass range of the mass spectrometer. The effect of formic

acid concentration on ESI-MS signal intensity was investigated by direct infusion of 0.10 mg/ml lysozyme solutions containing 0.050–0.50% formic acid. From Fig. 2 it can be deduced that the concentration of formic acid had only a minor effect on signal intensity. The maximum and minimum signal intensities were $14.3 \cdot 10^6$ and $8.5 \cdot 10^6$ counts, respectively, in the investigated concentration range. The increase in signal intensity after increasing the formic acid concentration from 0.050% to 0.10% can be explained by more efficient protonation of the proteins at higher hydronium ion concentration whereas the decrease in signal intensity at formic acid concentrations higher than 0.10% may be rationalized by competition of analyte ions and hydronium ions in the conversion process from solution to gas phase ions [26].

Formic acid has been demonstrated to be an alternative to trifluoroacetic acid as additive for RP-HPLC of proteins [27]. Because of the strong absorptivity of formic acid in the low UV-range proteins cannot be monitored at 215 nm with eluents containing formic acid. Therefore, 280 nm has to be used as detection wavelength resulting in significantly decreased detection sensitivity for proteins. Fig. 3 compares the chromatographic signals obtained from 1.5 pmol injections each of cytochrome *c*, lysozyme and carbonic anhydrase with simultaneous UV and ESI-MS detection using a gradient of acetonitrile in 0.50% aqueous formic acid to elute the proteins. It can be seen in the UV chromatogram that only lysozyme is detected at 280 nm, whereas cytochrome *c* and carbonic anhydrase show very small peaks that are hardly distinguishable from baseline noise (Fig. 3a). ESI-MS, on the other hand, allowed the detection of all three proteins with similar detection sensitivity (Fig. 3b). Moreover, eluted proteins were readily identified on the basis of their molecular masses calculated from the extracted ESI mass spectra. In contrast to UV detection, where a baseline drift still exists at 280 nm, the baseline is

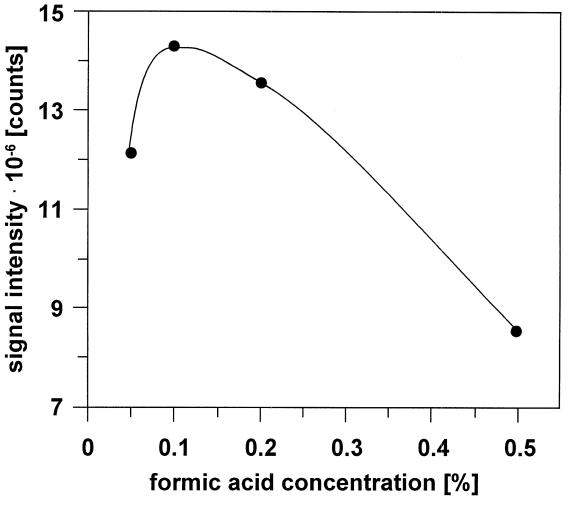

Fig. 2. Influence of formic acid concentration on ESI-MS signal intensity. Direct infusion of 0.10 mg/ml lysozyme in acetonitrile–water (50:50) containing 0.050–0.50% formic acid. Flow-rate, 3.0 μl/min; scan, 1000–2400 u in 1.0 s,

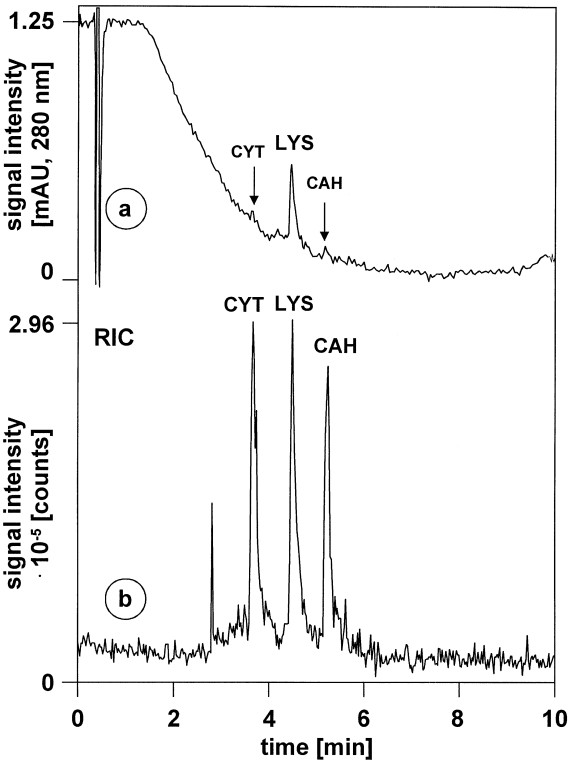

Fig. 3. Comparison of (a) UV and (b) ESI-MS detection with 0.50% formic acid as mobile phase additive. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid, acetonitrile–water (60:40); linear gradient, 0–100% B in 10 min; flow-rate, 50 μl/min; temperature, 80°C; detection, (a) UV, 215 nm, (b) ESI-MS, electrospray voltage, 5.0 kV; scan range, 600–2100 u in 1.5 s; sample, 1.6 pmol CYT, 1.4 pmol LYS, 1.2 pmol MYO.

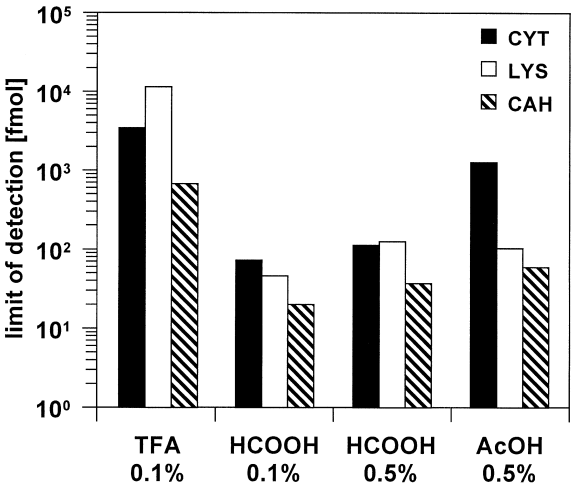

Fig. 4. Influence of mobile phase additive on limits of detection of proteins. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); linear gradient, 19.5–60% acetonitrile in 0.10% aqueous trifluoroacetic acid, 0.10% aqueous formic acid, 0.50% aqueous formic acid, and 0.50% aqueous acetic acid, respectively, in 10 min; flow-rate, 30 μl/min; temperature, 80°C; detection, ESI-MS, electrospray voltage, 4.5 kV; scan range, 500–2500 u in 2.0 s; sample, CYT, LYS, CAH.

not affected by the acetonitrile gradient in aqueous formic acid with ESI-MS detection.

The influence of mobile phase additive on detectability of proteins by HPLC–ESI-MS is summarized in Fig. 4. The lower limits of detection for cytochrome *c*, lysozyme, and carbonic anhydrase in full scan mode (500–2500 u in 2 s) were determined at a signal-to-noise ratio of 3 with eluents containing 0.10% trifluoroacetic acid, 0.10% formic acid, 0.50% formic acid, or 0.50% acetic acid. With 0.10% trifluoroacetic acid, limits of detection were in the 0.70–11 pmol range, whereas 0.10% formic acid allowed the detection of 20–70 fmol protein. This represents an improvement in detectability of proteins by a factor of 35–160. The limits of detection with 0.50% formic acid and 0.50% acetic acid were quite similar with values of 37–130 fmol. An exception was cytochrome *c* where the limit of detection with 0.50% acetic acid was more than one order of magnitude higher than with 0.50% formic acid. The detectability of proteins could be further improved by a factor of 10–15 by operating the mass spectrometer in the selected ion monitoring mode. With 0.50% formic acid as additive, observation of the four most abundant multiply charged signals of cytochrome *c* (*m*/*z* 728.0, 1374.3, 1545.9, 1766.6), lysozyme (*m*/*z* 1431.6, 1590.6, 1789.3, 2044.7), and carbonic anhydrase (*m*/*z* 807.2, 830.2, 854.6, 880.5) allowed their detection with a lower limit of detection of 10, 8.5 and 2.3 fmol, respectively.

3.2. Influence of mobile phase additive on column efficiency

To quantitate the effect of the mobile phase additive on column efficiency the peak widths at half height were measured with eluents containing 0.10% trifluoroacetic acid, 0.10% formic acid, 0.50% formic acid, and 0.50% acetic acid under otherwise identical chromatographic conditions. With 0.50% formic acid, for example, the values for the peak widths at half height in the UV chromatograms were between 13.0 and 15.5 s (Table 2). These values are 69–104% larger than those obtained with 0.10% tri-

Table 2
Peak widths at half height w_h observed with trifluoroacetic acid, formic acid and acetic acid as mobile phase additives using UV and full-scan ESI-MS detection

Additive	w_h[a] (s)					
	UV[b]			MS[c]		
	CYT	LYS	CAH	CYT	LYS	CAH
Trifluoroacetic acid, 0.1%	7.6	7.7	8.0	8.0	8.0	12.0
Formic acid, 0.1%	19.3	16.5	21.9	26.2	21.2	14.0
Formic acid, 0.5%	15.5	13.0	14.5	18.5	17.5	15.5
Acetic acid, 0.5%	27.6	29.4	24.0	30.0	36.7	32.5

[a] Gradient, 19.5–60% acetonitrile in 10 min, 30 μl/min, 80°C.
[b] Wavelength, 215 nm.
[c] Scan, 500–2500 u in 2 s.

fluoroacetic acid (Table 2). A decrease in formic acid concentration from 0.50% to 0.10% entailed an increase in peak widths at half height of 25–51% (Table 2). Of the three different additives tested, acetic acid resulted in most pronounced band broadening with peak widths at half height in the range of 24–29 s (Table 2). The peak width measured in a chromatogram not only depends on chromatographic column efficiency but also on the rate of generating and acquiring data by the utilized detector and data system. With UV detection, where data acquisition rates of 5–10 data points per second are generally used, the effect of acquisition rate on band width is insignificant. However, with ESI-MS detection using scanning mass analyzers such as quadrupoles or ion-traps, scan times of at least 1.0 s per 1000 u corresponding to a data acquisition rates of 0.5 or less points per second for a 500–2500 u mass range have to be applied in order to allow sufficient sampling time for each m/z value. Therefore, the observed chromatographic peak width with ESI-MS detection also depends on the data acquisition rate adjusted at the mass spectrometer [7]. Increasing the data acquisition rate, i.e., decreasing the scan time, results in narrower peaks and more accurate definition of a chromatographic peak profile at the cost of poorer signal-to-noise ratio in the acquired mass spectra. The effect of scanning ESI-MS detection on band broadening can be deduced from a comparison of the peak widths at half height measured with simultaneous UV and ESI-MS detection. Compared to the UV chromatograms, an average increase in peak widths at half height of 17% was observed in the reconstructed ion chromatograms which were recorded with a scan time of 2.0 s and a scan range of 500–2500 u (Table 2).

3.3. Separation and identification of proteins under optimized chromatographic and mass spectrometric conditions

From the results discussed in the two preceding sections it follows that substitution of trifluoroacetic acids by formic acid as mobile phase additive permits the chromatographic separation and mass spectrometric detection of proteins with significantly enhanced detectability at the cost of only a moderate loss in separation efficiency. To demonstrate the performance of the RP-HPLC–ESI-MS system with 0.50% formic acid as mobile phase additive, the mixture of eight proteins (see Fig. 1) containing 0.7–1.6 pmol per component was chromatographed on the PS–DVB–C_{18} column. For comparability of the two chromatograms in Figs. 1 and 5 and in order to obtain roughly the same retention times in both chromatograms, the gradient was ramped from 19.5–48.8% in 10 min with 0.50% formic acid as additive whereas the gradient steepness was kept the same in both chromatograms. Due to the combined effects of formic acid as additive and scanning data acquisition, the average peak width at half height with 0.50% formic acid was 32% larger than that with 0.20% trifluoroacetic acid (11.2 s in Fig. 5 versus 8.5 s in Fig. 1). Moreover, changes in chromatographic selectivity were observed upon exchange of trifluoroacetic acid with formic acid. Myoglobin, formerly eluting between α-lactalbumin and β-lactoglobulin B, eluted between trypsin and α-lactalbumin with formic acid. The difference in retention time between β-lactoglobulin B and carbonic anhydrase was 39.6 s with trifluoroacetic acid and only 20.5 s with formic

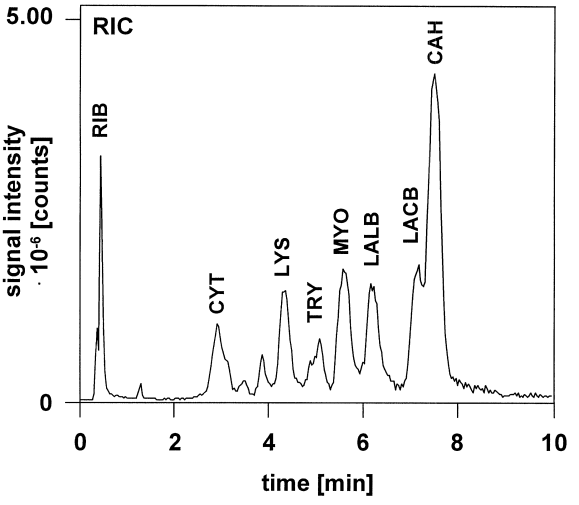

Fig. 5. Separation and ESI-MS detection of eight proteins with 0.50% formic acid as mobile phase additive. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid acetonitrile–water (60:40); linear gradient, 10–75% B in 10 min; flow-rate, 50 μl/min; temperature, 90°C; detection, ESI-MS, electrospray voltage, 5.0 kV; scan range, 1000–2500 u in 2.0 s; sample, 1.6 pmol RIB, 1.6 pmol CYT, 1.4 pmol LYS, 0.90 pmol TRY, 1.2 pmol MYO, 1.4 pmol LALB, 1.1 pmol LAC B, 0.70 pmol CAH.

acid resulting in poor separation of the two proteins with the latter additive. Nevertheless, the high specificity and information content of mass spectrometry more than compensated for the decrease in separation efficiency and enabled the mass analysis even of simple protein mixtures eluting as unresolved peaks such as β-lactoglobulin B and carbonic anhydrase. Furthermore, all proteins yielded mass spectra of high quality from which the molecular masses could be calculated with excellent accuracy. Table 3 compares some of the measured molecular masses with the theoretical masses that have been calculated from the amino acid sequence. The high accuracy of mass determination by RP-HPLC–ESI-MS is demonstrated by relative deviations of the measured masses from the theoretical masses ranging from 0.0042 to 0.025%.

3.4. Reproducibility and quantitation

Retention times and peak areas of proteins were reproducible with relative standard deviations of 1% and 5%, respectively, using UV detection at 215 nm and trifluoroacetic acid as additive ($n=12$) [23]. The reproducibility of retention times and peak areas with ESI-MS detection and formic acid as additive in full-scan mode was checked by four consecutive injections of 3.0 ng each of cytochrome *c*, lysozyme and carbonic anhydrase. Although the peak profiles with ESI-MS detection are not as well defined as with UV detection resulting in slight shifts of the peak maxima, the relative standard deviations in retention times measured with ESI-MS detection were only 0.97% for cytochrome *c*, 0.59% for lysozyme, and 0.57% for carbonic anhydrase ($n=4$). The peak areas of four consecutive injections were reproducible with relative standard deviations of 6.7%, 3.4% and 6.8%, respectively. These values were not significantly different from the values with UV detection (*F*-test at 95% level of significance) and make ESI-MS detection highly suitable for qualitative and quantitative analysis. The plots of peak area versus concentration for β-lactoglobulin B, β-lactoglobulin A, and α-lactalbumin measured by selected-ion monitoring of the five most abundant signals of the proteins showed good linearity linear over at least two-orders of magnitude (0.010–1.0 mg/ml) with the equations of the linear regression line being $y=9.90\cdot10^{9}x+7.61\cdot10^{7}$, $R^2=0.9985$ for α-lactalbumin, $y=9.38\cdot10^{8}x+4.86\cdot10^{7}$, $R^2=0.9983$ for β-lactoglobulin B, and $y=7.76\cdot10^{8}x+4.97\cdot10^{7}$, $R^2=0.9974$ for β-lactoglobulin A (*y*, peak area in counts; *x*, protein concentration in mg/ml).

The reproducibility and accuracy of mass determination by RP-HPLC–ESI-MS was tested by 6-pmol injections of cytochrome *c* (500–1500 u in 1.5 s; the molecular mass was calculated by deconvolution of the sum of nine scans; chromatographic conditions as in Fig. 5). The run-to-run reproducibility and accuracy of the molecular mass determined from five analyses on two consecutive days were characterized by a relative standard deviation of 0.0078% and a relative mass deviation of 0.0094%, respectively. The day-to-day reproducibility and accuracy was calculated from 11 measurements spread over 16 days. The value for the relative standard deviation was 0.011% with a relative mass deviation of 0.0042%.

3.5. Application of RP-HPLC to on-line sample preparation prior to mass analysis of proteins

The molecular mass of a protein is an important parameter in the biochemical characterization of a

Table 3
Molecular masses of proteins determined by HPLC–ESI-MS

Protein	Theoretical mass	Measured mass±SD[a]	Relative deviation[b] (%)
CYT	12 359.3	12 357.9±2.6	0.011
LYS	14 306.2	14 306.8±2.0	0.0042
MYO	16 951.5	16 949.5±2.9	0.012
LACB	18 277.3	18 274.6±4.3	0.015
CAH	29 022.7	29 030.0±5.0	0.025

[a] SD=Standard deviation calculated by the deconvolution program from the series of mutiply-charged ions.
[b] Deviation from the theoretical mass calculated from the sequence.

protein. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is a universal technique in protein molecular mass determination. Although biological samples and even purified commercial preparations of proteins often contain relatively high concentrations of non-volatile buffers, salts and detergents, proteins usually do not have to be purified before analysis because SDS–PAGE is a highly efficient separation technique. However, the accuracy of mass determination by SDS–PAGE ranging from a few percent to about 30% (depending on the three-dimensional structure of the analyzed protein [10]) is relatively low compared to the accuracy of 0.01% attainable with ESI-MS. Impurities and matrix components in protein samples can interfere with ESI and severely impair spectral quality, hence, purification prior to mass determination by ESI-MS in usually inevitable. Off-line purification techniques such as solid-phase extraction, ion-exchange, ultrafiltration and size-exclusion chromatography are effective in removing interferences but they are labor intensive and often result in low analyte recovery. After isolation and purification, the analysis of proteins by ESI-MS has traditionally been achieved by direct infusion of an acidified protein solution by means of a syringe pump. This method provides fast analysis and allows signal averaging to improve the signal-to-noise ratio for weak signals. Fig. 6a shows the resulting mass spectrum from an infusion of 19 pmol of a commercial transferrin preparation without prior sample purification. It is apparent that impurities present in the sample preparation are efficiently suppressing the analyte signals resulting in a spectrum void of any useful mass information. The chemical background can be efficiently eliminated by using on-line RP-HPLC separation prior to mass analysis. Fast on-line purification of 5 pmol transferrin was accomplished by elution with a gradient of 19.5–48.8% acetonitrile in 0.5% aqueous formic acid in 5 min. The dramatic increase in spectral quality upon on-line cleanup by RP-HPLC is illustrated by the mass spectrum depicted in Fig. 6b which was obtained by averaging 10 scans under the peak eluting at 5.2 min and gave a molecular mass of 79 601 for human transferrin.

Another example of on-line RP-HPLC purification is illustrated in Fig. 7. A mouse monoclonal anti-TNP IgE antibody fragment [28], which has been

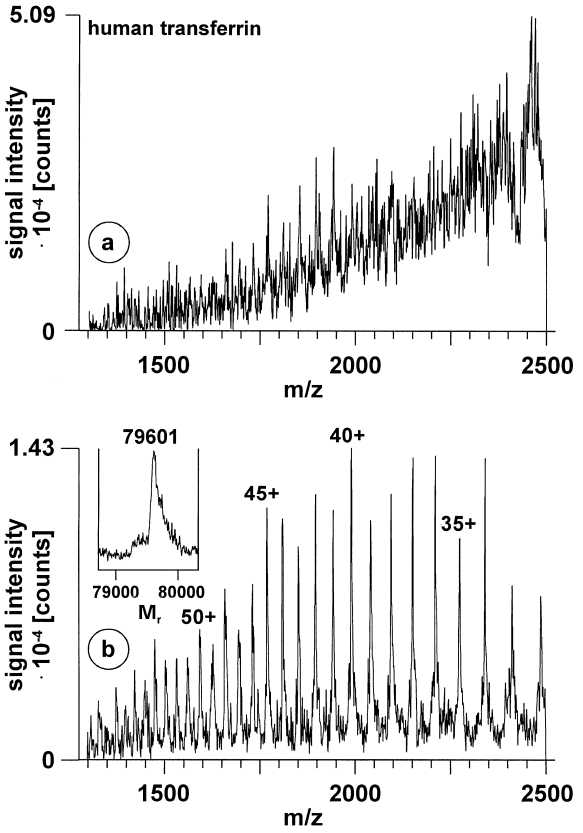

Fig. 6. Comparison of direct infusion–ESI-MS and RP-HPLC–ESI-MS for the determination of the exact molecular mass of an M_r 79 000 protein. (a) Mass spectrum obtained by direct infusion of a 1.0 mg/ml solution of transferrin in acetonitrile–water (50:50) containing 0.50% formic acid. Flow-rate, 3.0 μl/min; acquisition time, 30 s; sample, 19 nmol transferrin (analytical-reagent grade). (b) Mass spectrum obtained by RP-HPLC–ESI-MS analysis. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid, acetonitrile–water (60:40); linear gradient, 10–75% B in 10 min, 75–100% B in 5 min; flow-rate, 50 μl/min; temperature, 90°C; sample, 5.0 pmol transferrin (analytical-reagent grade); in (a) and (b): electrospray voltage, 4.5 kV; scan range, 1300–2500 u in 2.0 s.

prepared by gene cloning technology, was purified by RP-HPLC and mass analyzed. Considerable amounts of buffer components, detergents and a protein byproduct present in this sample made direct mass spectrometry impossible. During the chromatographic separation with a gradient of 15–46.5% acetonitrile in 0.50% formic acid in 7.0 min, the byproduct and the main product eluted at 4.4 min

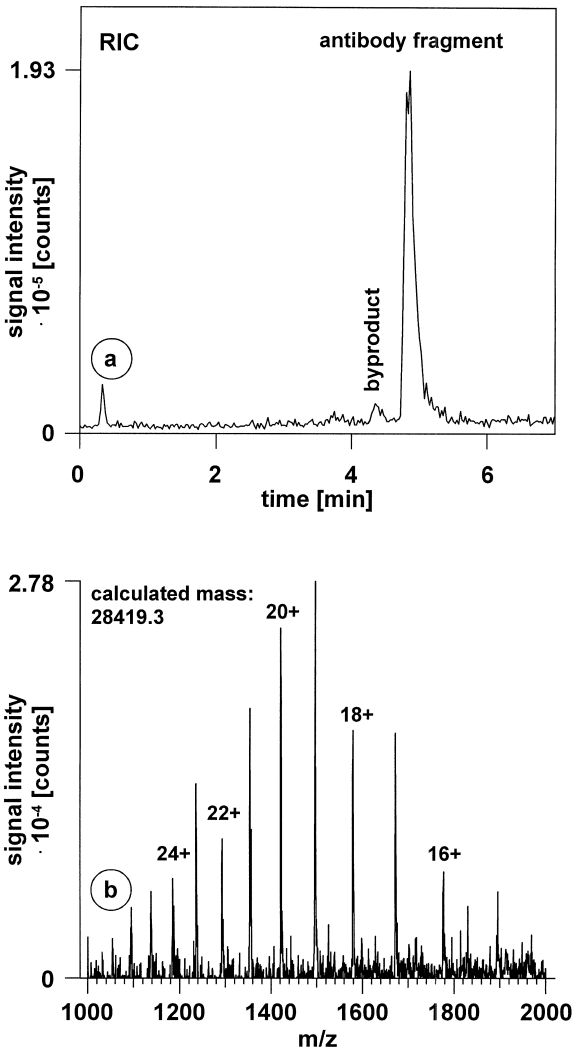

Fig. 7. Characterization of a monoclonal mouse anti-TNP IgE antibody fragment by RP-HPLC–ESI-MS. Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid, acetonitrile–water (60:40); linear gradient, 0–70% B in 10 min; flow-rate, 50 μl/min; temperature, 80°C; electrospray voltage, 5.0 kV; scan range, 1000–2000 u in 1.5 s. (a) Reconstructed ion chromatogram, (b) average of 7 mass spectra under the peak at 4.9 min.

and 4.8 min, respectively (Fig. 7a). Analysis of the extracted mass spectra gave molecular masses of 15 505.3 and 28 419.3 for the protein impurity (spectrum not shown) and the antibody fragment (Fig. 7b), respectively. This preparation was used to characterize the day-to-day reproducibility of mass determination by RP-HPLC–ESI-MS with a real sample. Five measurements of the molecular mass spread over nine days gave an average molecular mass of 28 420.2 with a relative standard deviation of only 0.0023% which clearly demonstrates the high reproducibility of mass determination by RP-HPLC–ESI-MS.

3.6. Application of RP-HPLC–ESI-MS to the analysis of proteins in complex matrices

RP-HPLC can not only be used to purify protein preparations that contain only one or a few target compounds but also to separate proteins in rather complex mixtures of biological origin. Fig. 8a shows the reconstructed ion chromatogram of a crude allergen extract from house dust mite. Although the chromatogram is very complex and separation is incomplete, extraction of mass spectra from selected sections of the chromatogram enabled the direct identification of at least one major protein component in the extract. Averaging 26 scans at 3.93–4.36 min yielded the mass spectrum depicted in Fig. 8b and allowed the calculation of a molecular mass of 14 103.6 for this protein. Fig. 8c also demonstrates the limits of RP-HPLC as separation method for highly complex samples. The fine structure of the peak eluting between 4.5 and 8.5 min indicates that several substances elute from the column with only partial resolution. The complexity of the effluate is also reflected in the extracted mass spectrum where the multitude of unresolved signals in the range of 700–1700 u did not allow successful mass assignments. To be able to analyze the proteins eluting in this section of the chromatogram, more selective sample preparation methods or multidimensional separation are necessary.

3.7. Application of RP-HPLC–ESI-MS to the qualitative and quantitative analysis of whey proteins

Bovine milk contains 3.0–3.5% of protein, of which 80% consists of caseins, insoluble at pH 4.6. The remaining 20% of so called whey proteins are classified as milk proteins that are soluble at pH 4.6 and include α-lactalbumin, β-lactoglobulin A and B,

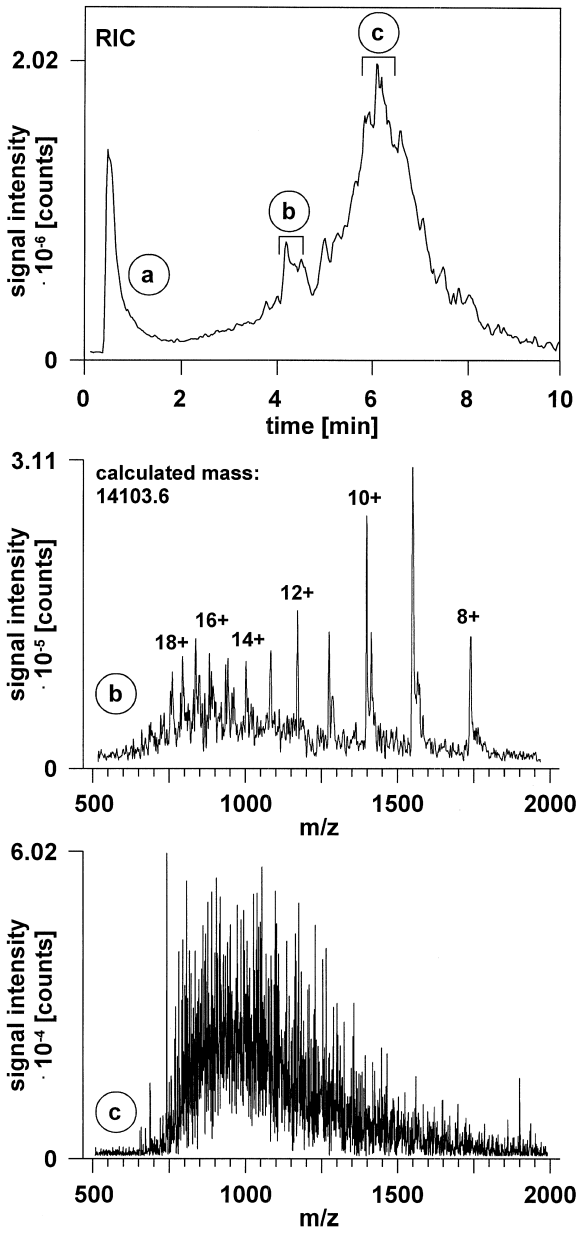

Fig. 8. RP-HPLC–ESI-MS analysis of proteins in an extract of house dust mite (*Dermatophagoides pteronyssinus*). Column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid, acetonitrile–water (60:40); linear gradient, 10–75% B in 10 min, 75–100% B in 5 min; flow-rate, 50 μl/min; temperature, 80°C; electrospray voltage, 5.0 kV; scan range, 500–2000 u in 1.5 s; sample, 2.4 pg house dust mite extract in 200 nl water. (a) Reconstructed ion chromatogram, (b) and (c) averaged mass spectra at 3.9–4.8 min and 5.8–6.5 min.

bovine serum albumin and immunoglobulins. The analysis of whey proteins has traditionally been performed by PAGE, HPLC and capillary electrophoresis [29,30]. Leonil et al. analyzed milk proteins by RP-HPLC–ESI-MS on a 150×2.1 mm I.D. Zorbax 300 SB RP-8 column using 0.10% trifluoroacetic acid as additive [31]. Fig. 9a shows the separation of whey proteins in a commercial whey drink by microbore RP-HPLC on a PS–DVB–C_{18} column using formic acid as mobile phase additive. α-Lactalbumin eluted at 6.2 min and was readily identified by its molecular mass of 14 184.6 from the mass spectrum depicted in Fig. 9b. Whereas β-lactoglobulins A and B could be separated to baseline on alkylated PS–DVB with 0.20% trifluoroacetic acid as additive (see Fig. 6 in Ref. [23]) the two proteins coeluted when the eluent contained 0.50% formic acid. Nevertheless, because of the ability of ESI-MS to analyze simple protein mixtures, both proteins were unequivocally identified by the extracted mass spectrum (Fig. 9c) yielding molecular masses of 18 283.4 and 18 369.7, respectively. Moreover, selected ion monitoring allowed the quantitative analysis of all three proteins without inference from other coeluting species. Using the calibration curves mentioned in Section 3.4 and the average peak area from three injections of the whey drink sample, the measured concentrations of α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A in the whey drink were 0.684, 1.839 and 1.599 mg/ml, respectively.

4. Conclusions

The potential of RP-HPLC–ESI-MS with different organic acids as mobile phase additives for qualitative and quantitative analysis of proteins in a variety of samples has been demonstrated. Formic acid was shown to be a real alternative to trifluoroacetic acid as mobile phase additive, which is known to efficiently suppress ion formation in ESI-MS resulting in poor detectability of proteins with the latter additive. Although separations with formic acid as mobile phase additive were less efficient than separations with trifluoroacetic acid, the high selectivity and sensitivity of ESI-MS detection allowed

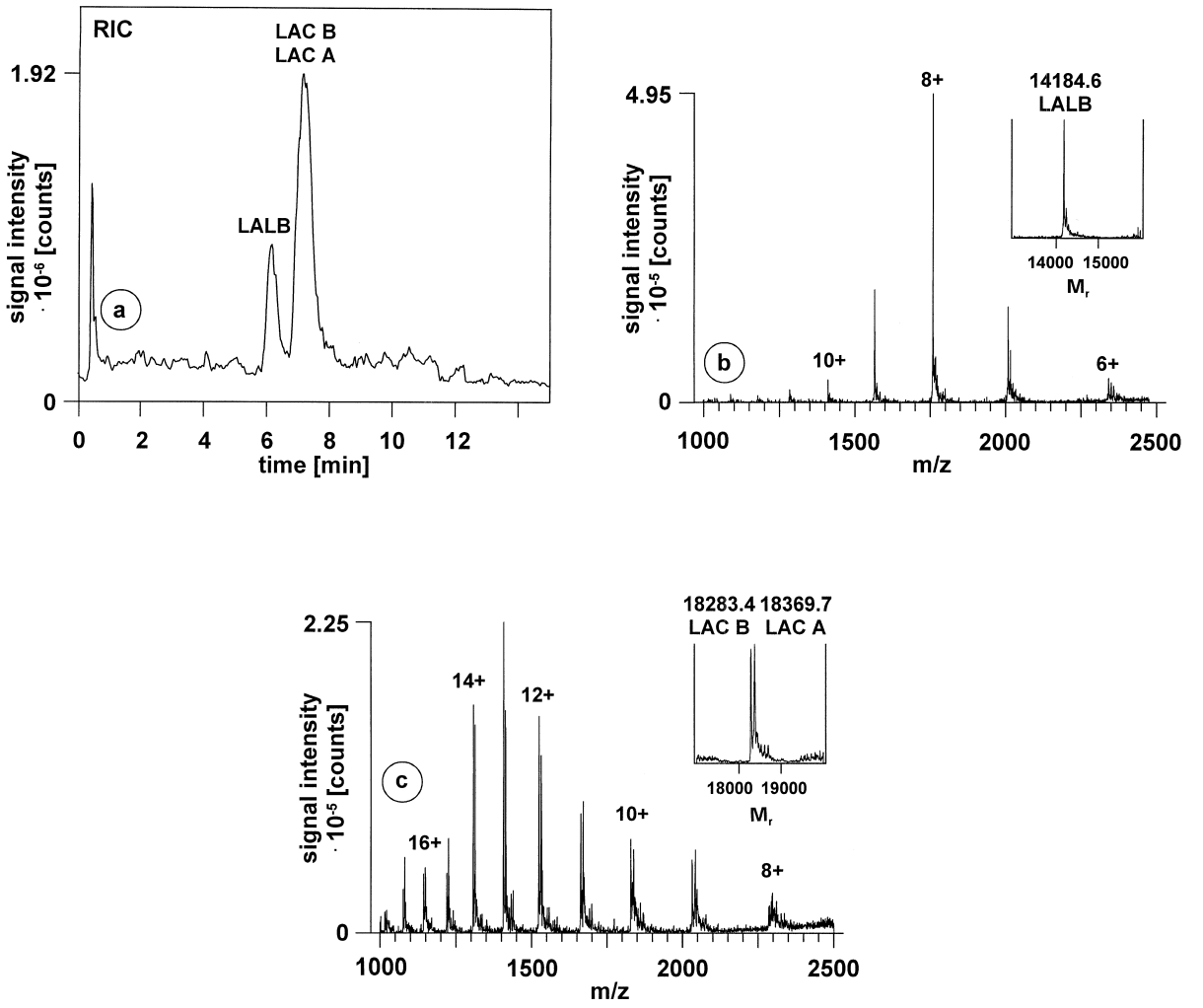

Fig. 9. Separation, identification and quantitation of proteins in a whey drink by RP-HPLC–ESI-MS. (a) Reconstructed ion chromatogram: column, PS–DVB–C_{18} (2.3 μm, 60×1.0 mm I.D.); mobile phase, (A) 0.50% formic acid, acetonitrile–water (15:85), (B) 0.50% formic acid, acetonitrile–water (60:40); linear gradient, 10–75% B in 10 min, 75–100% B in 5 min; flow-rate, 50 μl/min; temperature, 90°C; electrospray voltage, 4.5 kV; scan range, 1000–2500 u in 1.5 s; sample, 200 nl whey drink, 1:5 diluted with water. (b) Extracted ESI-spectrum of α-lactalbumin; inset, deconvoluted mass spectrum. (c) Extracted ESI-spectrum of β-lactoglobulin B and A; inset, deconvoluted mass spectrum.

the unambiguous identification and quantitation even of coeluting proteins with a limit of detection in the low femtomole range.

Acknowledgements

The authors are grateful to Janos Varga (Leopold-Franzens University, Innsbruck) for providing the monoclonal mouse anti-TNP antibody fragment. Part of this work was supported by a grant from the Austrian Science Fund (P13442).

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