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The impact of buffers and surfactants from micellar electrokinetic chromatography on matrix-assisted laser desorption ionization (MALDI) mass spectrometry of peptides
Effect of buffer type and concentration on mass determination by MALDI-time-of-flight mass spectrometry

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Abstract

This paper describes the effect of various buffers, surfactants, and organic additives commonly encountered in capillary zone electrophoresis and micellar electrokinetic chromatography on the molecular weight determination of peptides by matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry. Signal-to-noise ratio generally decreased with increasing buffer concentration without affecting mass accuracy, but the type of buffer was also important. Good spectra were obtained with an ammonium acetate buffer up to a concentration of 500 m*M* without impacting ionization of either peptides or other mobile phase constituents. Ionization of organic additives, such as anionic surfactants, non-ionic surfactants, and cyclodextrins was buffer dependent and presented a problem when the mass of the additive was in the range of the peptide mass. Brij®-35, Tween®-80, and cyclodextrins all produced prominent spectra of their own in the presence of sodium or potassium containing buffers, but not with ammonium acetate. Cationization of these neutral species with sodium or potassium ions allowed them to acquire a positive charge and produce spectra. In contrast, the ammonium ion appears to be a poor cationizating agent. Ionization of neutral surfactants was suppressed in ammonium acetate without impacting the spectra of peptides. Ammonium acetate buffers containing 30 m*M* sodium dodecyl phosphate also gave spectra with good signal intensity and no interference from the surfactant. Suppression of peptide ionization in MALDI was a problem when methanol, tetrabutyl amine, or poly(vinyl alcohol) were used with either ammonium acetate, sodium phosphate, and *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethansulfonic acid). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Buffer composition; Micellar electrokinetic chromatography; Matrix-assisted laser desorption/ionization mass spectrometry; Mass spectrometry; Peptides; Cyclodextrins; Surfactants

1. Introduction

Genomics and DNA databases have transformed the way we think about protein characterization. Instead of chemically sequencing proteins, DNA databases are now being used to predict the sequence [1]. Following trypsin digestion of a protein, masses of a few of the peptide fragments are being used to identify the parent gene in a DNA database, and the protein sequence is derived from the DNA sequence of the gene. Proteins in complex mixtures can even be identified and sequenced in this way with the aid

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of separation systems to fractionate the tryptic peptides. The two critical elements in this approach to protein characterization are highly selective and rapid separation systems and a mass spectrometer capable of analyzing mixtures of peptides. Capillary electrophoresis (CE) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry are important tools in this approach to protein characterization.

CE has the advantage of being a high efficiency microtechnique that is rapid. This is particularly useful when separating the peptides in large numbers of fractions derived from two-dimensional electrophoresis or liquid chromatography. The disadvantage is that it sometimes lacks selectivity. Micellar electrokinetic chromatography (MEKC) extends the range of electrophoretic separations to include partitioning into micelles [2]. It provides unique selectivity by simultaneously selecting on the basis of molecular size and shape, charge, hydrophobicity, and even the tendency to hydrogen bond in some cases [3]. MEKC has been found to be efficient in the separation of peptides [4] by providing alternative selectivity to that of CE alone. Non-ionic, anionic, and cationic micelles have all been used for peptide separations [4–6]. One of the problems of MEKC is that it is difficult to couple to electrospray ionization (ESI) mass spectrometers. Surfactants used in micelle formation are a major problem.

MALDI [7,8] is unique in being capable of providing the mass of 30 or more peptides in a mixture. The great advantage of MALDI over ESI [9] in the analysis of polypeptides is the general absence of multiple charged states of the analyte. This allows the analysis of more complex samples with MALDI. The major problem with MALDI is quenching of ionization, either from other analytes in the mixture or components of the sample matrix. Quenching by other analytes is overcome by increasing the degree of fractionation before MALDI mass spectrometry, i.e., by reducing the number of components in the sample presented to the mass spectrometer. Matrix quenching is generally not a problem unless it arises from some component in the separation system. It is in this context that the surfactants used in MEKC could be a problem in MALDI. This paper examines the impact of surfactants commonly used in MEKC on peptide ionization in MALDI.

2. Materials and methods

2.1. Materials

The peptides used in these studies were a gift from Steve Kates at PerSeptive Biosystems. Reagents were of reagent grade and used as received. Ammonium acetate, ammonium bicarbonate, and sodium acetate were obtained from Fisher Scientific (New Jersey, USA). *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethansulfonic acid) (HEPES), guanidine, urea, 2-acetoamido-2-iminodiacetic acid (ADA), 2-(*N*-morpholine)ethane sulfonic acid (MES), sodium borate, *N*-(2-hydroxyethyl)piperazine-*N*-l,3-propane sulfonic acid (EPPS), 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), Tris–(hydroxymethyl)-aminomethane (Trizma base), SDS lauryl sulphate, β-cyclodextrin, γ-cyclodextrin, Brij®-35, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Glycine was obtained from Bio Rad (Richmond, CA, USA). Sodium phosphate and sodium citrate were purchased from Mallinckrodt (Paris, KY, USA). Tween®-80 and sodium carbonate were from Aldrich (Milwaukee, WI, USA). Imidazole was purchased from ACROS (New Jersey, USA). Poly(vinyl alcohol) M_w 78000 was acquired from Polyscience (Warrington, PA, USA). Tetrabutyl ammonium phosphate was obtained from Kodak (Rochester, NY, USA).

2.2. Buffer and sample preparation

Stock buffer solutions were prepared at a concentration of 500 m*M* with deionozed water. ADA and sodium borate were prepared at a concentration of 100 m*M* owing to their limited solubility. The stock solutions were later diluted with water to the concentrations of interest. The stock solutions of cyclodextrins, surfactants, and other additives were prepared by adding appropriate amounts of these compounds to ammonium acetate (50 m*M*), sodium phosphate (50 m*M*), and HEPES (50 m*M*). Samples were prepared by mixing 1 μl of the peptide solution

(2.5 μ*M* dissolved in deionized water) with 99 μl of buffer at the concentration of interest. A 1-μl aliquot of the mixture was mixed with 24 μl of the matrix solution and was used for MALDI. The matrix solution for peptides was prepared by making a saturated solution of α-cyano-4-hydroxycinnamic acid in a water–ACN (1:1, v/v) and 3% TFA solution. The wells of the MALDI plate were spotted with 1-μl samples and allowed to air dry before being placed in the mass spectrometer.

2.3. Instrumentation

MALDI-TOF-MS was performed with a Voyager-Delayed Extraction BioSpectrometry Workstation from PerSeptive Biosystem (Framingham, MA, USA) in the positive ion mode for analysis of the peptide. The total accelerating voltage was set to 20 kV, the laser intensity was adjusted to 1350 at 337 nm. The gride and guide wire voltages were 68 and 0.05% of the applied voltage, respectively. Delay time was 75 ns and roughly 150 laser shots were averaged. The MALDI-TOF-MS parameters were kept constant throughout the study in order to make the comparison between the obtained results at different conditions possible.

Capillary electrophoresis experiments were performed using a BioFocus™ 3000 Capillary Electrophoresis System (Bio Rad, CA, USA), equipped with a UV detector monitoring a wavelength of 214 nm. A fused-silica capillary column of 38 cm (34 cm effective length)×75 μm I.D.) (Polymicro Technologies, Phoenix, AZ, USA) was operated at a constant temperature of 25.0°C and an applied voltage of 10 kV. The background electrolyte (BGE) was made by dissolving 20 m*M* sodium dodecyl phosphate (SDS) in 10 m*M* ammonium acetate at pH 7.01.

3. Results and discussion

3.1. Impact of buffers and salts on MALDI spectra

A variety of buffers and salts are used to moderate selectivity in MEKC at concentrations ranging to 100 m*M* or more. The concern is that these additives will either impact mass accuracy or quench the production of ions. It was found that neither the type of buffer nor concentration altered mass accuracy to an unacceptable level.

The observed mass was within 0.01–0.06% of the theoretical mass in all cases (data not shown). The major impact of buffers in MALDI appears to be on ionization, as seen in Table 1. Buffers and additives, such as ammonium acetate, glycine, and guanidine could be used at concentrations ranging up to 500 m*M*. Hydroxymethylaminomethane (Tris), ammonium bicarbonate, imidazole, EPPS, CAPS, and sodium carbonate could be used to at least 200 m*M* in concentration. The rest of the salts and buffers examined allowed production of acceptable spectra at concentrations up to 100 m*M*. Previous studies have shown that spectra obtained with Tris–acetate buffer are superior to those obtained with sodium phosphate or sodium acetate buffers [10]. Of all the buffers examined, only ADA had to be used at less than a 100 m*M* concentration. The contribution of amino acid composition and sequence to the ionization process of various buffers and salts was not examined.

The mechanism by which energy is transferred from the crystalline matrix to peptides to cause ionization in MALDI is not well understood. It is for this reason that an explanation of the results in Table 1 at a mechanistic level is impossible. Obviously the hydrophobicity and volatility of the buffer components impact both their concentration on the surface of matrix crystals and the degree to which they mediate direct interaction of peptides with the surface of the relatively hydrophobic crystal matrix. This process can he compared to adsorption chromatography where there is a distribution of an analyte, a peptide in this case, between a surface and a liquid mobile phase. The major difference is that the solvent is evaporated before analysis in the case of MALDI. As the solvent evaporates there will be a competition between all the components in the solvent for the crystal surface. Those left in direct contact with the surface are probably the most likely to be ionized.

In the context of interferring with energy transfer, additives absorbing at the frequency of the laser (337 nm) could impact the production of spectra. Imida-

Table 1
Effect of the salt concentration on the mass determination by MALDI-TOF-MS[a]

Buffer (salt)	Concentration (m*M*)									
	20	50	100	150	200	250	300	350	400	500
Ammonium acetate	+[b]	+	+	+	+	+	+	+	+	+
Guanidine	+	+	+	+	+	+	+	+	+	+
Glysine	+	+	+	+	+	+	+	+	+	+
TRIS	+	+	+	+	+	+	+	+	–	–
Ammonium bicarbonate	+	+	+	+	+	+	–	–	–	–
Imidazole	+	+	+	+	+	+	–	–	–	–
EPPS	+	+	+	+	+	+	–	–	–	–
Sodium acetate	+	+	+	+	+	–	–	–	–	–
CAPS	+	+	+	+	+	–	–	–	–	–
Sodium carbonate	+	+	+	+	+	–	–	–	–	–
Sodium citrate	+	+	+	+	–	–	–	–	–	–
HEPES	+	+	+	–	–	–	–	–	–	–
Sodium phosphate	+	+	+	–	–	–	–	–	–	–
Sodium borate	+	+	+	–	–	–	–	–	–	–
MES	+	+	+	–	–	–	–	–	–	–
ADA	+	+	–	–	–	–	–	–	–	–

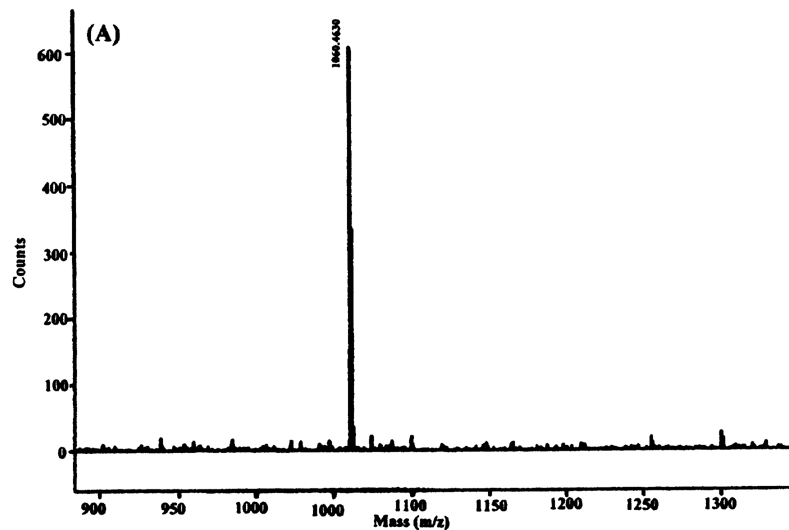
[a] Operating conditions: the salts were dissolved in deionized water at different concentrations. For other conditions see Section 2. All spectra were obtained using the peptide H-TRNLADQED-NH_2 (M_W=1059).
[b] The mass of the peptide could be determined. Percent deviation from the real mass [(theoretical mass–observed mass)/theoretical mass] was found to be 0.01–0.06%.

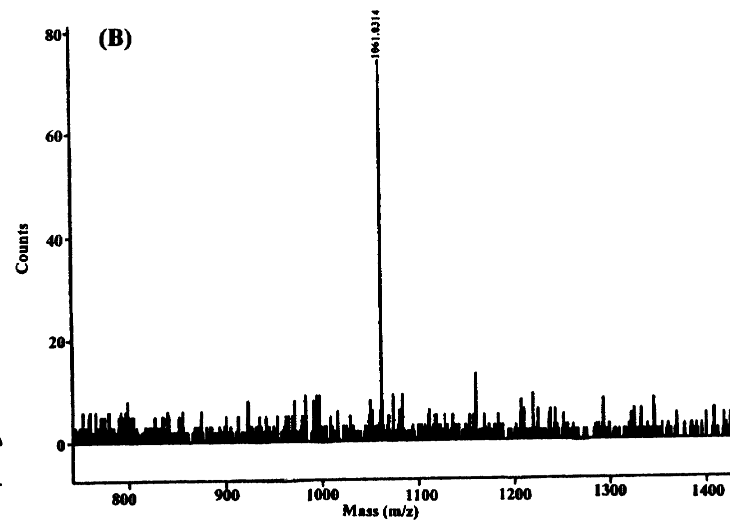
zole is such a case. The absorbance of 250 m*M* imidazole at 337 nm is 129 AU. It is seen in Table 1 that there is no special problem associated with imidazole at concentrations lower than 250 m*M*.

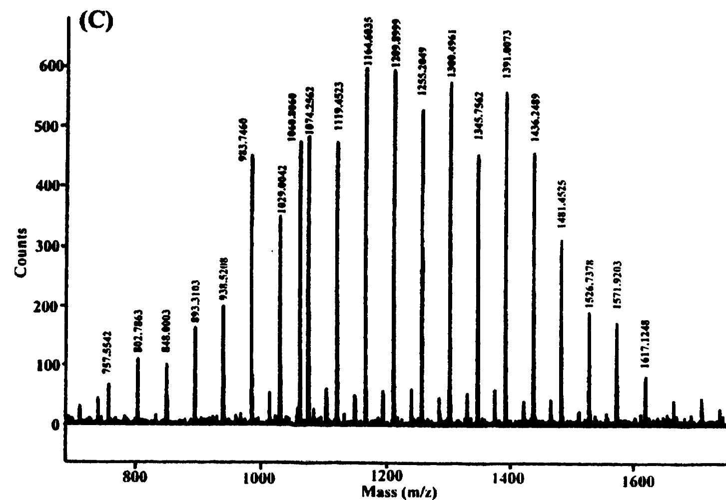
3.2. Effect of surfactants on MALDI-MS spectra

MEKC is based on the use of surfactants to create micelles. Variations of this technique also use additives such cyclodextrins and poly(vinyl alcohol) to either create secondary equilibria in the mobile phase to further enhance selectivity or suppress electroosmotically induced flow. Because the molecular weight of many surfactants and the cyclodextrins is greater than 500 and they are present at millimolar concentrations, there is concern they will hinder the MALDI-MS of peptides. The degree to which these reagents interfere with MALDI-MS was examined using a peptide sample designated Fr 14. MALDI mass spectra of Fr 14 with SDS and Brij®-35 are seen in Fig. 1. In agreement with previous studies [11], it was found that SDS could not be used at concentrations exceeding 30 m*M*. It is seen in Fig. 1A and B that acceptable spectra are obtained at a 30 m*M* concentration in the cases of both Brij®-35 and SDS using ammonium acetate as the buffer. The MALDI matrix itself produced ions approaching 500 amu, but no substantial ionization of Brij®-35 was observed with ammonium acetate. When either sodium phosphate or HEPES was substituted for ammonium acetate, the spectra changed dramatically in the case of Brij®-35 (Fig. 1C and D). The spectrum of Brij®-35 in these later two cases is very intense even through it is used at six times lower the concentration than with ammonium acetate. No spectra were obtained using SDS dissolved in these buffers (data not shown).

The interpretation of the Brij®-35 results are as follows: Brij®-35 is composed of a hydrophobic hexadecyl group attached to a polyoxyethylene oligomer. It has a mean moleclar weight of slightly over 1000 and a broad molecular weight distribution due to the heterogeneity of the polyoxyethylene tail. It is neutral and not easily ionized either directly or by cationization with ammonium ion in the ion source. In contrast, strong cations are known to form complexes with neutral species and produce spectra of the composite. Polystyrene is cationized by silver ions for example and produces spectra in which the isotopic contributions of silver are clearly identifiable







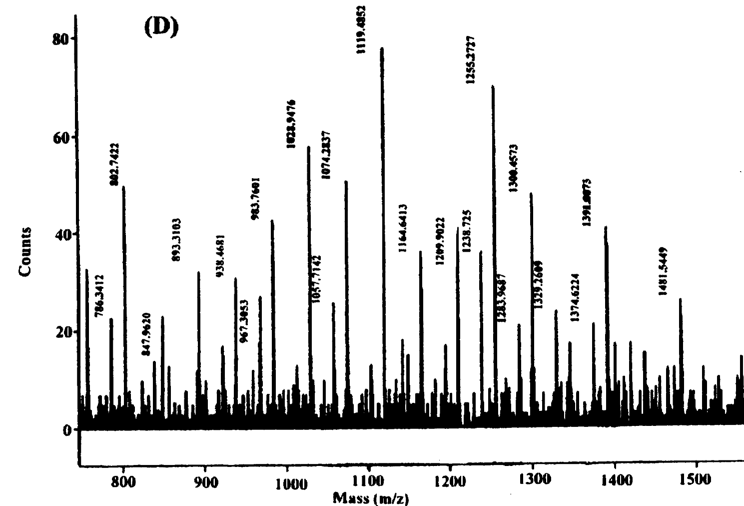


Fig. 1. Mass determination of Fr 14 in the presence of various surfactants dissolved in different buffer solutions. (A) 30 m*M* Brij®-35 in ammonium acetate (50 m*M*); (B) 30 m*M* SDS in ammonium acetate (50 m*M*); (C) 5 m*M* Brij®-35 in sodium phosphate (50 m*M*); (D) 5 m*M* Brij®-35 in HEPES (50 m*M*). Operation conditions as those given in Section 2.

[12]. This appears to be the case with Brij®-35 in sodium phosphate and HEPES, although the spectrum of Brij®25 is different with these two cationizating agents.

Cationization of neutral additives by buffers was further examined with Tween®-80 and cyclodextrins. Tween®-80 is similar to Brij®-35 except that stearic acid is linked to sorbitol through an ester linkage and there are multiple polyoxyethylene oligomers attached to the sorbitol moiety. Again it is seen that a neutral surfactant can produce a MALDI spectrum by cationization with sodium ions (Fig. 2). Ionization of cyclodextrins in the presence sodium ions but not ammonium ions is seen in Fig. 3. Although ionization of cyclodextrins occurs, they would only interfere if the spectrum of the peptide overlaps with the mass of the cyclodextrin.

The data presented above clearly demonstrates that low molecular weight surfactants are not a problem at concentrations usually lower than 30–40 m*M* with any of the buffers examined. It is also seen that high molecular weight, neutral species are only a problem in special cases. An example is when strong cationizating agents are components of the buffer system. Ammonium ions were never found to cause ionization of neutral species. Even when neutral species are present and ionized, they are only a problem if they are of broad molecular weight distribution and in the mass range of the peptide sample being analyzed.

3.3. Effect of other additives MALDI-MS spectra

A variety of other additives are used in CE as either ion pairing agents, mediators of analyte adsorption in micelles, modifiers to modulate electroosmotic flow, or to control surface adsorption. Tetrabutylamine and other related amphiphilic compounds are added as ion pairing agents and to change the electroosmosis [13,14]. When Fr 14 was analyzed in different concentrations of tetrabutylamine (10–100 m*M*), spectra were suppressed at all concentrations. Ionization was also suppressed by poly(vinyl alcohol) at concentrations of 0.01–0.1%. Poly(vinyl alcohol) is used to decrease the electroosmotic flow as well as to decrease the absorption of

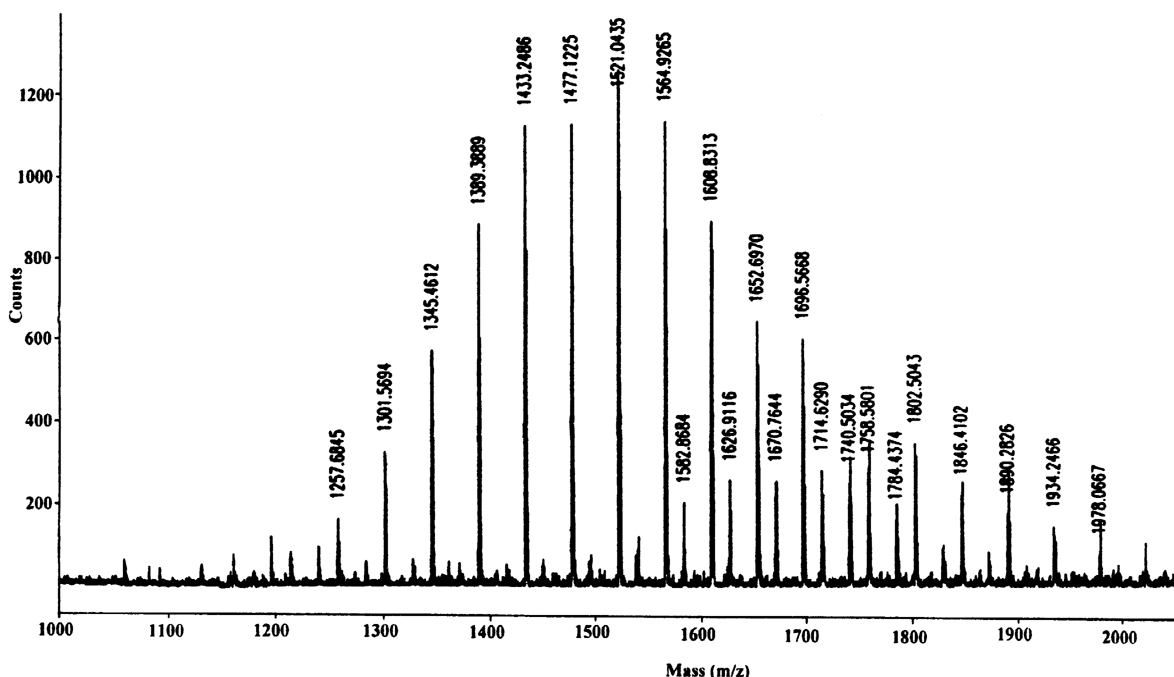


Fig. 2. Mass determination of Fr 14 in the presence of 15 m*M* Tween®-80 dissolved in 50 m*M* sodium phosphate. Operation conditions as those given in Section 2.

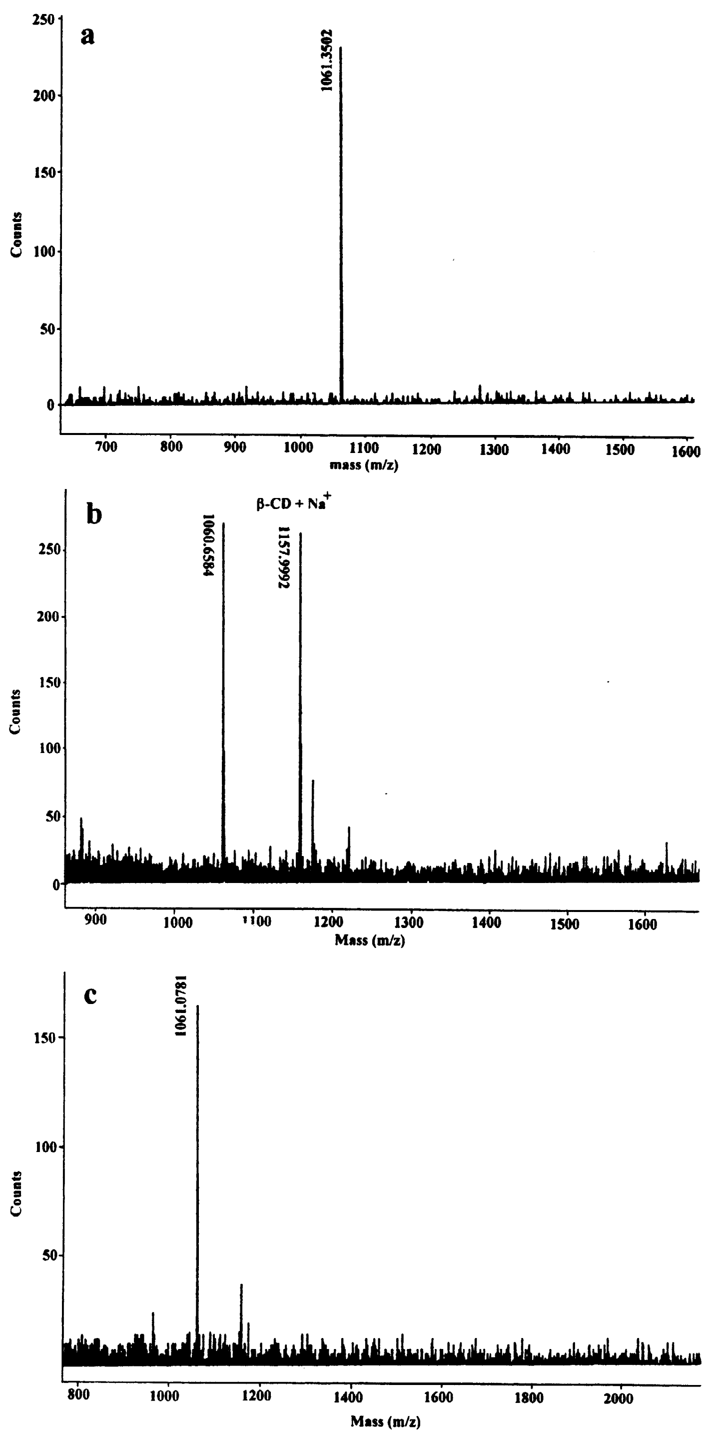


Fig. 3. Effect of γ-CD and β-CD on the MALDI-TOF analysis of Fr 14. (a) γ-CD at a concentration of 40 m*M* in ammonium acetate (50 m*M*); (b) β-CD at a concentration of 15 m*M* in sodium phosphate (50 m*M*); (c) β-CD at a concentration of 15 m*M* in ammonium acetate (50 m*M*). Operation conditions as those given in Section 2.

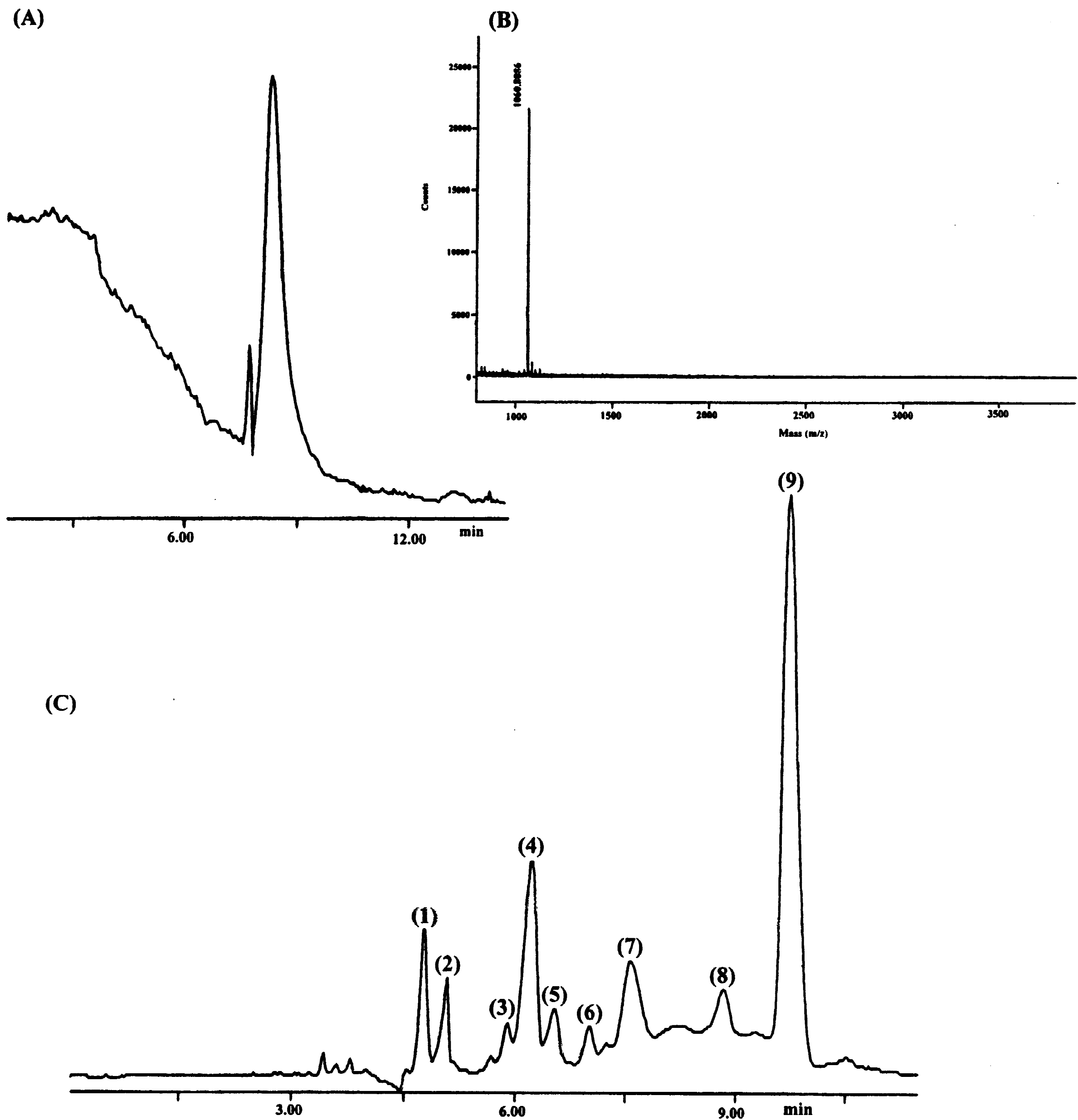


Fig. 4. Electrophergrams obtained running the peptide mixture in capillary. Operation conditions: BioFocus™ 3000 Capillary Electrophoresis System (BIO RAD, CA, USA), equipped with a UV detector monitoring a wavelength of 214 nm. A fused-silica capillary 38 cm (34 cm effective length)×75 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) was kept at a constant temperature of 25.0°C and the applied voltage was 10 kV. (A) CZE separation of the peptide mixture, BGE was 10 m*M* acetate buffer, pH was measured to 7.01; (B) Mass spectra obtained analysing CZE fraction (A) by MALDI-TOF; (C) MEKC analysis of Fr 14; BGE was 20 m*M* SDS in ammonium acetate (10 m*M*).

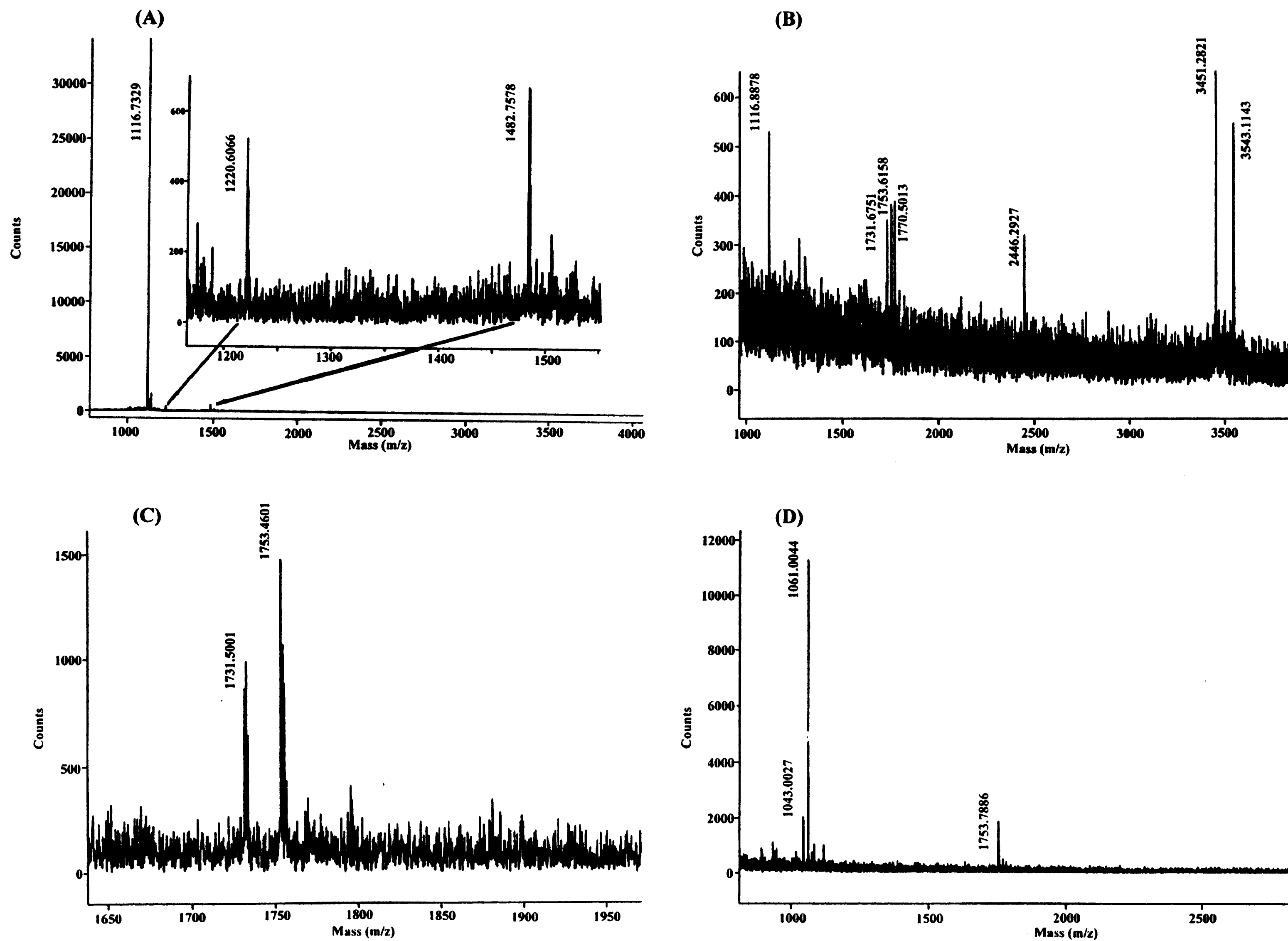


Fig. 5. MALDI analysis of the collected fractions from MEKC analysis of Fr 14. Operation conditions as those given in Table 2 and Fig. 4. Mass spectra obtained analyzing (A) fraction 1 (peaks 1, 2, 3, and 4); (B) analyzing fraction 2 (peaks 4, 5, 6, 7, and 8); (C) fraction 3 (peak 8); (D) fraction 4 (peaks 8 and 9) by MALDI-TOF-MS.

Table 2
Mass determination of Fr 14 by MALDI-TOF analyzing collected fractions from MEKC using SDS as a pseudo stationary[a]

Fraction	Peaks (number)	Observed masses (Da)
1	1–4	1116.7329; 1220.0606; 1482.7578
2	4–8	1116.7329; 1731.6751; 2446.2927; 3451.2821; 3543.1143
4	8	1731.5001
5	8, 9	1753.7886 (1731.5001+Na); 1060.0502

[a] Separation conditions: ammonium acetate (10 m*M*, pH=7.0); SDS as surfactant was dissolved in the BGE at a concentration of 20 m*M*; column: 40 cm (36 cm effective length)×75 μm I.D.; fused-silica capillary thermostated at 25°C; detection: 214 nm; applied voltage: 15 kV: current: 18 and 30 μA in the presence of SDS; MALDI conditions as those given in Section 2.

proteins to the capillary wall [15,16]. Urea is occasionally used in MEKC to mediate hydrogen bonding. It was found to have no impact on ionization at concentrations up to 500 m*M*. Organic solvents, such as methanol and acetonitrile, are employed as additives to improve solubility, reduce interactions with the capillary wall, and to alter the partition coefficient with micelles. Concentrations of methanol from 5 to 50% suppressed ionization. Again a mechanistic interpretation of these results is not possible other than the speculation that they interfere with the adsorption of analytes to matrix crystals.

4. Micellar electrokinetic chromatography off-line coupled to MALDI

An analysis of the peptide sample Fr 14 by capillary zone electrophoresis (Fig. 4A) and MALDI-MS (Fig. 4B) produced single peaks in both cases, appearing to be a single component. The only thing in this data that would bring this conclusion into doubt would be the fact that the peak from electrophoresis is broad, suggesting other partially resolved species might be present. This possibility was examined using MEKC with 20 m*M* SDS in 10 m*M* ammonium acetate at pH 7.0. Fig. 4C shows that Fr 14 is indeed a mixture, containing at least nine components. Fractionation of this sample with the MEKC system as shown in Table 2 with subsequent analysis of the fractions by MALDI-MS (Fig. 5) revealed that each of these components differed in molecular weight.

This raises the question of how Fr 14 could appear to be a pure compound in all the spectra presented in the first part of this paper when it was actually a mixture. Clearly this is a case of the widely reported phenomenon of analyte quenching, i.e., one analyte species present at higher concentrations suppressing the ionization of others. From this it may be concluded that fractionation of all components in a mixture is critical if it is the objective to determine sample purity.

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