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Analysis of histones by on-line capillary zone electrophoresis–electrospray ionisation mass spectrometry

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Abstract

The on-line combination of capillary electrophoresis and electrospray ionisation mass spectrometry was applied for the determination of some basic histones from calf thymus. The separation performance of those basic proteins was significantly improved by coating the capillaries with hydroxypropylmethylcellulose. The coating appeared to mask effectively the underlying silanol groups thus avoiding undesirable adsorption of the histones onto the capillary walls, while it was also shown to be an effective way to avoid contamination of the mass spectrometer. Finally, capillary electrophoresis–electrospray ionisation mass spectrometry with coaxial sheath liquid was successfully applied to the analysis of histones using a simple dialysis step of the sample as sample pretreatment.

Keywords: Coated capillaries; Histones; Proteins

1. Introduction

Histones are a group of proteins which are found to be associated with DNA in the chromosomes. They are small proteins (with molecular masses in the range of 12 000–20 000) and extremely alkaline (isoelectric points (p*I* values) of ~11.5). Their alkaline character can be explained by the content of lysine (Lys) and arginine (Arg) resulting in highly positive charges, which are responsible for their electrostatic interactions with the DNA chains to form the nucleosomes. There are several types of histones and their classification is based on the relative amounts of lysine and arginine which they contain [1].

Histones play an important role as the major structural proteins of chromatin but they also serve as regulators of gene expression [2,3]. Because of their importance, there is a need for analytical techniques enabling the rapid separation and sensitive detection of these proteins. High-performance liquid chromatography (HPLC) represents a good approach for the separation of histones, but although the achieved resolution can be good for some of these compounds, the complete separation for others has not been possible yet [4–6]. Recently, capillary zone electrophoresis (CZE) has been shown as an effective alternative for the determination of various kinds of basic proteins including the histones [7–10].

Separation of basic proteins such as histones by capillary electrophoresis with fused-silica capillaries is impaired by interactions of the proteins with the silanol groups present on the inner wall of the fused-silica capillary resulting in band broadening and tailing which reduces the separation efficiencies

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and leads to poor reproducibilities [11–13]. There are several ways to overcome the problem of protein adsorption, for instance the use of acidic or alkaline electrophoresis buffers. However, the use of extreme pH values tends to denature the proteins [13]. Another strategy to circumvent these problems is the use of a suitable additive in the electrophoretic buffer to dynamically mask the silanol groups. Various compounds have been used as electrolyte additives including amines, cationic surfactants, etc. [12,13]. However, when these kind of additives are used, the main problem encountered with the on-line coupling between CE and mass spectrometry (MS) is the risk of contamination of the MS source since the non-volatile additives will rapidly accumulate on the surfaces of electrospray source parts and deteriorate the sensitivity of detection [14,15].

Coating the capillary surface with a polymeric layer either by covalent binding or by adsorption has been demonstrated as a good alternative to prevent more efficiently interactions with the wall and to avoid MS contamination [16,17].

The on-line detection of the analytes separated by CE is often carried out by means of a UV–visible detector. However, in the last few years the use of MS has increased [18–21]. On-line coupling of capillary electrophoresis with electrospray ionisation-mass spectrometry (CE–ESI-MS) is a promising combination of two analytical techniques; while CE provides highly efficient separations of different kinds of analytes, MS can provide molecular mass information as well as structurally related information for the analytes. Currently, electrospray ionisation serves as the most common ionisation method for CE–MS applications. The most widely used interface for commercial CE–ESI-MS instrumentation is the sheath-flow arrangement [20,21]. In this interface, a coaxial sheath liquid is introduced and it provides electrical contact at the CE capillary terminus and also generates the necessary flow for a stable electrospray.

To date, there are few examples showing the determination of histones by using the off-line combination between CZE and ESI-MS [6]. In this paper we report on the on-line coupling between CZE, using a new developed coating based on hydroxypropylmethylcellulose (HPMC), and ESI-MS. Different parameters affecting the separation and detection have been investigated. The introduction of a dialysis step prior to the separation has been studied to eliminate low-molecular-mass analytes present in the samples, which can interfere in the electrospray process.

2. Experimental

2.1. Reagents and standards

Acetic acid and formic acid were purchased from J.T. Baker (Deventer, The Netherlands). Glycidoxypropyltrimethoxysilane was obtained from Aldrich Chemie (Steinheim, Germany). Boron trifluoride diethyletherate (BF_3) was from Riedel-de Häen (Seelze-Hannover, Germany). HPMC with a viscosity of 4000 cP for a 2% aqueous HPMC and the histone standards, II-AS, III-S and VII, all from calf thymus, were obtained from Sigma (St. Louis, MO, USA). Deionised water (Millipore, Bedford, MA, USA) was used for the preparation of the samples and electrolyte solutions.

2.2. Capillary coating procedure

The capillaries were coated according the procedure for coating methylcellulose described by Liao et al. [22]. In the present report, methylcellulose was changed for HPMC. Briefly, first the capillary was extensively washed with acetone, water and then with NaOH and HCl, both at a concentration of 0.1 *M* during 5 min. Afterwards, the capillary was left overnight in a 5% (v/v) solution of glycidoxypropyltrimethoxysilane in chloroform. After washing with acetone the capillary was then filled with 0.5% (w/v) HPMC solution in water and placed in an oven at 120°C for 50 min. These steps leave a thick HPMC layer dried onto the capillary wall. After flushing the capillary with acetone, the HPMC was chemically bonded to the epoxy group of the silane by filling the capillary with a solution of 3% (v/v) BF_3 in water, which acts as a catalyst, and in this way the stationary coating was created. The capillary was finally rinsed with acetone and water and it was stored in 0.1% (v/v) acetic acid until use.

2.3. Sample pretreatment

Samples containing the proteins under study were pretreated using dialysis prior to the separation. To do that, samples were filtered by using a dialysis membrane, which was placed into a conventional filtration system for a period of 3 h. After that, the samples were ready for their analysis.

The molecular mass cut-off of the dialysis membranes (Dianorm, München, Germany) used for the desalting of the protein samples was 5000.

2.4. Capillary zone electrophoresis

High-performance capillary electrophoresis was performed on a Beckman P/ACE 2200 system with a UV detector with wavelength filters of 200, 214, 254 and 280 nm (Beckman, Fullerton, CA, USA). Data collection and post-run data analysis were carried out using System Gold software (Beckman Instruments). For the coupling with the MS a PrinCE (Prince Technologies, Deventer, The Netherlands) CE system was used. A fused-silica capillary (LC-Service, Emmen, The Netherlands) or an HPMC coated capillary column of 77-cm total length and 75-μm I.D. were used for the separation. A detection window was made 70 cm from the capillary inlet by burning off the polyimide coating when non-coated capillaries were used and by scraping off the polyimide layer by using a scalpel for the coated capillaries.

New uncoated capillaries were conditioned sequentially with 0.1 *M* NaOH for 30 min, water for 20 min and electrolyte for 20 min. At the beginning of each experimental day, the capillary was washed sequentially with NaOH 0.1 *M* for 10 min, deionised water for 10 min and electrolyte for 15 min. Between the runs the capillary was flushed with the background electrolyte for 5 min. The HPMC coated capillaries were pretreated before their first use with the run electrolyte for 30 min and prior to each injection the capillaries were rinsed for 5 min by flushing with the running electrolyte.

The protein samples were dissolved in deionised water at a final concentration of 1 mg/ml and were injected in the hydrodynamic mode for 5 s. After a sample was loaded, the run was initiated by applying 15 kV across the capillary. Monitoring the UV absorbance at 200 nm performed on-column detection. Runs were carried out in 5% acetic acid or 0.1 *M* formic acid containing 0.02% HPMC as the background electrolyte (BGE) when uncoated capillaries were used. When coated capillaries were used the electrolytes were 5% acetic acid or 0.1 *M* formic acid.

All solutions were filtered through a 0.2-μm nylon acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA) prior to their use.

2.5. CE–ESI-MS

MS experiments were performed on a Finnigan MAT TSQ 700 mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray interface (Analytica, Brandford, CT, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid which consisted of methanol–water (80:20, v/v) containing 1% acetic acid and was delivered at a flow rate of 2 μl/min by a Model 2200 syringe pump (Harvard, Holliston, MA, USA). The mass spectrometer was operated in the positive ion mode. The spectrometer was scanned from m/z 500 to 1000 at 3 s/scan during separation and detection.

For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation capillary was fitted into the electrospray needle of the ion source and a continuous flow of conductive sheath liquid established electrical contact between capillary effluent and electrospray needle. MS operating conditions were optimised by adjusting the needle-counter electrode distance, liquid sheath flow rate and applied electrospray potentials while a standard solution of histone III-S, after its dialysis, was continually introduced through the CZE–ESI-MS interface. The same optimised conditions were subsequently used for the analysis of the histones.

2.6. Matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI–TOF-MS)

MALDI–TOF-MS was used for the confirmation of the molecular masses of the histones under study. MALDI–TOF-MS was performed on a Vision 2000 instrument (Finnigan MAT). The instrument oper-

ated with a nitrogen laser at 337 nm, while 2,5-dihydroxybenzoic acid (DHB) was used as matrix (Aldrich Chemie).

3. Results and discussion

3.1. Optimisation of the capillary electrophoretic separation

The first goal in this paper was to find the optimum electrophoretic conditions for the separation of the histones included in this study. In previous applications, CZE combined with UV detection was applied to the separation of histones by using a phosphate buffer [7–10]. In the coupling of CE with ESI-MS, the selection of the buffer system should consider not only the CE resolution but also the compatibility of the buffer with the detection system [20–23]. Therefore, we tried formic acid and acetic acid as alternatives to phosphoric acid.

By using 0.85 *M* acetic acid or 0.1 *M* formic acid, migration of the histones was not initially observed, even after 1 h. This indicates a strong adsorption of the histones onto the fused-silica surface, even at the low pH values used in these experiments, as a result of the interaction between the different functional groups of these proteins with the active sites on the inner surface of the fused-silica capillaries giving rise to peak broadening and asymmetry and irreproducible migration times. After several injections under the above-mentioned conditions we observed excessive peak tailing and migration time repeatability was rather poor. Fig. 1 shows the electropherogram of the histones obtained by using 0.1 *M* formic acid as the electrophoresis buffer combined with an uncoated capillary.

It has been reported that the interaction between the proteins and the capillary wall can be eliminated by using an acidic pH of the electrophoresis buffer [13]. Unfortunately, from our experiments it appeared that the histone binding to the capillary walls could not be prevented in this manner.

There are several other ways to suppress or minimize the problem of the protein adsorption onto the surface of the capillary walls [12,13]. One of these ways is the use of an additive into the background electrolyte for a dynamic wall coating.

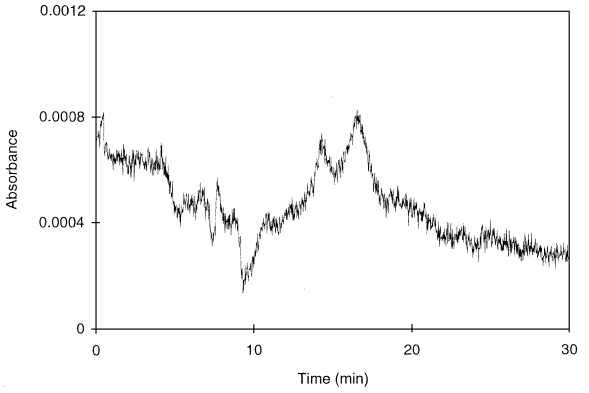

Fig. 1. Electropherogram of a standard histone solution of 1 mg/ml. Experimental conditions: electrolyte, 0.1 *M* formic acid; applied voltage, 15 kV; uncoated capillary of 77 cm (effective length 70 cm)×75-μm I.D.; hydrodynamic injection for 5 s.

Therefore, we tested the suitability of some neutral polymers such as HPMC and poly(vinyl alcohol) (PVA) since it has been reported previously that HPMC can improve the resolution in the separation of some histones when it is added to the electrolyte [8,9].

After having added HPMC to the electrophoresis buffer, sharper peaks and better separation were obtained in comparison with the previously reported results when this additive was not used. We studied the effect of adding different concentrations of this additive in the range between 0.005 and 0.1% and from the obtained results it could be concluded that the best separation between the histones was obtained when a concentration of 0.02% was used showing that even low levels of HPMC provide sufficient dynamic coating for histone separations thus preventing the protein–wall interactions. Higher concentrations did not improve the results.

Under the best conditions obtained for the HPMC addition, with amounts of 0.02%, we investigated the influence of the type of electrolyte and its concentration on the resolution and the peak shape of the compounds to be separated. Acetic acid and formic acid were chosen as electrolytes due to their volatility to avoid any contamination of the ESI device. The effect of the concentration of both acids in the separation of the histones was studied. UV absorbance detection was used for these experiments to expedite the evaluation process and to avoid com-

plexities of MS detection. When acetic acid was used, changing the electrolyte concentration from 50 m*M* to 1.5 *M* had a negligible effect on the mobility order of the compounds. However, as expected, changing the acetic acid concentration significantly influenced the peak shapes, since as the electrolyte concentration increased, resolution also improved. At concentrations below 400 m*M* the peaks of the proteins became seriously broadened and the theoretical plate numbers were dramatically decreased. Li described that silanol groups on the fused-silica wall can exist in ionic form (SiO^-) at a higher pH than 2 [24]. We therefore assumed that the broad peaks were due to some adsorption of the protonated histones onto the capillary wall by ion exchange. On the other hand, at higher concentrations of acetic acid, the efficiency of the histones was increased and a good resolution between these proteins was obtained at 1 *M*.

We also investigated formic acid as the background electrolyte in the same concentration as used for acetic acid. In this case we chose 0.1 *M* as the electrolyte concentration since by using this concentration a good separation of the histones was obtained. When concentrations higher than 0.5 *M* were used, CE currents were unstable throughout the separation, probably due to Joule heating.

Both acids enable the highly efficient separation of the proteins under study. Finally, we choose an electrolyte containing 0.1 *M* formic acid since this was regarded as optimum for the separation of the histones included in this study with respect to maximum separation efficiency in combination with minimum analysis time.

The addition of an organic solvent was also tested to improve the separation between the histones. It has been reported by some authors that these substances can affect the conductivity and viscosity of the BGE and as a consequence both the electrosmotic flow and the electrophoretic mobility of the analytes are affected [25]. The influence of methanol and acetonitrile was investigated. To do that, both solvents were added in a concentration ranging from 0 to 60% (v/v) to the BGE. The general trend was a decrease in the electroosmotic mobilities with increasing the organic content of the BGE; this effect was more pronounced with methanol because of the increased buffer viscosity. After studying the organic solvent effect it could be concluded that no significant differences were observed with respect the results obtained previously when the organic modifiers were not added except than migration times were slightly longer, but the addition of organic solvent provided no improvement in either peak shape or resolution.

Rinsing of the capillary between analyses is important in achieving run-to-run reproducibility since the proper selection of this intermediate step has a serious impact on the consistency of the migration times and also on the efficiency of the separation between the analytes. In this step, the inner surface of the capillary is reconditioned and adsorbed molecules are eliminated. To study the influence of the rinsing conditions on the response, different combinations were examined: rinsing by deionised water only, by 1 *M* or 500 m*M* acetic acid, by BGE and by water or acetic acid followed by BGE. Each rinsing step was studied at different periods of time, ranging from 2 to 10 min. After all the experiments it appeared that rinsing with BGE only or by the mixture with water or acetic acid followed by the BGE were the best conditions. When only deionised water was used the peaks of the histones were very broad and the separation was degraded meaning that the adsorption of the histones was not avoided. By using only acetic acid or formic acid the response was also affected; in general we obtained lower migration times but some of the peaks co-eluted, although the acids apparently removed the adsorbed proteins from the capillary wall. Finally, we chose electrophoresis buffer for the rinsing step since it leads to highly reproducible migration times and also the efficiency of the separation is not affected.

The efficiency of PVA as an additive was also studied. PVA is often used as a dynamic coating in the separation of proteins [13]. We investigated the effect of adding PVA in different concentrations, ranging from 0.005 to 0.1%, to the electrophoresis buffer. As expected, the electroosmotic mobility decreased as the concentration of PVA became higher; this effect could be partly due to increased viscosity. However, the separation efficiency of the system containing PVA was poor in comparison with that obtained when HPMC was the additive, since broader peaks were observed. Although PVA was

noticed to reduce the interactions between the analytes and the capillary wall, HPMC appeared to be more effective.

3.2. Coating performance

Based on the good results we obtained with the addition of the HPMC to the electrophoresis buffer, we tested the performance of a new developed coating by using this polymer to overcome the inherent problems of the introduction of involatile buffer additives into the mass spectrometer.

The coating procedure was based on that proposed previously by Liao et al. [22] for a methylcellulose coating. In our case, to determine the optimum HPMC concentration for the coating, the performance of different coated capillaries with HPMC in a concentration range from 0.05 to 1% (w/v) was investigated. Higher concentrations of HPMC were not suitable to produce a polymer solution with adequate viscosity for coating a capillary. According to the obtained results it could be concluded that when low concentrations of HPMC were used, broad peaks for the histones were observed, which is indicative of adsorption of the compounds on the capillary wall, so in those conditions the underlying silanol groups were not completely masked. The best results were obtained for an HPMC concentration of 0.5%.

Using this type of coating, the electroosmotic flow was completely suppressed.

When HPMC coated capillaries were used for the separation of histones good results were obtained which were comparable to those previously obtained when HPMC was used as dynamic coating. Fig. 2 shows the electropherogram of a standard mixture of histones. Comparison with Fig. 1 clearly demonstrates the enormous gain in efficiency. The long-term stability of the coating was evaluated by performing repetitive analyses; no drift in migration times due to loss in separation efficiency was observed even after 70 analyses.

An important requirement for the longevity of a coating is the washing procedure between runs. In the present case, rinsing with the electrophoresis buffer gave the best results since the reported migration times were very stable.

The use of an organic solvent was also tested but in this case the HPMC coating was destroyed when methanol or acetonitrile were added to the BGE.

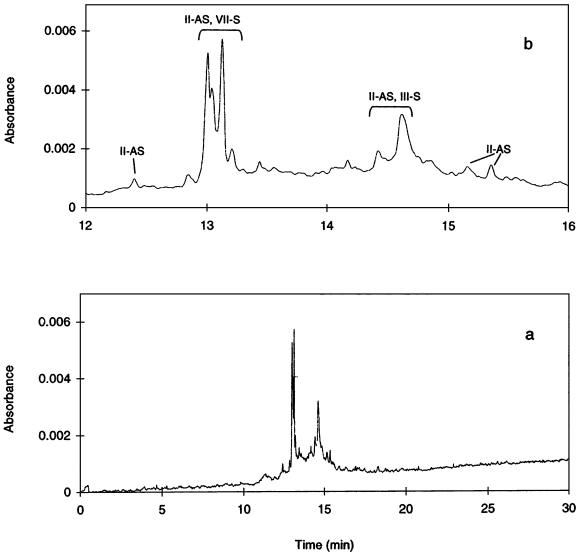

Fig. 2. Electropherogram of a standard histone solution of 1 mg/ml in an HPMC-coated capillary using 0.1 *M* formic acid as background electrolyte. Capillary dimensions: 77-cm total length, effective length 70 cm, 75-μm I.D.; applied voltage, 15 kV; hydrodynamic injection for 5 s; UV detection 200 nm. (b) Region between 12 and 16 min.

3.3. On-line ESI-MS

For the direct coupling of capillary electrophoresis with mass spectrometry we used a coaxial sheath-flow interface, which is described in detail in a previous study by Smith et al. [26].

In the first series of experiments, standard solutions of the histones were directly injected into the MS. The sample solutions of each individual histone standard were infused directly into the electrospray interface at 3 μl/min with the syringe pump. MS conditions were optimised to produce the highest signal intensity but unfortunately, histones were not detected by positive ESI. The possible reason was the salt content in the standards since in the procedure employed to isolate the histones from calf thymus some salts are used and consequently, matrix constituents can affect the spray stability as well as the ionisation efficiency. For this reason these sam-

ples required treatment before ESI-MS [27]. Some experiments were also carried out by MALDI–TOF-MS because MALDI is a relatively robust technique with regard to such sample components as salts and counter-ions, but unfortunately, no answer was obtained for the histones.

Several sample pretreatment techniques were tested to remove those compounds, which can interfere in the MS response. Among the different approaches tested, such as solid-phase extraction by using a C_{18} zip-tip, dialysis was shown to be the best. In the dialysis procedure, the proper selection of the molecular mass cut-off (MWCO) of the membrane is extremely important since if the pores are too large, the analyte ions will migrate through the membrane together with the matrix ions. On the other hand, if the membrane pores are too small, the ions to be eliminated will remain in the sample solution. Initially, in our case we tried dialysis of the samples with a membrane of an MWCO of 10 000, since, as reported by Sigma, the molecular mass of the histones is in the range between 11 000 and 20 000 and ideally, to avoid losses of the analytes, the MWCO of the membranes should be chosen according to the M_r of the analytes. Unfortunately, when using that membrane we did not obtain any response on the MS, probably due to loss of the analytes during dialysis because the nominal MWCO of the membrane is larger than the molecular mass of the histones. Finally, a membrane of an MWCO of 5000 was tested. In this case, when the samples were introduced into the MS system, we could obtain a response for the histones. Apparently, as a consequence of the desalting step, the levels of non-volatile salts in the samples were reduced to that extent that the ionization efficiency of the histones was sufficiently increased.

The next step in this study was the optimisation of the operating conditions of the ESI-MS to produce a signal of maximum stability and sensitivity. Different parameters such as the distance between the outlet end of the capillary and the electrospray needle, the liquid sheath flow rate and composition, and the applied electrospray potentials were studied. These parameters were optimised by monitoring the signal of histone III-S, which was infused through the CE capillary into the ESI source.

The composition of the sheath liquid affects the ESI sensitivity. In addition, it also played an important role for the degradation of the CE resolution. Volatile reagents such as formic acid or acetic acid in methanol–water solutions have been proven to be good combinations as sheath liquids in CE–ESI-MS [28]. We tested the influence of methanol composition in the sheath liquid over the range between 20 and 90%. A value of 80% was finally chosen since with this the highest sensitivities were obtained for the histones.

By keeping the methanol percentage constant, different sheath liquid modifiers, such as acetic acid and formic acid, were also investigated. In our case, the presence of acetic acid gave the highest signals for our compounds; moreover, the use of formic acid resulted in a negative effect on the sensitivity because of increased background noise. Thus, for subsequent CE–ESI-MS experiments an aqueous solution of 80% methanol in the presence of 1% acetic acid was used as sheath liquid.

Fig. 3 shows the total ion electropherogram obtained under optimised CE–ESI-MS conditions. As can be observed, no significant band broadening was obtained in comparison with the CE–UV electropherograms. For the molecular mass determination of the histones under study scans were extracted from the obtained electropherogram and averaged to give the mass spectrum for each peak. However, in this case, for every peak we obtained rather complex spectra thus indicating the presence of additional compounds. To illustrate this, Fig. 4 shows the positive ion spectra of some of the peaks obtained in the total ion electropherogram for the mixture of histones. Different algorithms have been developed to resolve the complex mixtures, but unfortunately for the studied histones, the deconvolution of the series of multiply charged ions obtained for each peak to get a pure spectrum was very difficult due to the complexity of the sample. It was only possible to determine the molecular mass of some of the peaks. The reported values are shown in Table 1. It should be mentioned that for histone II-AS this task was specially difficult due to the high inhomogeneity of this compound since it is a mixture of all kind of histones as reported by Sigma.

To verify the data obtained for the histones with ESI-MS, MALDI–TOF-MS was used. Fig. 5 shows the corresponding mass spectrum for the mixture of

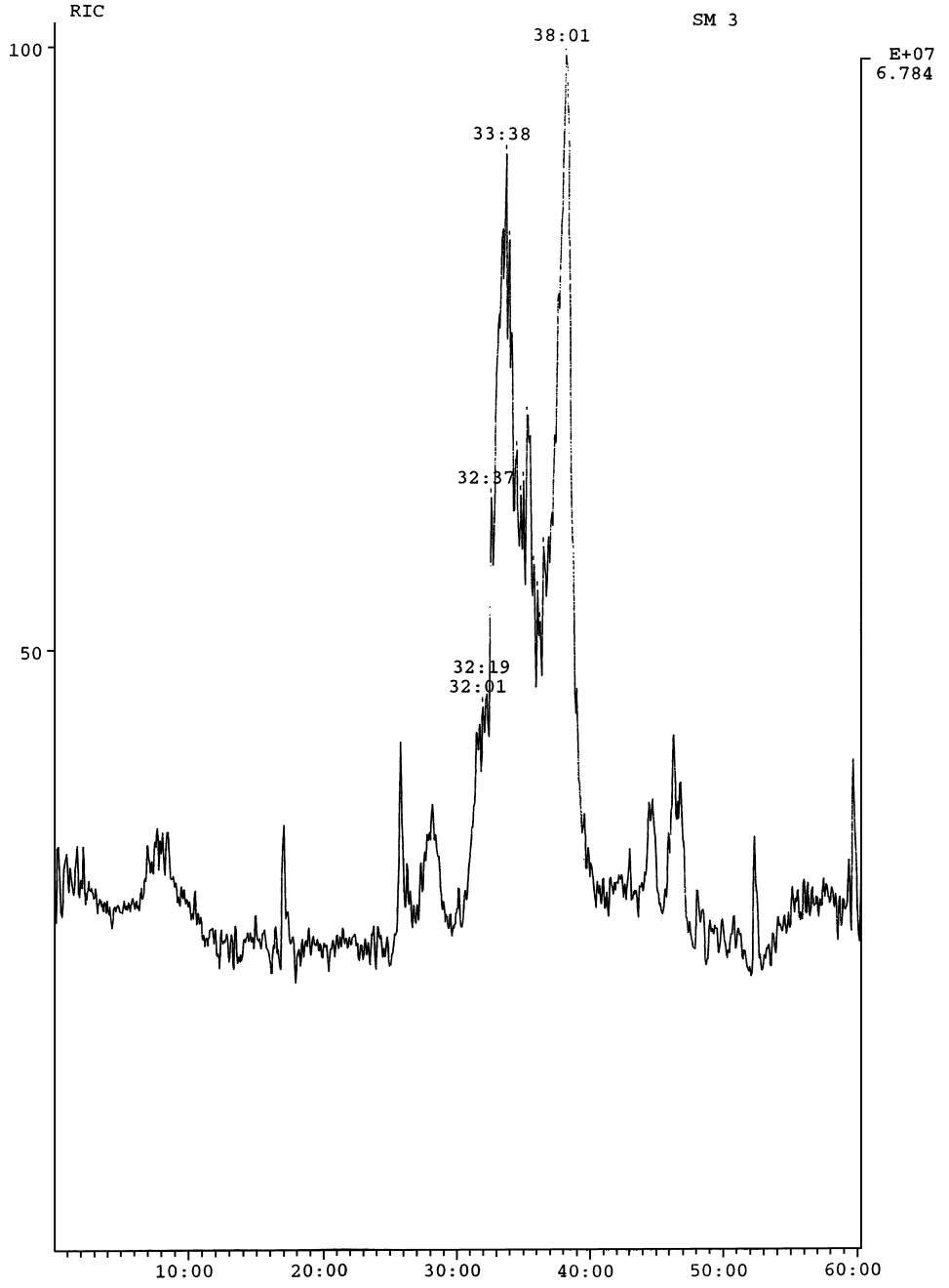

Fig. 3. On-line CE–ESI-MS analysis of histones. The CE conditions are the same as reported in Fig. 2. MS conditions: sheath liquid, methanol–water (80:20, v/v) containing 1% acetic acid; sheath flow, 3 μl/min; MS scan range, m/z 500–1000.

the histones. As can be observed, several peaks were obtained and the assigned molecular masses found were in the range between 10 000 and 22 600. Through the comparison of the molecular masses from both methods we observed systematic differences especially for the lower masses.

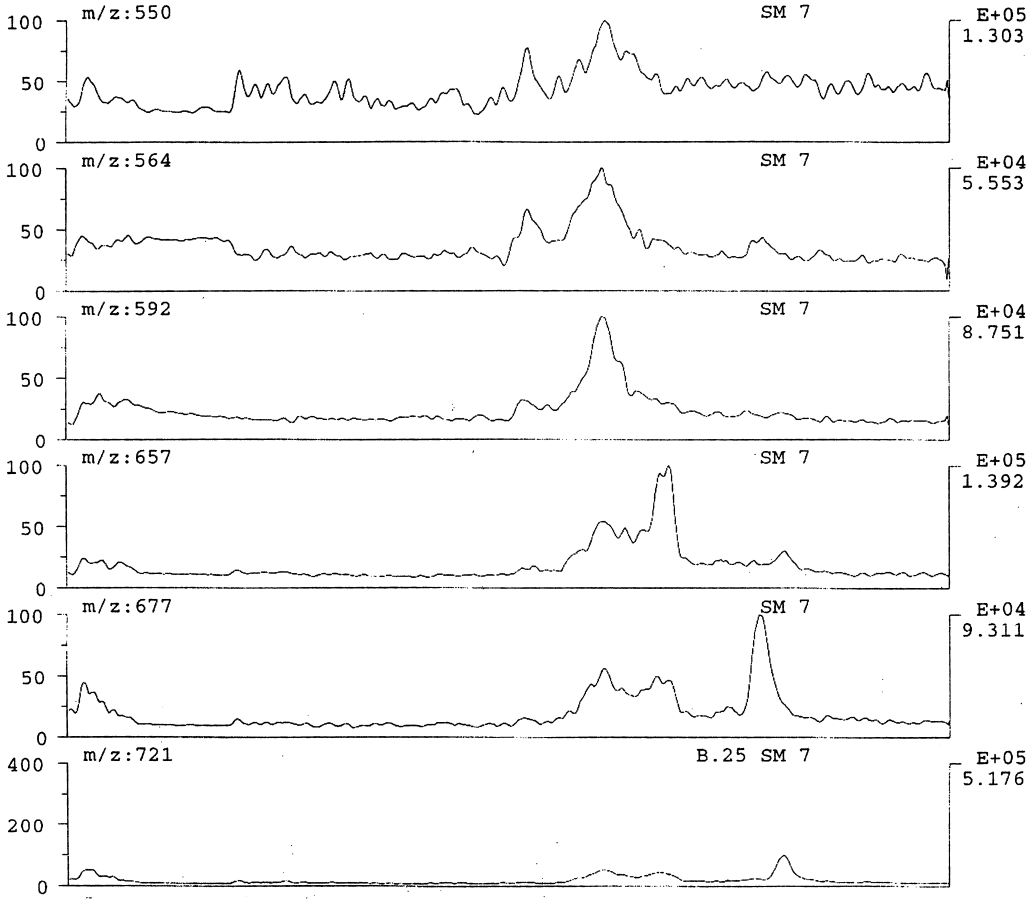

Fig. 4. Extracted multiply charged ion from the CZE–ESI-MS analysis of histones. Number of charges ranging from 24 to 36.

The use of a mass spectrometric detector is very useful in determining the masses of the analysed proteins. Through them it is possible to determine post-translational and chemical modifications of the proteins since they induce mass changes. So, a mass spectrometric detector provides an excellent tool in the proteomics field and this is a clear advantage over other conventional detectors such as UV.

Table 1
Molecular masses of calf thymus histones obtained by ESI

Histone	Molecular mass
H-III-S	21 264
	21 795
	22 065
	22 598
H-VII	13 250
	13 774

4. Conclusions

The HPMC coated capillaries used in this study were found to be quite effective at minimizing the problems of adsorption associated with the solute–wall interactions. These capillary characteristics are highly desirable for on-line CE–ESI-MS coupling since the contamination of the ion source for the introduction of additives in the background electrolyte is avoided.

The developed on-line CE–ESI-MS method allows the separation and identification of some histones from calf thymus although a dialysis step was

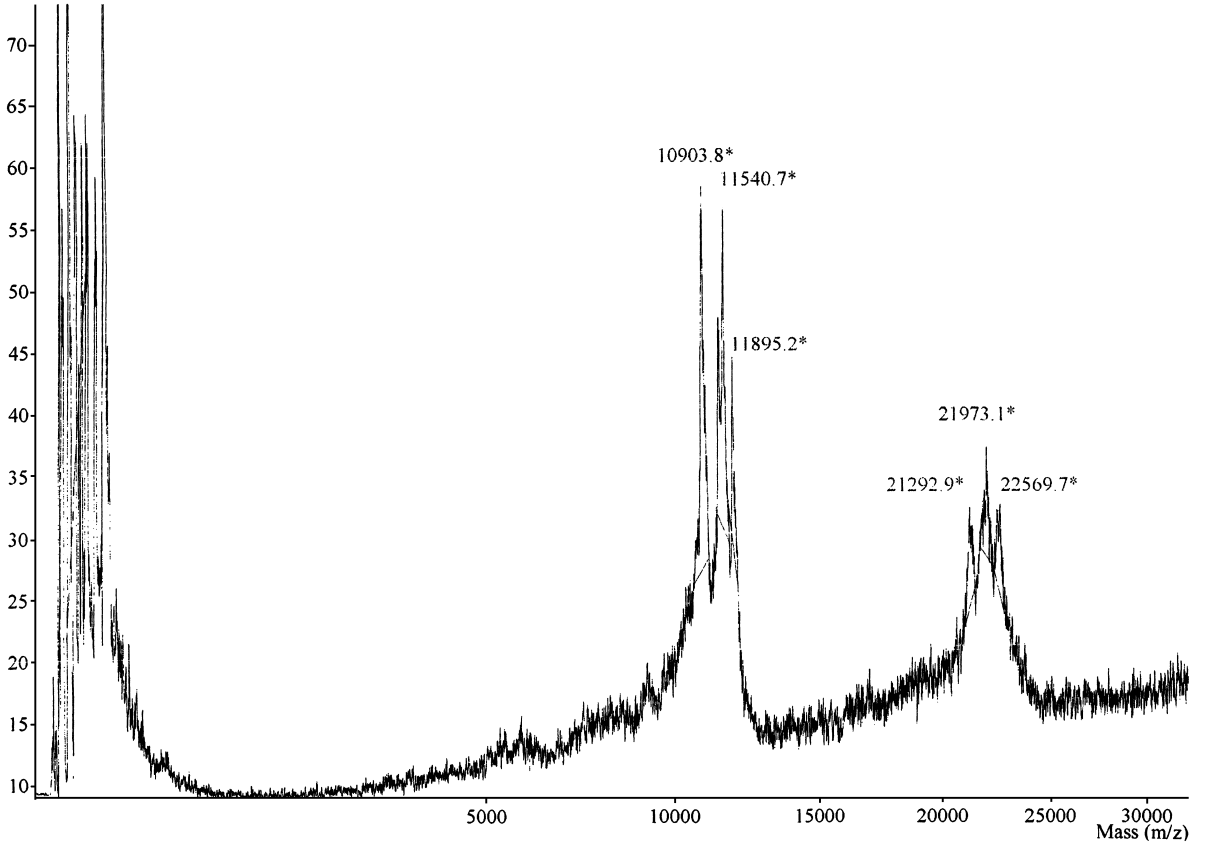

Fig. 5. MALDI–TOF-MS spectrum of a standard histone solution of 1 mg/ml. Dihydroxybenzoic acid was used as matrix and laser wavelength was 337 nm.

required prior to the analysis to eliminate undesirable compounds in the sample matrix.

These preliminary results obtained for CZE–ESI-MS of histones clearly show promise and further work will be focused on expanding this preliminary data.

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