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Monolithic capillary columns for liquid chromatography–electrospray ionization mass spectrometry in proteomic and genomic research

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Abstract

Peptides, proteins, single-stranded oligonucleotides, and double-stranded DNA fragments were separated with high resolution in micropellicular, monolithic capillary columns prepared by in situ radical copolymerization of styrene and divinylbenzene. Miniaturized chromatography both in the reversed-phase and the ion-pair reversed-phase mode could be realized in the same capillary column because of the nonpolar character of the poly-(styrene/divinylbenzene) stationary phase. The high chromatographic performance of the monolithic stationary phase facilitated the generation of peak capacities for the biopolymers in the range of 50–140 within 10 min under gradient elution conditions. Employing volatile mobile phase components, separations in the two chromatographic separation modes were on-line hyphenated to electrospray ionization (tandem) mass spectrometry, which yielded intact accurate molecular masses as well as sequence information derived from collision-induced fragmentation. The inaccuracy of mass determination in a quadrupole ion trap mass spectrometer was in the range of 0.01–0.02% for proteins up to a molecular mass of 20 000, and 0.02–0.12% for DNA fragments up to a molecular mass of 310 000. High-performance liquid chromatography–electrospray ionization mass spectrometry utilizing monolithic capillary columns was applied to the identification of proteins by peptide mass fingerprinting, tandem mass spectrometric sequencing, or intact molecular mass determination, as well as to the accurate sizing of double-stranded DNA fragments ranging in size from 50 to 500 base pairs, and to the detection of sequence variations in DNA fragments amplified by the polymerase chain reaction.

Keywords: Monolithic columns; Proteomics; Genomics; Proteins; Peptides; Nucleic acids

1. Introduction

The introduction of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) as soft ionization techniques for biomole-

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cules initiated the broad application of mass spectrometry (MS) to the investigation of biological problems [1]. Moreover, advances in mass spectrometric methodology enabled the intact molecular mass determination of biological macromolecules far beyond several hundred thousand Da [2,3], as well as the study of supramolecular complexes [4,5]. Peptide mass fingerprinting [6] and sequencing [7,8] by MS and tandem MS, respectively, have laid the groundwork for modern approaches to whole proteome analysis [9–11]. Likewise, MS is playing a constantly growing role in nucleic acid characterization, ranging from the characterization of synthetic DNA and RNA [12–14], the sequencing of oligonucleotides [15,16] for the detection of mutations [17,18].

In view of the high complexity of mixtures of biological origin and the fact that the mixtures are frequently too complex to be handled simultaneously by MS, pre-MS separation technology becomes the bottleneck for the successful study of biomolecules. Although single-stage chromatographic or electrophoretic separation systems offer high selectivity and resolving power for biopolymers [19,20], the total peak capacity may not suffice to separate all the components of interest contained in complex mixtures. A practical means of increasing the selectivity of a separation system is the use of two or more separation stages, resulting in the multidimensional separation of a biopolymer mixture [9,20–22].

Two-dimensional gel electrophoresis currently represents the most efficient two-dimensional separation technique, generating peak capacities of 1000–5000 in 1 day. Nevertheless, despite considerable advances, reproducibility, quantitation, automation, and post-separation analyte identification remain challenging and have triggered the search for alternative two-dimensional separation methodology based on chromatographic techniques. The combination of ion-exchange high-performance liquid chromatography (HPLC) and reversed-phase HPLC has been demonstrated to increase the peak capacity for proteins and peptides by at least one order of magnitude [23,24]. Additionally, mass spectrometry can be viewed as a separation technique distinguishing between different mass-to-charge ratios. Therefore, the on-line conjugation of liquid-phase separation methods and mass spectrometry yields multidimensional separation systems as well [23]. In practice, mass spectrometry is most frequently interfaced to chromatographic separation methods because they offer very rugged interface technology and easier optimization of experimental conditions compared to electrophoretic separations.

Despite considerable advances in multidimensional separation, improvements in separation selectivity and column efficiency still remain the major objectives in the design of novel stationary phases for HPLC of biopolymers. One promising approach to the synthesis of packing materials with enhanced mass transfer properties is the micropellicular, monolithic column configuration, in which the chromatographic bed consists of a single piece of a polymer having macroporosity for convective flow [25,26]. Diffusional distances are significantly reduced because of the high interconnectivity of the channels in the monolithic packing, resulting in higher chromatographic efficiency [27]. Monolithic columns can be readily prepared in the capillary format which makes them ideally suited for the on-line interfacing to ESI-MS [28,29].

ESI-MS has turned out to function most efficiently with analytes electrosprayed from solutions containing volatile components and having low conductivity and surface tension [30], although some interface designs can tolerate considerable amounts of nonvolatile additives. Because of the applicability of volatile, hydro-organic mobile phases, reversed-phase HPLC represents the most common chromatographic mode for HPLC–ESI-MS of peptides and proteins [31,32], while ion-pair reversed-phase HPLC has turned out to be highly suitable for HPLC–ESI-MS of nucleic acids [33]. Both chromatographic modes were realized with monolithic columns based on hydrophobic copolymers, such as poly(styrene-co-divinylbenzene) [28,34] or poly-(norborn-2-ene-co-1,4,5,8,8*a*-hexahydro-1,4,5,8,*exo,endo*-dimethanonapthalene) [35,36] and successfully hyphenated to ESI-MS. In this communication, we report on the optimization of separation systems employing monolithic separation media for peptide, protein, and nucleic acid analysis. The characteristics of the separation systems as well as some examples of application for the investigation of proteomic and genomic problems are discussed.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (HPLC gradient grade), acetic acid (analytical reagent grade), and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). Butyldimethylamine (analytical reagent grade), trifluoroacetic acid (TFA, for protein sequence analysis) and triethylamine (analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). A 1.0 *M* stock solution of triethylammonium acetate, pH 7.0, was prepared by adding acetic acid to a 1.0 *M* aqueous solution of triethylamine until pH 7.0 was reached. A 0.50 *M* stock solution of butyldimethylammonium bicarbonate, pH 8.5, was prepared by passing carbon dioxide gas (AGA, Vienna, Austria) into a 0.50 *M* aqueous solution of the amine at 5 °C until pH 8.5 was reached.

All standard proteins were obtained from Sigma (St. Louis, MO, USA). Trypsin (sequencing grade modified) and the 25-basepair DNA step ladder were obtained from Promega (Madison, WI, USA). The tryptic digest of carboxymethylated bovine β-lactoglobulin A was prepared according to the published protocol [37] and dialyzed against water in dialysis sacks (Sigma). Proteins from the major and minor antenna system of the photosystem II were extracted and prepurified from spinach leaves as described in Ref. [38]. The size standards of double-stranded DNA fragments were obtained from Sigma (pUC18 DNA–*Hae*III digest and pBR322DNA–*Hae*III digest) and MBI Fermentas (Vilnius, Lithuania, pUC19 DNA–*Msp*I digest and pBR322 DNA–*Alu*I digest). The standards of phosphorylated oligodeoxyadenylic acids (pdA_{12-18} and pdA_{40-60}) were purchased as sodium salts from Pharmacia (Uppsala, Sweden).

An 82-bp fragment of the STS G-107954 (tgttcagaatatcctcttccctgCTTCTG(T / delT)GGGTAATTTGTTT(A / G)TTCCTGTCAATCAGCT(A / G)Tcaggaggactgattgggcag, primer sequences in lower case letters) was amplified by polymerase chain reaction according to the protocol described in the SNP database of the NCBI at http://www.ncbi.nlm.nih.gov/SNP, No. G66893.

2.2. High-performance liquid chromatography and interfacing with electrospray ionization mass spectrometry

The Ultimate™ fully integrated capillary HPLC system (LC-Packings, Amsterdam, The Netherlands) with a column oven (model CTO-2A, Shimadzu, Kyoto, Japan) was used for all chromatographic experiments with UV detection. The detection cell was a 3-nl ULT-UZ-N10 cell from LC-Packings. The system used for HPLC–ESI-MS experiments consisted of a low-pressure gradient micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3-mm O.D. copper tubing which was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), and a microinjector (model C4-1004, Valco Instruments, Houston, TX, USA) with a 500-nl internal sample loop. ESI-MS was performed on a quadrupole ion trap mass spectrometer (LCQ, Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ion source.

Monolithic capillary columns (60×0.20 mm I.D.) were prepared according to the published protocol [34]. The capillary column was directly connected to the spray capillary (fused-silica, 90 μm O.D., 20 μm I.D., Polymicro Technologies, Phoenix, AZ, USA) by means of a microtight union (Upchurch Scientific, Oak Harbor, WA, USA). For analysis with pneumatically assisted ESI, an electrospray voltage of 3–5 kV and a nitrogen sheath gas flow were employed. The temperature of the heated capillary was set to 250 °C. Total ion chromatograms and mass spectra were recorded on a personal computer with the Xcalibur software version 1.2 (Thermo Finnigan). Mass calibration and tuning were performed in the positive ion mode by direct infusion of a solution of caffeine (Sigma), methionyl-arginyl-phenylalanyl-alanine (Finnigan), and Ultramark 1621 (Finnigan).

Fine tuning for ESI-MS of proteins in the positive ion mode was accomplished by infusion of 3.0 μl/min of a 0.4-pmol/μl solution of cytochrome C or a 6.9-pmol/μl solution of carbonic anhydrase in 0.050% aqueous TFA solution containing 20% acetonitrile (v/v). Fine tuning for ESI-MS of oligo-

nucleotides in the negative ion mode was performed with a 30 pmol/μl solution of $(dT)_{24}$ (Microsynth, Balgach, Switzerland) in 25 m*M* butyldimethylammonium bicarbonate, 20% acetonitrile (v/v). For all direct infusion experiments, cations present in the oligonucleotide samples were removed by on-line cation-exchange using a 20×0.50-mm I.D. cation-exchange microcolumn packed with 38–75-μm Dowex 50 WX8 particles (Serva, Heidelberg, Germany) [39].

Protein identification and assignment of peptides were accomplished with the help of the ExPASy Molecular Biology Server of the Swiss Institute of Bioinformatics (http://www.expasy.org).

3. Results and discussion

3.1. Selectivity and peak capacity of micropellicular capillary monoliths for biopolymer separations

One of the unique properties of monolithic capillary columns based on poly-(styrene/divinylbenzene) (PS/DVB) is the variety of different biopolymers that can be separated with high efficiency on one single stationary phase (Fig. 1). The high efficiency is a consequence of polymerization in the presence of porogens such as decanol/tetrahydrofuran that favor the formation of macropores, resulting in a micropellicular configuration which lacks micropores accessible only by diffusional mass transport [40]. In contrast to ion-exchange chromatography, where the necessity of high salt concentration to elute the sample components impairs its direct conjugation with ESI-MS, the monolithic PS/DVB stationary phase is suitable for the chromatographic separation of peptides, proteins, oligonucleotides, and double-stranded nucleic acids in the reversed-phase or ion-pair reversed-phase mode utilizing mobile phase compositions that are particularly suited for the direct interfacing with ESI-MS [33,34,41].

Although the separation efficiency of HPLC is lower compared to capillary electrophoresis, one- and two-dimensional gel electrophoresis, HPLC is the only separation method that is suitable for the online coupling with mass spectrometry without a loss of sensitivity like in capillary electrophoresis.

Polypeptides were separated with a gradient of 2–50% acetonitrile in aqueous, 0.050% trifluoroacetic acid solution. Fig. 1a illustrates the chromatogram of a tryptic digest of β-lactoglobulin A, which was separated within 8 min into 24 peaks with peak widths (at base) between 1.9 and 6.3 s. Since complete digestion of β-lactoglobulin A by trypsin yields 18 tryptic peptides, the additional peptides have to be due to incomplete digestion of the protein (see below). The elution of the analytes as very narrow bands is highly advantageous for generating high peak capacity in a short period of time and it can be deduced from the chromatogram of Fig. 1a that there is plenty of space between most of the eluting species for the separation of even more complex peptide mixtures. Assuming an average peak width of 3.5 s and an effective gradient time of 8 min (excluding the dead volume and the peak of unretained compounds), as many as 137 peaks are separable in principle during a 10-min gradient run. The time required to generate this peak capacity is substantially shorter than that with conventional peptide separation columns packed with porous, silica-based particles [37], in which separations at comparable resolution take 45–120 min.

The fractionation of intact proteins was feasible in PS/DVB monoliths under very similar mobile phase conditions compared to those used for peptides. As shown in Fig. 1b, 11 standard proteins ranging in molecular mass from 12 360 (cytochrome C) to 57 586 (catalase) were separable with a steep gradient of 16–48% acetonitrile in 10 min. The distribution of the separated proteins over the total solvent gradient demonstrates the good selectivity of the polymeric stationary phase for the separation of proteins of widely differing properties, ranging from small and hydrophilic (cytochrome C) to large and rather hydrophobic (catalase). Since the gradient was ramped up to only 48% acetonitrile, there is still space left for the analysis of even more hydrophobic proteins. The average peak width was 5.7 s which yields an estimate of 84 for the peak capacity in a 10-min gradient run (excluding the dead volume and the peak of unretained compounds).

The sample probably most suitable to characterize the peak capacity of the monolithic columns for nucleic acid separation is a ladder of homologous oligodeoxynucleotides. Reversed-phase HPLC is not

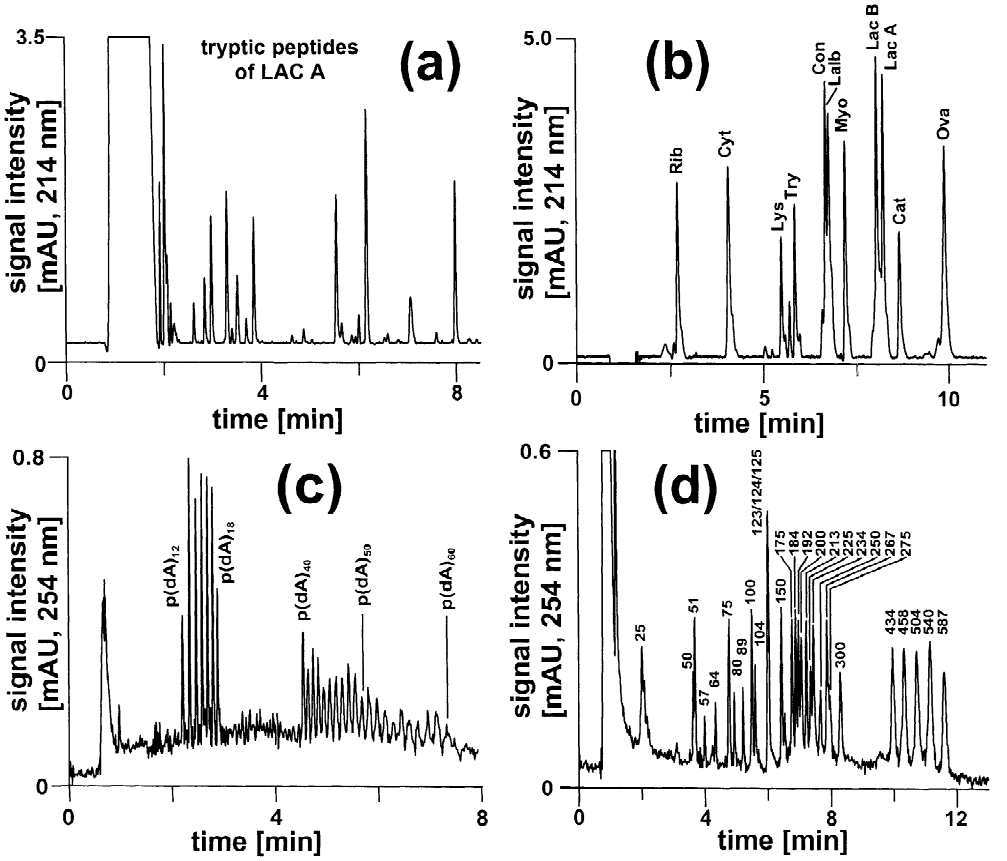

Fig. 1. High-resolution separations of (a) peptides, (b) proteins, (c) single- and (d) double-stranded DNA in a monolithic capillary column. Column, PS–DVB monolith, 60×0.20 mm I.D.; mobile phase in (a,b), (A) 0.050% trifluoroacetic acid in water, (B) 0.050% trifluoroacetic acid, 80% acetonitrile in water; gradient, (a) 2.5–63% B and (b) 20–60% B in 10 min; mobile phase in (c,d), (A) 100 m*M* triethylammonium acetate, pH 7.0, (B) 100 m*M* triethylammonium acetate, pH 7.0, 25% acetonitrile; gradient in (c) 12–32% B in 3 min, 32–37% B in 12 min and (d) 25–60% B in 6 min, 60–68% B in 9 min; flow-rate, 2.0 μl/min; temperature in (a,c,d), 50 °C, (b) 70 °C; detection, UV, (a,b), 214 nm, (c,d), 254 nm; sample, in (a), tryptic digest of 167 fmol bovine β-lactoglobulin A, in (b) RIB, ribonuclease A; CYT, cytochrome C; LYS, lysozyme; TRY, trypsin; CON, conalbumin; LALB, α-lactalbumin; MYO, myoglobin; LAC A and B, β-lactoglobulin A and B; CAT, catalase; OVA, ovalbumin; 10–60 fmol of each protein, in (c), 76 fmol of each $p(dA)_{12-18}$ and 19 fmol of each $p(dA)_{40-60}$, in (d), 1.8 fmol pBR322 DNA *Hae*III and 3.6 ng of 25-basepair ladder.

suitable for the chromatography of unmodified oligodeoxynucleotides because of their highly polar character. Nevertheless, nonpolar stationary phases can be used for the separation of nucleic acids in the ion-pair reversed-phase mode, in which an amphiphilic ion-pair reagent added to the mobile phase and carrying positive charge effects electrostatic interactions between the surface of the stationary phase and the polyanionic analytes [42]. Similar to reversed-phase HPLC, elution of the nucleic acids is accomplished by a gradient of increasing acetonitrile concentration.

Fig. 1c illustrates the separation of phosphorylated oligodeoxyadenylic acids, ranging in size from the 12-mer to the 60-mer. All components of the ladder were separated with resolution to baseline within 7.5 min. From the excellent resolution even of the long-chain oligomers, it can be extrapolated that single nucleotide resolution is feasible up to approximately 100-mers, provided that the gradient steepness is

gradually decreased with increasing molecular size of the analytes. The separation of 60 components in 7.5 min corresponds to a maximum peak capacity of approximately 80 during a 10-min gradient run.

Finally, a mixture of a 25-basepair ladder and a restriction digest of the pBR322 plasmid was utilized to evaluate the peak capacity of the monolithic capillary columns for the separation of double-stranded nucleic acids. From the chromatogram depicted in Fig. 1d it is evident that 28 out of the 30 fragments detected were at least partially separated and that a relative difference in length of 4–6% suffices to enable the separation of the DNA fragments. Only the 123-, 124-, 125-basepair fragments coeluted because of their small difference in length. With an average peak width of 9.4 s for the fragments ranging in size from 25 to 587 base pairs, about 55 fragments are separable within 10 min.

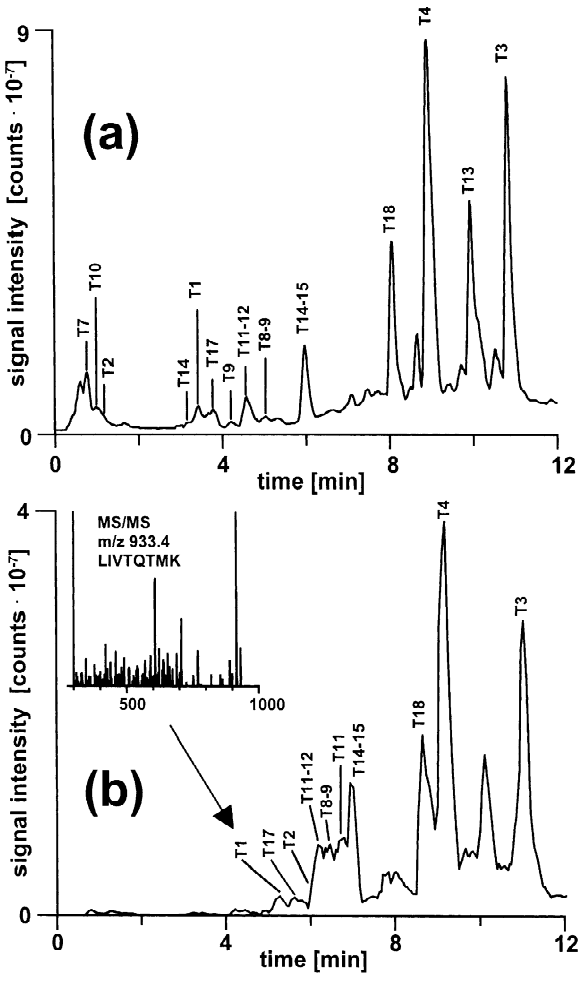

Fig. 2. Peptide mass fingerprinting (a) and tandem mass spectrometric identification (b) of tryptic peptides from β-lactoglobulin A. Column, PS–DVB monolith, 60×0.20 mm I.D.; mobile phase, (A) 0.050% trifluoroacetic acid in water, (B) 0.050% trifluoroacetic acid in acetonitrile; linear gradient, (a) 0–48% B, (b) 0–50% B in 15 min; flow-rate, 2.0 μl/min; temperature, 50 °C; scan, (a) full scan, 500–2000 amu, (b) full scan followed by data-dependent MS–MS of the first–third most intense ions; electrospray voltage, 4.0 kV; sample, tryptic digest from bovine β-lactoglobulin A, 12.5 pmol.

3.2. Examples of application of monolithic column technology in proteome analysis

3.2.1. Protein identification by peptide mass fingerprinting and tandem mass spectrometry

Today, the most successful protein identification procedures for proteomics are based on accurate molecular mass determinations and/or tandem mass spectrometric investigation of a set of tryptic peptides generated from the proteins of interest. In both cases, the mass spectrometric data are matched against protein databases with the aid of computer-based tools, yielding information about the identity of the protein(s) present in the sample. Fig. 2a illustrates an example for mass fingerprinting of the tryptic peptides of β-lactoglobulin A from bovine milk utilizing the monolithic separation system. Complete digestion of the protein yields 18 peptides, of which 15 could be detected in the chromatogram (Table 1). Processing of the measured molecular mass data using the PeptIdent tool of the EXPASy Molecular Biology Server returned the data summarized in Table 1. The measured molecular masses of the peptides corresponded excellently with the theoretical masses and the observed relative mass deviations of less than 200 ppm are typical for ion trap mass spectrometers. The smallest fragment retained on the column and detected by ESI-MS was a pentapeptide of the sequence IIAEK (T7), whereas smaller peptides such as T16 (ALK), T15 (FDK), T16 (ALK), T8 (TK), T12 (K), and T6 (K) passed the PS/DVB-based column in the void volume. The loss of such small peptides is usually not an issue because they are of only limited relevance for protein identification. However, two of the small fragments, namely T12 and T15, were represented in partially

Table 1
Mass fingerprinting and MS–MS identification of tryptic peptides from β-lactoglobulin A separated in a monolithic capillary column

Retention time (min)[a]	m/z (charge)	Measured mass	Theoretical mass	Fragment no.	MS–MS	Sequence
0.61	573.3 (1+)	572.30	572.36	T7	−	IIAEK
1.51	916.4 (1+)	915.40	915.47	T10	−	LDAINENK
1.80	673.3 (1+)	672.30	672.39	T2	+	GLDIQK
5.70	623.3 (2+)	1244.60	1244.59	T14	−	TPEVDDEALEK
5.70	1245.5 (1+)	1244.50	1244.59	T14	−	TPEVDDEALEK
5.70	933.5 (1+)	932.50	932.54	T1	+	LIVTQTMK
6.03	837.5 (1+)	836.50	836.48	T17	+	ALPMHIR
6.37	674.3 (1+)	673.30	673.42	T9	−	IPAVFK
6.63	597.4 (2+)	1192.80	1192.68	T11–12	−	VLVLDTDYKK
6.63	1193.7 (1+)	1192.70	1192.68	T11–12	+	VLVLDTDYKK
6.88	903.5 (1+)	902.50	902.57	T8–9	+	TK IPAVFK
7.11	1065.5 (1+)	1064.50	1064.58	T11	+	VLVLDTDYK
7.44	818.6 (2+)	1635.20	1634.78	T14–15	+	TPEVDDEALEKFDK
7.44	1636.8 (1+)	1635.80	1634.78	T14–15	−	TPEVDDEALEKFDK
9.04	859.5 (2+)	1717.00	1715.79	T18	+	LSFNPTLQEEQCHI
9.04	1717.7 (1+)	1716.70	1715.79	T18	−	LSFNPTLQEEQCHI
9.61	1157.6 (2+)	2313.20	2312.26	T4	+	VYVEELKPTPEGDLEILLQK
10.42	1425.9 (2+)	2849.80	2847.27	T13	−	YLLFCMQNSAEPEQSLVCQCLVR
11.39	1354.7 (2+)	2707.40	2706.38	T3	+	VAGTWYSLAMAASDISLLDAQSAPLR

[a] From chromatogram in Fig. 2a.

digested peptides (T11–12, T14–15). The total coverage of the sequence with tryptic peptides was 93.8%, which was more than sufficient for unequivocal identification of the protein in the database.

Whereas mass fingerprinting is efficient for the identification of relatively simple protein mixtures extracted from 2-D gels or prefractionated by other suitable separation procedures, identification on the basis of sequence information gained from MS–MS experiments represents the method of choice for the detection of hundreds or even thousands of proteins required for whole proteome analysis. The high specificity of MS–MS data is due to partial sequence information, where a sequence of eight to 10 amino acids can be unique for a single protein and, hence, suitable for its unequivocal identification.

The analysis of the tryptic peptides of β-lactoglobulin A by reversed-phase HPLC–ESI-MS–MS is shown in Fig. 2b. In this experiment, MS–MS data were collected in the so-called data-dependent mode, in which first a full scan spectrum serves to automatically select ions for subsequent fragmentation and mass analysis of the fragment ions, resulting in alternating full scan MS and full scan MS–MS data acquisition. The inset in Fig. 2b illustrates an example for the MS–MS spectrum of the fragment T1, having the sequence LIVTQTMK. The number of peaks detected in both chromatograms of Fig. 2 is quite similar. Shifts in retention times are due to differences in gradient delay and flow through the monolithic columns. The lower resolution of the peaks in Fig. 2b is a consequence of the smaller number of data points used to generate the reconstructed ion chromatogram of Fig. 2b, where only the datapoints of the full MS scans are represented, while the datapoints related to MS–MS scans are filtered out [43].

Although a peptide sequence can be deduced from MS–MS spectra by manual interpretation using fragmentation reactions well described in the literature [44,45] this is rarely done today since the sequence can be readily retrieved by means of a computer-based database search, as long as the sequence is present in one of the publicly accessible protein or DNA sequence databases [10]. Evaluation of the HPLC–ESI-MS–MS data was performed with the SEQUEST program, which automatically identifies peptides by cross-relating uninterpreted, experimental MS–MS data with simulated peptide

mass spectra extracted from standard protein and DNA databases. Ten peptides were successfully identified upon analyzing the MS–MS data, which represent 11 peptides expected from complete tryptic digestion or 69.8% of the total sequence of β-lactoglobulin A. The small peptides T7 and T10 were only found by mass fingerprinting, whereas fragment T13 was obviously present in both chromatograms but could not be positively identified by the SEQUEST search, possibly because of insufficient fragmentation of the relatively large 23-mer peptide. The peptide T2 was detected in both chromatograms at two significantly different elution positions. Because of the high specificity of MS–MS identification, we assume the T2 was correctly identified in Fig. 2b at an elution time of 6 min, while its identification in Fig. 2a constitutes a coincidental false-positive with a mass difference between the experimental and the predicted molecular mass of only 130 ppm.

3.2.2. Identification of proteins based on accurate intact molecular mass determinations

The methods of mass fingerprinting and MS–MS sequencing are potent tools for protein identification and comparative quantitation of proteomes using isotope-labeled internal standards [9]. Nevertheless, the protein sequence is usually merely partially covered and posttranslational modifications and/or sequence variations in the missed peptides may remain undetected. Moreover, absolute quantitation of protein expression is prone to error because of the multiple steps involved during sample preparation. Consequently, the identification of proteins by their intact molecular mass is advantageous for the investigation of numerous biological problems, because it reflects the full sequence of the protein and all its posttranslational modifications. Moreover, accuracy and reproducibility of quantitation strongly benefit from the direct analysis of the proteins in the biological samples.

However, identification of proteins by accurate mass measurements using HPLC–ESI-MS is nontrivial because the peak capacity of a single dimension of separation is not sufficient for the resolution of highly complex protein mixtures. The ESI mass spectra of coeluting proteins become considerably complex due to overlapping series of multiply charged ions that are difficult to deconvolute if the number of coeluting species exceeds four to five and/or if they are present in widely differing amounts. Multidimensional separation, using on-line ion-exchange chromatography [23], off-line isoelectric focusing [46], or sucrose gradient ultracentrifugation [38] in the first dimension, followed by reversed-phase chromatography in the second dimension have been successfully employed to increase peak capacity.

By virtue of their excellent peak capacity in the reversed-phase dimension (see Fig. 1), monolithic PS/DVB capillary columns can help to further improve the total peak capacity of multidimensional separation systems, to reduce the total analysis times, and to increase the on-line sampling frequency of fractions coming from the first dimension of separation. Fig. 3 illustrates examples of protein mass spectra extracted from the reconstructed ion chromatogram of a separation of eight analytes in a monolithic column. In order to enhance the mass spectrometric detectabilities of the proteins, the concentration of trifluoroacetic acid was reduced from 0.10 to 0.050% at the cost of an only minor increase in peak widths. A change to formic acid as acidic additive for reversed-phase HPLC did not offer any advantage, because at a concentration offering the same chromatographic efficiency no improvement in mass spectrometric detectability could be achieved.

Due to the high quality of mass spectra recorded during the reversed-phase HPLC–ESI-MS analysis, highly accurate molecular masses were obtained for the standard proteins. It can be deduced from Table 2 that the relative deviations of the molecular masses determined with a quadrupole ion trap mass analyzer ranged between −180 and 206 ppm. This mass accuracy compares favorably with the mass accuracies reported for intact molecular mass measurements obtained with reflectron time-of-flight mass analyzers [28,47]. Such high mass accuracy is a prerequisite for the applicability of intact molecular masses as protein tags, although additional information, such as isoelectric point or hydrophobicity may be necessary for unequivocal protein identification [48].

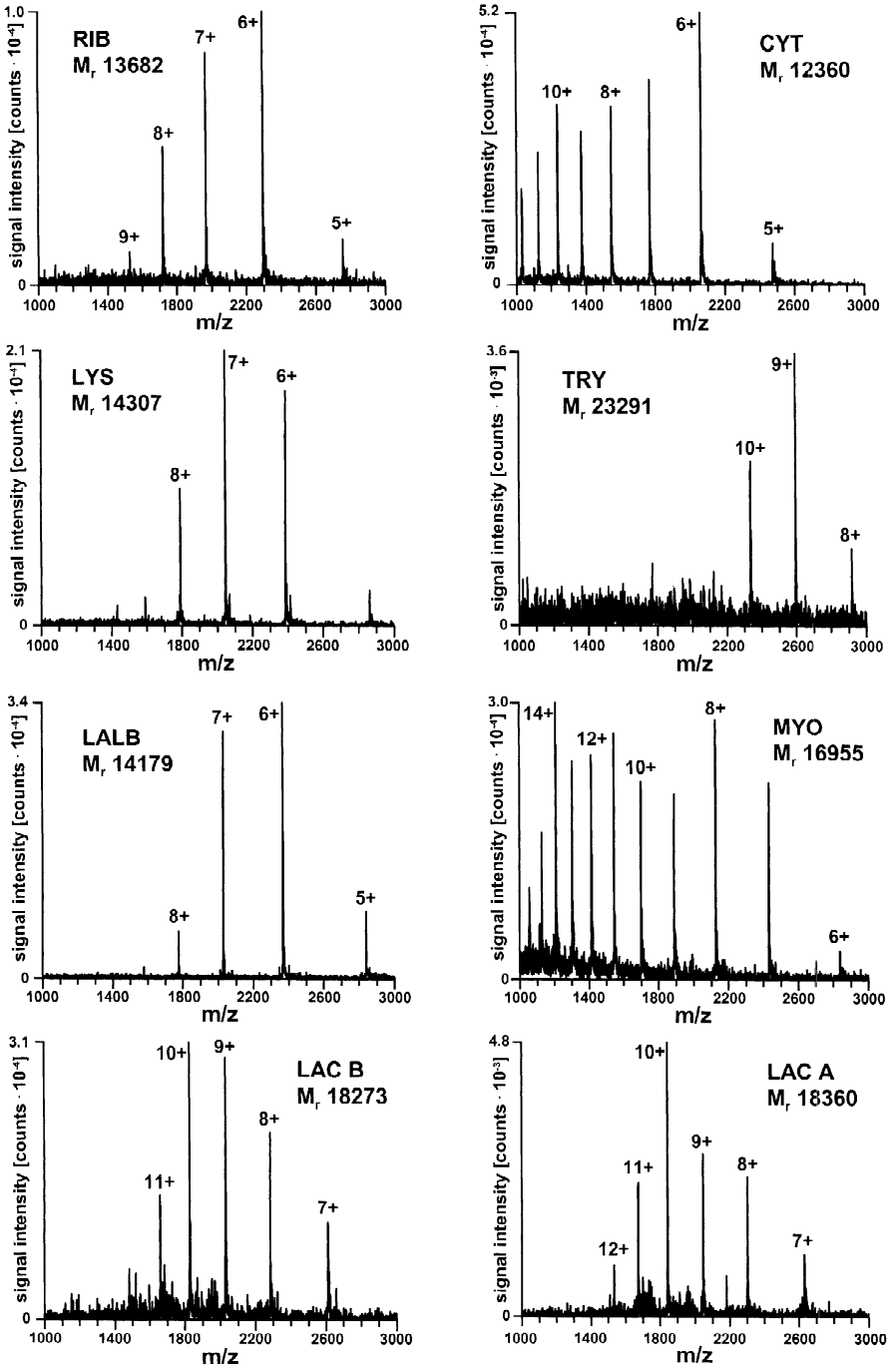

Fig. 3. ESI mass spectra of standard proteins separated on a PS/DVB monolith. Column, PS–DVB monolith, 60×0.20 mm I.D.; mobile phase, (A) 0.050% trifluoroacetic acid in water, (B) 0.050% trifluoroacetic acid, 80% acetonitrile in water; linear gradient, 20–60% B in 15 min; flow-rate, 1.8 μl/min; temperature, 80 °C; scan, 1000–3000; electrospray voltage, 3.0 kV; sample. RIB, ribonuclease A; CYT, cytochrome C; LYS, lysozyme; TRY, trypsin; LALB, α-lactalbumin; MYO, myoglobin; LAC A, β-lactoglobulin A; LAC B, β-lactoglobulin B; 809–429 fmol of each protein.

Table 2
Protein molecular masses and relative errors of the measured masses

Protein	Measured mass	Theoretical mass	Relative error (ppm)
Ribonuclease A	13 682	13 682.33	−24
Cytochrome C	12 360	12 360.14	−11
Lysozyme	14 307	14 305.16	129
Trypsin	23 291	23 293.34	100
α-Lactalbumin	14 179	14 180.18	−83
Myoglobin	16 955	16 951.50	206
β-Lactoglobulin A	18 360	18 362.38	−130
β-Lactoglobulin B	18 273	18 276.29	−180

3.2.3. Separation and identification of hydrophobic membrane proteins using monolithic columns

Due to their interfacial position in cells, membrane proteins play important roles in various cellular processes, including signal transduction, metabolite and ion transport, and photosynthesis. Two-dimensional gel electrophoresis has been successfully applied to the analysis of membrane proteins [49]. However, intact molecular mass determinations of gel-separated membrane proteins are hampered due to the difficulties in extracting the hydrophobic proteins from the gel and the necessity to add detergents to keep the membrane proteins in solution. Liquid chromatography-based separation has been shown to significantly alleviate the problem of signal suppression in the presence of detergents, since they are efficiently removed before investigation by ESI-MS [38,50].

The separation and intact molecular mass determination of the light-harvesting antenna proteins of photosystem I extracted from pea leaves in a 100-μm I.D. monolithic PS/DVB capillary column is illustrated in Fig. 4a. For this analysis, the gradient was ramped from 31 to 39% acetonitrile in 8 min and from 39 to 44% acetonitrile in 17 min. The antenna proteins are very similar in amino acid sequence and in molecular mass, making it difficult to identify the proteins by SDS–PAGE and/or immunoblotting. However, reversed-phase HPLC–ESI-MS readily enabled their identification by comparison of the experimental intact molecular masses obtained upon deconvolution of the raw mass spectra shown in Fig. 4b–e with the molecular mass expected from the corresponding DNA sequences.

3.3. Examples of application of monolithic column technology in genome analysis

3.3.1. Sizing of nucleic acids by HPLC–ESI-MS

The size of double-stranded DNA fragments, usually expressed as the number of basepairs, is a basic information about a DNA molecule. It cannot only be used for the identification of nucleic acids generated by DNA restriction digestion or polymerase chain reaction (PCR) [51], but also for the detection of insertions or deletions in DNA sequences [18]. Gel electrophoresis can be used to determine the size of DNA fragments by using the reciprocal relationship between fragment length and electrophoretic mobility [52]. However, because the electrophoretic mobility of a DNA fragment is affected by its sequence-dependent secondary structures, local sizing accuracy will be compromised by standard fragments with anomalous mobility. The influence of AT content on retention of DNA fragments resulting in occasional inversions of eluted peaks as a function of molecular size prevented also the employment of anion-exchange chromatography for size accurate fragment identification [53]. So far, ion-pair reversed-phase HPLC has been the most accurate separation method for the determination of the size of double-stranded DNA fragments, which allowed the calculation of fragment size from retention data with an accuracy of better than 3.2% [51].

With the demonstration of the capability of ESI to ionize nucleic acid molecules having molecular masses far beyond 100 000, a new era in the sizing of DNA in terms of sizing accuracy has begun

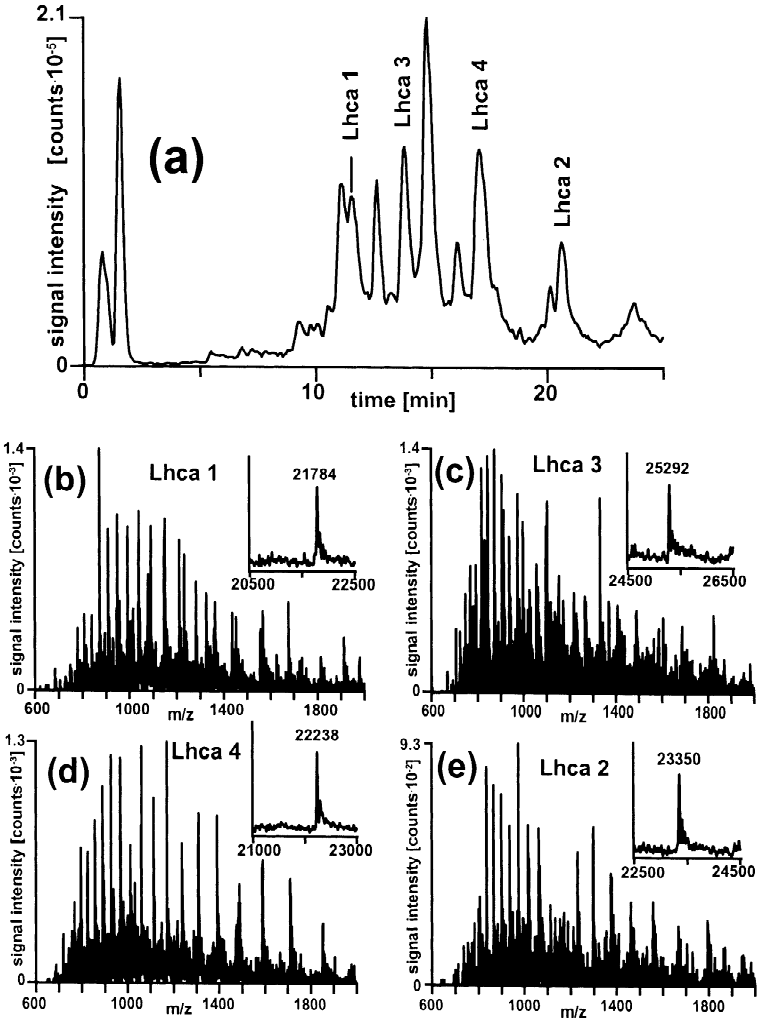

Fig. 4. Analysis of the antenna proteins from photosystem I of pea. Column, PS–DVB monolith, 60×0.10 mm I.D.; mobile phase, (A) 0.050% trifluoroacetic acid, 20% acetonitrile in water, (B) 0.050% trifluoroacetic acid, 95% acetonitrile in water; linear gradient, 15–25% B in 8 min, 25–32% B in 17 min; flow-rate, 0.5 μl/min; temperature, 80 °C; scan, 600–2000; electrospray voltage, 3.0 kV; sheath gas, nitrogen; sample, membrane proteins of the photosystem I of pea.

[3,54]. The quality of mass spectra obtained by ESI-MS strongly depends on the purity of the sample introduced into the mass spectrometer [12,55]. Hence, on-line desalting and purification of DNA fragments before mass spectrometric investigation has proven to be essential for the investigation of small amounts of nucleic acids [18,34]. For the evaluation of the sizing accuracy of ion-pair-reversed-phase HPLC–ESI-MS, we determined the molecular masses of DNA restriction fragments from four different commercial DNA sizing standards.

Table 3 summarizes the molecular mass and size data for the 40 restriction fragments. The relative deviations in molecular mass ranged from 0 to 0.12% (average 0.049%) and did not show any tendency towards positive or negative bias or dependence on molecular mass. The size in basepairs was calculated from the molecular mass by subtracting

Table 3
Molecular masses and deduced fragment sizes of double-stranded DNA fragments

Fragment length (bp)[a]	Source[b]	Theoretical molecular mass	Measured molecular mass	Relative deviation in mass (%)	Calculated length (bp)	Relative deviation in size (%)
46	3	28 501.7	28 476	−0.090	46.03	0.06
49	3	30 316.8	30 299	−0.059	48.98	−0.05
51	4	31 559.6	31 565	0.017	51.03	0.05
57	4	35 263.0	35 252	−0.031	56.99	−0.01
57	3	35 260.1	35 240	−0.057	56.97	−0.05
63	3	38 964.5	39 006	0.106	63.07	0.11
64	4	39 592.8	39 573	−0.050	63.99	−0.02
67	2	41 438.1	41 423	−0.036	66.98	−0.03
80	1	49 475.4	49 535	0.121	80.11	0.13
80	4	49 475.4	49 494	0.038	80.04	0.05
89	4	55 039.0	55 058	0.035	89.05	0.05
90	3	55 658.4	55 690	0.057	90.07	0.08
100	3	61 825.5	61 803	−0.036	99.96	−0.04
102	1	63 067.3	63 123	0.088	102.10	0.10
104	4	64 313.0	64 391	0.121	104.15	0.14
110	2	68 005.6	67 977	−0.042	109.95	−0.04
111	2	68 623.0	68 623	0.000	111.00	0.00
123	4	76 045.8	76 059	0.017	123.03	0.03
124	4	76 675.1	76 731	0.073	124.12	0.10
147	2	90 883.4	90 862	−0.024	146.99	−0.01
174	1	107 566.3	107 640	0.068	174.14	0.08
184	4	113 747.4	113 802	0.048	184.12	0.06
190	2	117 438.0	117 424	−0.012	189.98	−0.01
192	4	118 668.8	118 722	0.045	192.08	0.04
213	4	131 674.0	131 733	0.045	213.13	0.06
226	3	139 688.5	139 718	0.021	226.06	0.03
234	4	144 646.6	144 708	0.042	234.13	0.06
242	2	149 566.0	149 596	0.020	242.04	0.02
256	1	158 221.7	158 220	−0.001	256.00	0.00
257	3	158 848.0	158 849	0.001	257.02	0.01
267	1	165 018.1	165 221	0.123	267.33	0.12
267	4	165 019.1	165 091	0.044	267.12	0.05
281	3	173 693.5	173 522	−0.099	280.76	−0.08
298	1	184 198.4	184 262	0.035	298.15	0.05
331	2	204 588.8	204 611	0.011	331.08	0.02
403	3	249 099.7	248 874	−0.091	402.71	−0.07
404	2	249 643.9	249 750	0.043	404.13	0.03
434	1	268 240.4	268 340	0.037	434.22	0.05
489	2	302 222.7	302 391	0.056	489.32	0.07
501	2	309 574.2	309 567	−0.002	500.94	−0.01

[a] Actual length derived from the sequence.
[b] 1, pUC18 DNA-*Hae*III digest; 2, pUC19 DNA-*Msp*I digest; 3, pBR322 DNA-*Alu*I digest; 4, pBR322DNA-*Hae*III digest.

the mass of H_2O from the molecular mass and dividing by the average mass of an AT and GC basepair (mass of (AT-H_2O)=617.4, mass of (GC-H_2O)=618.4, average=617.9). Since the mass of an AT basepair differs from that of a GC basepair only by 1 mass unit, the length of a double-stranded DNA fragment in basepairs is essentially independent of base composition. It can be deduced from the last column of Table 3 that the measured sizes were in excellent agreement with the actual sizes (maximum relative deviation of 0.13%) and that the sizing accuracy is more than one order of magnitude better

than that of mass measurements based on chromatographic retention data [51].

3.3.2. HPLC–ESI-MS for the detection of dna sequence variations

The high accuracy of mass determinations by ESI-MS readily facilitates the detection of sequence variations that cause a change in the size of double-stranded nucleic acids, e.g., insertions/deletions [18], or short tandem repeats (STRs) [56,57]. Single or multiple base substitutions, however, are difficult to detect in double-stranded DNA due to the small mass difference between AT and GC basepairs. In such cases, denaturation of the double-stranded DNA into the corresponding single strands prior to mass spectrometry becomes obligatory [58]. Because both the intact molecular mass and the charge states of single-stranded nucleic acids are smaller compared to those of double-stranded nucleic acids, the molecular mass of the former can be measured with higher accuracy. As a consequence, the relative mass deviations for single-stranded DNA were found to be approximately one order of magnitude lower than those of double-stranded DNA (compare the average mass deviation of 0.05% in Table 3 with an mass deviation of 0.005% in Table 4). The smallest mass difference due to a single base substitution in a DNA single strand is 9 mass units (substitution of A by T), which can be usually resolved by mass spectrometry in polynucleotides comprising up to 100–150 nucleotides with quadrupole ion trap [59] or time-of-flight mass analyzers, and in even longer nucleic acids with Fourier-transform ion-cyclotron resonance mass analyzers [60].

The characterization of multiple single nucleotide polymorphisms (SNPs) in an 82-basepair DNA fragment amplified by PCR is demonstrated in Fig. 5. The amplicon included three SNPs, namely T> deletion of T, A>G, and A>G, located at positions 30, 44, and 61, respectively, from the 5′ end of the forward primer. The PCR product was chromatographed in the monolithic column under denaturing conditions (Fig. 5a), and the eluting single strands were on-line introduced into the mass spectrometer for mass determination. Fig. 5b,c depicts the deconvoluted mass spectra that were obtained for the

Table 4
Genotyping of multiple SNPs in an 82-basepair amplicon by accurate mass measurements

Measured molecular mass	Theoretical molecular mass	Relative deviation in mass (%)	Identification[a]
25 262	25 262.8	0.0032	$(T,G,G)_{rev}$
25 291	25 292.8	0.0071	$(T,A,A)_{rev}$
25 574	25 576.0	0.0078	$(T,G,G)_{rev}+dA$[b]
25 605	25 606.0	0.0039	$(T,A,A)_{rev}+dA$
25 245	25 246.6	0.0063	$(T,A,A)_{for}$
25 277	25 278.6	0.0063	$(T,G,G)_{for}$
25 559	25 559.8	0.0031	$(T,A,A)_{for}+dA$
25 592	25 591.8	0.0008	$(T,G,G)_{for}+dA$

[a] Polymorphisms: T>deletion of T at position 30, A>G at position 44, and A>G at position 61 relative to the 5′ end of the forward primer.

[b] Non-template addition of dA by Taq polymerase.

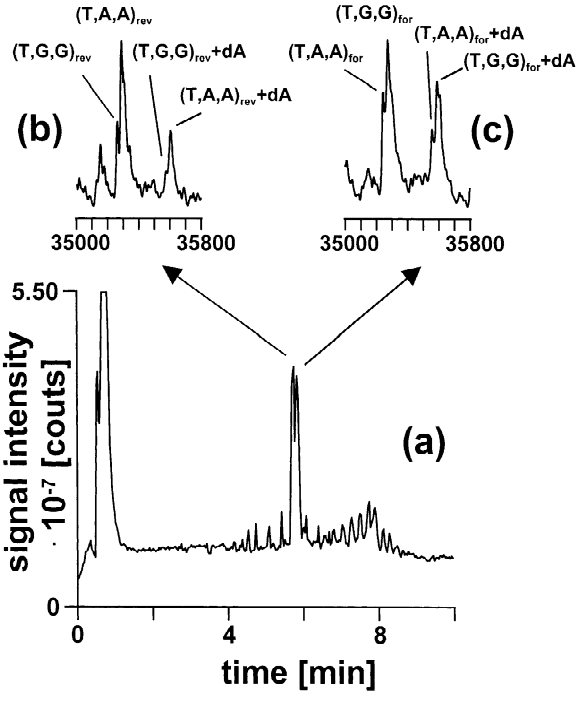

Fig. 5. Genotyping of SNPs in an 82-basepair fragment amplified from SNP G-107954. Column, PS–DVB monolith, 60×0.20 mm I.D.; mobile phase, (A) 25 m*M* butyldimethylammonium bicarbonate, pH 8.4, (B) 25 m*M* butyldimethylammonium bicarbonate, pH 8.4, 20% acetonitrile; linear gradient, 5–70% B in 10 min; flow-rate, 2.0 μl/min; temperature, 70 °C; scan, 500–2000 amu; electrospray voltage, 3.4 kV; sheath gas, nitrogen; post-column addition of 3.0 μl/min acetonitrile; sample, in 500 nl PCR reaction solution.

reverse (identified by the subscript rev) and the forward (identified by the subscript for) strands. A comparison of the measured molecular masses with the masses calculated from the DNA sequence clearly identified the sample as heterozygous with the following allele combinations: TGG on one chromosome and TAA on the other chromosome (Table 4).

4. Conclusions

The major advantage of the micropellicular, monolithic capillary columns based on PS/DVB copolymer rests within their excellent chromatographic separation efficiency for biopolymers, including peptides, proteins, and nucleic acids. Moreover, monolithic capillary columns are capable of generating high peak capacity in a short period of time, resulting in a considerable reduction in analysis time. Such rapid, high-resolution analysis is essential primarily for routine and high-throughput applications in proteomics and genomics. Nevertheless, the maximum peak capacity can usually not be fully exploited, since the peaks are not uniformly spread across the gradient. For problems involving the analysis of more complex mixtures, shallower gradients can be applied to increase the peak capacity even further at the cost of an increase in analysis time. Due to their miniaturization, robustness, and the applicability of volatile mobile phases, separation systems using PS/DVB monoliths can be readily hyphenated to ESI-MS, yielding highly valuable information about the structure and identity of the separated analytes.

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