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Applicability of predictive models to the peptide mobility analysis by capillary electrophoresis–electrospray mass spectrometry

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Abstract

The prediction of peptide mobility by capillary electrophoresis (CE) coupled to electrospray mass spectrometry (MS) is studied in order to verify the validity of the semi-empirical models developed in classical CE. This work relies on the experimental determination of the electrophoretic mobilities of 68 peptides, different in charge and in size. The results indicate that the prediction is possible in CE–MS experiments, in spite of the restraints inherent in the coupling conditions. The best fit of experimental data was obtained with the Offord's model. The efficiency of the model was confirmed by the analysis of a peptide mixture in CE–MS.

Keywords: Electrophoretic mobility; Mathematical modelling; Peptides

1. Introduction

Short peptides (relative molecular mass (M_r) < 1000), obtained from protein hydrolysates, present a great interest for food, pharmaceutical and cosmetic industries because of their nutritional and biological properties [1,2]. Often, these peptides stand in some complex mixtures of structurally related compounds. In order to favor their valorization, these complex solutions need to be well characterized, by a precise identification of the different peptides. That need requires some powerful analytical technique, able to separate the peptides and to identify them. Until nowadays, the most common technique to achieve that operation was the coupling between liquid chromatography (LC) and mass spectrometry (MS), conducted on reversed-phase columns [3]. Peptides were separated according to their hydrophobia coefficient, then identified by MS. Nevertheless, this technique suffers from a deficiency to well-separated short peptides because of the very small differences of hydrophobia between most of them.

In the last 15 years, the advantages of the capillary electrophoresis (CE) in the separation of peptides have been largely demonstrated [4]. This technique combines actually a high-resolution power, a high sensitivity and a low analysis time. CE is also efficient to obtain some information about the identity, the purity and some structural changes of peptides. Moreover, some theoretical models have been developed, directly linked to the physicochemical properties of peptides [5]. In all these models, the electrophoretic mobility is derived from Stocke's law and is related to q/r_s, where q is the charge of the peptide and r_s its Stocke radius. Most of the semi-empirical models calculate q from the ionization constants of the amino acids and relate r_s to the M_r of peptides. From that common basis, there are some discrepancies in the literature for the dependence degree of mobility on charge and on peptide size. Actually, according to the model, the calculation of the charge, which is mainly based on the Henderson–Hasselbalch equation, takes into account, or not, the effects of the electrostatic charge suppression. The other difference between the models stands in the assumptions involved in the relation between r_s and M_r [6]. These assumptions are established in function of the peptide size, the charge density and the ionic strength of the buffer [7]. As a consequence, the best model changes theoretically in function of, on one hand the nature of peptides used to establish the correlation and, on the other hand, the buffer system chosen. For this reason, no model is universally accepted.

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In classical CE–UV, these models offer the possibility to predict the electrophoretic mobility of a peptide from knowledge of its amino acid content. Yet, the opposite, i.e. the prediction of the amino acid content from the electrophoretic mobility is not possible since the experimental electrophoretic mobility depends on two parameters: the charge and the size. So, in CE–UV, these models do not allow the identification of unknown peptides. For this reason, we have investigated the coupling of CE with MS. Several authors have previously studied the capacity of CE–MS to characterize some standard peptides [8–10] and some digests of standard proteins [11–13]. Actually, MS offers the advantage to supply the molecular mass of the peptides. For each peptide, the determination of its amino acid content would be then possible from the knowledge of its electrophoretic mobility and its mass, which allows estimation of its charge.

The association of CE with MS is so become an important, if not necessary, analytical tool to identify peptides using a semi-empirical model. Recently, such a model has been applied in CE–MS, which allowed the identification of peptides with post-translational modifications in digest of human myelin basic protein [14]. Yet, no work has focused on the possible variation between the model quality between CE–UV and CE–MS. In fact, compared to CE–UV, the coupling of CE with MS generates some technological restraints, which can end in some differences in the accuracy of prediction models. Firstly, the experimental conditions differ in the buffer composition and the capillary length. Secondly, the absence of the capillary temperature regulation from the exit of CE to the entrance in MS can lead to a disruption in the peptide mobility in CE–MS mode. Finally, a loss of resolution when the coupling is carried out by a sheath–liquid interface has been described, due to the migration of liquid sheath counterions into the separation capillary [15].

Then, in the present study, we have investigated the applicability of the semi-empirical models, currently used in classical CE–UV, for the coupling CE–MS. The electrophoretic mobilities from a set of 68 peptides have been used to establish the correlation level of these models, just as well in CE–UV as in CE–MS. These results are firstly discussed as a function of the nature of the model and as a function of the influence of some other parameters, able to affect the separation of peptides. In a second time, the quality of the prediction is compared between CE–UV and CE–MS. Finally, the ability of CE–MS to predict the electrophoretic mobility of ten peptides contained in a mixture is verified and discussed.

2. Experimental

2.1. Chemicals and reagents

Table 1 lists the 68 peptides used to establish the correlation straight line. Since the final objective of this work is

Table 1
Sequence and characteristics of the peptides used to establish the correlation

Peptide No.	Peptide sequence	N	M_r	q	HC
1	G	1	75.1	0.29	−1.6
2	K	1	146.2	1.21	−1.3
3	H	1	155.2	1.1	−1.9
4	R	1	174.2	1.13	0.1
5	GG	2	132.0	0.74	−3.2
6	AG	2	146.2	0.74	−1.6
7	AA	2	160.2	0,74	0.0
8	GP	2	172.1	0.74	−0.8
9	AV	2	188.2	0.74	4.2
10	LA	2	202.5	0.74	8.1
11	GM	2	205.9	0.74	−0.9
12	PP	2	212.3	0.74	1.6
13	VV	2	216.3	0.74	8.4
14	AM	2	220.3	0.74	2.5
15	GF	2	222.3	0.74	9.4
16	FA	2	236.3	0.74	11
17	GY	2	238.2	0.74	0.6
18	LL	2	244.3	0.74	16.2
19	AY	2	252.4	0.74	2.1
20	GW	2	261.0	0.74	12.1
21	LH	2	268.6	1.74	6.2
22	AW	2	275.4	0.74	13.7
23	LF	2	278.4	0.74	19.1
24	MM	2	280.4	0.74	5.0
25	LY	2	294.4	0.74	10.2
26	PL	2	302.4	0.74	8.9
27	MY	2	312.4	0.74	4.6
28	FF	2	312.4	0.74	22.0
29	LW	2	317.4	0.74	21.8
30	GGG	3	189.0	0.74	−4.8
31	AGG	3	203.3	0.74	−3.2
32	GGP	3	229.5	0.74	−2.4
33	PGF	3	319.4	0.74	10.2
34	AAY	3	323.3	0.74	2.1
35	GLY	3	351.6	0.74	8.6
36	LLL	3	357.5	0.74	24.3
37	GFF	3	369.4	0.74	20.4
38	KYK	3	437.6	2.74	−0.6
39	AAAY	4	394.4	0.74	2.1
40	KKKKK	5	659.0	5.74	−6.5
41	EHWSY	5	702.6	1.72	12.0
42	TISYDL	6	710.6	0.59	17.2
43	TVTYKL	6	723.6	1.74	13.1
44	TVTFKF	6	741.6	1.74	24.9
45	TVSYKF	6	744.0	1.74	15.4
46	TITFDY	6	758.4	0.59	20.7
47	TITYDF	6	758.4	0.59	20.7
48	TVTYDY	6	760.6	0.59	10.4
49	TISYDY	6	760.6	0.59	11.2
50	TITYKF	6	771.6	1.74	17.4
51	TITYEY	6	788.7	0.72	17.2
52	FKNKEF	6	811.8	2.72	14.5
53	RKRSRKE	7	958.1	5.72	−5.8
54	RYVFYFV	7	993.2	1.74	34.8
55	RPPGFSPL	8	870.0	1.74	19.3
56	VHLTPVEK	8	922.1	2.72	11.2
57	VQGEESNDK	9	1005.0	1.55	−7.3
58	WGNFAVFNGV	10	1110.3	0.74	36.9
59	AMGSKGNATDSA	12	1109.0	1.59	−3.2
60	AMGSAGNRTDSA	12	1137.0	1.59	−1.8
61	AMGSKGNRTASA	12	1150.0	2.74	−5.1
62	AMGSKGARTDSA	12	1151.0	2.59	−1.1

Table 1 (*Continued*)

Peptide No.	Peptide sequence	N	M_r	q	HC
63	AMGSKGNRADSA	12	1164.0	2.59	−3.1
64	AMGSKGNRTDAA	12	1178.0	2.59	−2.5
65	APGYKAEIKYNA	12	1324.5	2.72	1.5
66	APGYKHEIKYAA	12	1347.5	3.72	1.6
67	AAGYKHEIKYNA	12	1364.5	3.72	−1.2
68	KPVGKKRRPVKVYP	14	1652.0	6.74	10.5

N is the number of amino acids residues, M_r the relative molecular mass, q the charge and HC the hydrophobia coefficient.

to identify some small peptides, 52 peptides of that list are constituted of <7 amino acids, among which 24 are dipeptides. Each peptide is listed with its characteristics, N, M_r, q and HC. N is the number of amino acids residues, M_r is the relative molecular mass, q is the charge and HC is the hydrophobia coefficient (calculations given below). Peptides were purchased from Sigma (St. Louis, MO, USA) or custom synthesized at the Laboratoire de Chimie Physique Macromoléculaire (Nancy, France). They were dissolved in Milli-Q water at a concentration of 1 mg/ml, and were stored in a freezer when not in use. Methanol (Merck, Darmstadt, Germany) and formic acid 98% (Sigma) were of analytical grade. All solutions, samples and buffers, were passed through 0.22 μm nylon filters prior to use.

2.2. Determination of the charge and the hydrophobia coefficient

The net charge of each peptide at pH 2.75 was calculated considering any dissociable group separately, using the Henderson–Hasselbach equation [16]. Thus, for peptides with j acidic groups, total positive charge is given by:

$$P_t = \sum_j \frac{1}{1 + 10^{(\mathrm{pH} - \mathrm{p}K_j)}} \quad (1)$$

On the other hand, for peptides with i basic groups, the total negative charge is expressed by:

$$N_t = \sum_i \frac{10^{(\mathrm{pH} - \mathrm{p}K_i)}}{1 + 10^{(\mathrm{pH} - \mathrm{p}K_i)}} \quad (2)$$

thus, q is obtained by algebraic sum:

$$q = P_t - N_t \quad (3)$$

The pK_a values were determined using a pK_a set related to mean values reported for peptides [17]. These values consider the absence of mutual electrostatic interaction between the charged groups. The neighbouring ionized groups affect theoretically the pK_a value of a given group [18]. Yet, the low pH of the buffer leads to fully protonated carboxy groups and minimizes the error in the prediction of the charge. Then, the peptide values of ionization constants allow the best agreement between calculated and actual charge. The hydrophobia coefficient of each peptide was estimated by the sum of each hydrophobia coefficient of the amino acids included in the peptide. The hydrophobia coefficient of each amino acid was previously determined by reversed phase chromatography according to a procedure described by Van der Ven [19] and adapted in our laboratory.

2.3. Apparatus and procedures

CE experiments were performed in a 100 cm × 50 μm i.d. × 365 μm o.d. fused-silica capillary (Alltech, Deerfield, IL, USA) on the Beckman P/ACE System 5000, equipped with an UV detector and a System Gold data station, supplied by Beckman. The running buffer was 50 mM formic acid (pH 2.75) which allowed a good quality of spray while maximizing the peptide resolution. The UV absorbance detection at 214 nm took place at 20 cm of injection end through a window created by removal of 1 cm of polyimide coating. The polyimide coating was also removed 2–3 mm from the other end to ensure a good quality of spray. Samples were hydrodynamically injected for 10 s at 0.5 psi (1 psi = 6894.76 Pa). The voltage applied over the capillary during CE analysis was 25 kV under a temperature of 23 °C. Between each run, capillary was regenerated with 0.1 M sodium hydroxide for 3 min, then washed with water for 3 min and with running buffer for 4 min.

The mass spectrometer was an API 150 EX (PE Sciex, Toronto, Canada) single quadrupole equipped with a pneumatically assisted ESI (ionspray) interface [20], operated at +5 kV. Data acquisition and processing were performed with a computer running the Masschrom application. Electrical contact at the electrospray needle tip was established with a sheath–liquid constituted of 100% methanol solution, containing 0.2% formic acid as conductive modifier. In CE–MS, water is generally added to methanol to increase the conductivity of the sheath–liquid. In our work, the sufficient conductivity of pure methanol can be explained by the absorption of atmospheric water by methanol [21]. Adding some water presents the disadvantage of increasing the background noise and, consequently, it reduces the analysis sensitivity. The sheath–liquid was delivered at a flow rate of 6 μl/min. The mass spectrometer was operated in the positive ion mode. Mass spectra were acquired at some different ranges, from 70 to 1700 mass units (m/z) according to the size of the analyzed peptide, using a step size of 0.2 mass units and a dwell time of 0.1 ms. The same optimized conditions (except the scanned range, which was adjusted from 160 to 900 mass units) were used for the analysis of the peptide mixture.

In CE–MS, peptides could be detected until about 5 pmol whereas in CE–UV the limit of the detection of peptides was of the order of fmol. The lower sensitivity in CE–MS is due to the sheath-flow configuration. In fact, sheath–liquid mixes with the CE buffer at the CE outlet through coaxial tubing and then dilutes the component. In the literature, a variety of sheathless interfaces have been studied to increase the sensitivity of the coupling [22]. Nevertheless, a sheath-flow interface presents several advantages like reliability, simple

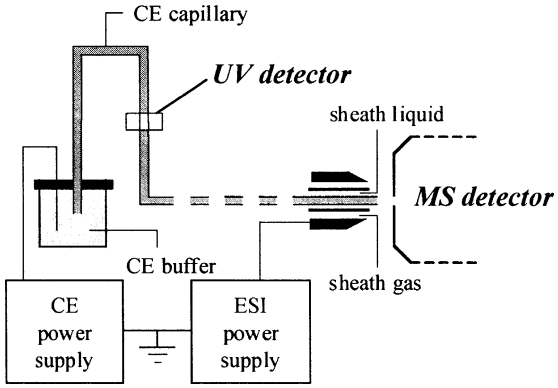

Fig. 1. Schematic illustration of online CE–MS setup. UV detector was at 20 cm of injection end and MS detector at 100 cm of injection end.

fabrication and use. Moreover, a limit of detection of the order of the pmol is enough to study the mobility of standard peptides and, also, to characterize peptides contained in a protein hydrolysate.

2.4. Determination of the electrophoretic mobility

The experimental electrophoretic mobility of each peptide (μ_{exp}(pep)) was measured as described elsewhere [23], from the migration time detected at the UV detector (CE–UV mode) and with the mass spectrometer (CE–MS mode) (Fig. 1). These electrophoretic mobilities were determined from the equation:

$$\mu_{exp}(pep) = \mu_{exp}(ref)\frac{t_m(ref)}{t_m(pep)} \quad (4)$$

In Eq. (4), t_m(pep) represents the migration time of peptide whereas t_m(ref) and μ_{exp}(ref) represent, respectively, the migration time and the electrophoretic mobility of a reference, 1,4-dimethylaminopyridine (DMAP) (Sigma). Prior each peptide, the electrophoretic mobility of this compound was systematically measured in order to take into account the possible day-to-day variations in the reproducibility of migration time, notably because of some modifications in capillary stability. This specific compound was selected as a reference because of its stability and of its high electrophoretic mobility at pH 2.75 [23].

The electrophoretic mobility of this compound was calculated as follows:

$$\mu_{exp}(ref) = \frac{Ll}{Vt_m(ref)} \quad (5)$$

where L is the total capillary length in cm, l the injector-to-detector capillary length in centimetres, V the applied voltage in volts and t_m(ref) is the migration time of DMAP in seconds. By this way, a value of 55.30×10^{-5} cm^2/(V s) was determined for μ_{exp}(ref) and corresponds to the average value of all the analyses, with a standard deviation of 5%. When the buffer pH value is below 3, the electroosmotic flow (μ_{eo}) has a very low intensity so it does not influence significantly the peptide mobility. As a consequence, this flow was neglected in the calculation of the mobility.

The theoretical mobility of each peptide was determined, in CE–UV and CE–MS, from the correlation established between the μ_{exp}(pep) of all the peptides and the Offord's model [24] (prediction model).

3. Results and discussion

3.1. Models

The aim of this study was to verify that models used to predict the electrophoretic mobility of peptides in CE–UV are applicable to CE–MS experiments. Among all the models commonly used, we have determined the most efficient in predicting peptide mobility, in CE–UV and CE–MS. Most of these models, based on semi-empirical approaches, estimate electrophoretic mobility in free solution CE according to some structural parameters (charge, size and shape). The general form of the equation is the following:

$$\mu_{exp} = \frac{q}{M_r^{\alpha}} \quad (6)$$

In this equation, q, M_r and α correspond, respectively, to the peptide charge, the peptide size and the peptide shape. The fractional coefficient α is the parameter by which the prediction takes into account the effect of frictional forces on the peptide mobility during electrophoretic motion [7]. As the intensity of these frictional forces is directly related to the peptide shape and ionization, α value varies theoretically from 1/3 to 2/3, according to the peptide size and buffer system (pH and ionic strength). However, from the reported results, some discrepancies on the choice of the model can be noticed, even among peptides of similar size [25]. The main reason for these divergences can be attributed to the differences between buffer systems used, never strictly identical from a work to another. For this reason, there is no generally accepted form for the dependence of mobility on peptide size and each new CE work must begin by the determination of the most appropriated model to its own electrophoretic conditions. Table 2 shows the correlation factor of the relation, established with the peptides listed in Table 1, between μ_{exp} and q/M_r^{α} using the semi-empirical models the mostly cited in the literature.

Table 2
Correlation factors obtained with different semi-empirical models relating peptide structure and mobility, for both UV and MS detection modes

	CE–UV	CE–MS
$q/M_r^{1/3}$	0.80	0.82
$q/M_r^{1/2}$	0.88	0.93
$q/M_r^{2/3}$	0.89	0.96
$\ln(1+q)/N^{0.435}$	0.80	0.85
$\ln(1+q)/M_r^{0.411}$	0.88	0.93

These results indicate that the charge-to-size parameter $q/M_r^{2/3}$ fits the best our experimental data, whatever in CE–UV or CE–MS. This model $q/M_r^{2/3}$ corresponds to the Offord's model, which consider that frictional forces depend on the surface area of the molecules, assuming some peptides with a large and rigid structure [7]. The best correlation was already obtained with Offord's model when large set of peptides with a wide molecular mass range was studied [26].

In the present work, there are a great variety of peptides but most of them contain <6 residues. Furthermore, the ionic strength of the buffer was relatively medium (50 mM). Models with $\alpha = 1/3$ (Stocke's law) and $\alpha = 1/2$ (Polymer law) are theoretically related to peptides with, respectively, small and intermediate sizes, with a separation performed with low to medium ionic strength buffer [27]. So the best correlation could be expected to be found with one of these two values. If the correlation factor is only slightly lower with $\alpha = 1/2$ compared to 2/3, the difference is great with $\alpha = 1/3$. In fact, with this value of α, the correlation was correct considering small peptides with a high electrophoretic mobility but was largely deficient when the peptides had low charge densities, whatever the number of amino acids residues in the peptide. Then, this model is mainly valid with small peptides of high charge densities [7]. The validity of this model is so limited to a little number of peptides and, then, it can not be used to characterize any complex protein digest. A α value of 1/2 gives a better correlation because the peptide is then considered as a polymer with an intermediate charge density, so peptides with low charge densities are taken into account in a better way with this model. Finally, the $q/M_r^{2/3}$ correlation is slightly superior in our electrophoretic conditions although a α value of 2/3 is supposed to well-correlated large peptides in high ionic strength buffer. This result illustrates well the difficulty to determine the most appropriated model without any experimental study. Nevertheless, it can be noticed that, in the literature, the best correlation is often obtained with the Offord's model [25,26,28].

The addition of a logarithmic dependence of q does not improve the correlation factor, even if the model proposed by Cifuentes and Hoppe [29] is relatively efficient ($r^2 = 0.93$). This extension of the current model was proposed to compensate for the charge suppression phenomena due to mutual electrostatic interactions of the charged groups [28]. This model is then efficient for highly charged peptides, but slightly less for small peptides without difference in charge intensity.

3.2. Influence of other parameters on mobility

The main deficiency of the Offord's model is that it takes into account only two physicochemical properties of peptides, the charge (q) and the relative molecular mass (M_r). The models with a logarithmic dependence of q use a third property, namely the phenomenon of shielding of charge in highly charged peptides. But, as seen above, the introduction

Table 3
Comparison of experimental electrophoretic mobility (μ_{exp}) obtained in CE–UV with theoretical value calculated by the equation $\mu_{theo} = (30.62 + 490.62(q/M_r^{2/3})) \times 10^{-5}\,cm^2/(V\,s)$

Peptide No.	$\mu_{exp} \times 10^5$	$\mu_{theo} \times 10^5$	% Deviation[a]
1	38.42	37.98	1.14
2	50.30	51.36	−2.11
3	44.10	48.66	−10.36
4	50.21	47.75	4.89
5	44.53	43.99	1.21
6	43.20	43.07	0.30
7	38.49	42.29	−9.89
8	42.31	41.72	1.40
9	41.08	41.04	0.08
10	40.47	40.52	−0.12
11	39.07	40.40	−3.39
12	41.10	40.19	2.22
13	41.27	40.06	2.93
14	39.47	39.94	−1.18
15	39.13	39.88	−1.93
16	39.96	39.49	1.19
17	38.02	39.44	−3.73
18	38.37	39.28	−2.37
19	38.14	39.08	−2.47
20	38.47	38.88	−1.06
21	50.12	50.48	−0.73
22	40.02	38.57	3.62
23	39.43	38.51	2.36
24	39.63	38.46	2.94
25	36.53	38.19	−4.56
26	39.62	38.05	3.96
27	37.39	37.88	−1.31
28	37.67	37.88	−0.55
29	38.28	37.79	1.27
30	42.88	41.01	4.37
31	39.41	40.49	−2.73
32	41.18	39.67	3.66
33	37.24	37.76	−1.40
34	36.45	37.70	−3.43
35	42.03	37.28	11.31
36	38.16	37.20	2.52
37	38.39	37.04	3.50
38	58.95	53.29	9.60
39	38.50	36.74	4.57
40	65.74	67.14	−2.13
41	42.68	40.66	4.73
42	33.60	33.63	−0.09
43	41.08	40.58	1.21
44	42.88	40.41	5.78
45	42.41	40.38	4.77
46	33.23	33.48	−0.74
47	33.40	33.48	−0.22
48	32.29	33.47	−3.66
49	31.27	33.47	−7.02
50	41.31	40.13	2.84
51	34.13	34.13	0.00
52	48.02	45.31	5.63
53	57.04	58.84	-3.15
54	40.77	38.56	5.40
55	41.66	39.36	5.53
56	47.79	44.07	7.79
57	40.46	37.57	7.14
58	33.21	33.38	−0.51
59	38.33	37.27	2.77
60	42.69	37.15	12.97
61	43.37	42.23	2.63

Table 3 (*Continued*)

Peptide No.	$\mu_{exp} \times 10^5$	$\mu_{theo} \times 10^5$	% Deviation[a]
62	47.80	41.56	13.06
63	43.69	41.47	5.08
64	43.58	41.38	5.05
65	39.26	41.05	−4.57
66	44.38	44.94	−1.27
67	42.75	44.82	−4.83
68	58.95	53.63	9.02

[a] % Deviation: $[(\mu_{exp} - \mu_{theo})/\mu_{exp}] \times 100$.

of that function type in the model leads to neglect uncharged small peptides.

Another physical property able to influence the peptide mobility is the secondary structure, which can affect the charge distribution and/or the peptide shape. However, the impact of the secondary structure can be only considered of importance with peptides constituted of more than 10 amino acids [30]. Below that number, peptides are unable to have any stable secondary structure. In our work, the objective being the characterization of a protein hydrolysate mostly composed of peptides with a relative molecular mass below 1000 (7–11 amino acids), we have focused the calibration on peptides with <10 amino acids. Then, only 10 peptides in the list of Table 1 (from Nos. 59 to 68) are expected to present a specific conformation. In Tables 3 and 4, the experimental mobility of each peptide is compared to its theoretical mobility, respectively, in CE–UV and CE–MS. Compared to the small peptides, most of these 10 large peptides have a similar deviation to the correlation straight line. The % deviation is only slightly more important for a few of these peptides, notably in CE–UV (+12.97% for No. 60 and +13.06% for No. 62, versus ± 4.67% as average deviation for the other peptides). However, in this work, these differences are not significant enough to consider the secondary structure like a predominant factor to be taken into account in the prediction of the peptide mobility.

The physical parameter mostly cited to explain the deviation between the experimental mobility and the theoretical mobility is the hydrophobia coefficient of the peptides. Indeed, hydrophobia can influence the peptide separation by some peptide–peptide or peptide–capillary interactions even, like in the present work, when no surfactant is added to the buffer. Focusing on the mobility of the most hydrophobic peptides can not determine the real impact of the hydrophobia on the quality of the correlation. Actually, if there is a hydrophobia effect during the separation, in any case the size and charge effects overshadow it [25]. In order to have a real estimation of the hydrophobia effect, the mobility of several peptides with similar sizes and charges but different in hydrophobia coefficients have been compared. Then, the four comparisons between peptides No. 17/18, 23/24, 47/48 and 54/55 did not reveal any significant variations in the electrophoretic mobility. This study shows that, in our electrophoretic

Table 4
Comparison of experimental electrophoretic mobility obtained in CE–MS with theoretical value calculated by the equation $\mu_{theo} = (31.07 + 507.21(q/M_r^{2/3})) \times 10^{-5}\,cm^2/(V\,s)$

Peptide No.	$\mu_{exp} \times 10^5$	$\mu_{theo} \times 10^5$	% Deviation[a]
1	36.79	38.31	−4.13
2	51.66	52.24	−1.12
3	49.78	49.43	0.70
4	46.73	48.48	−3.73
5	45.63	44.56	2.36
6	44.14	43.60	1.22
7	43.62	42.79	1.90
8	42.68	42.20	1.13
9	41.21	41.49	−0.68
10	41.82	40.94	2.10
11	41.26	40.82	1.06
12	42.53	40.60	4.52
13	41.75	40.47	3.06
14	41.91	40.35	3.73
15	39.97	40.28	−0.79
16	41.11	39.88	3.01
17	39.35	39.82	−1.19
18	38.87	39.66	−2.03
19	39.11	39.45	−0.87
20	39.49	39.24	0.64
21	51.97	51.32	1.26
22	40.13	38.92	3.03
23	39.96	38.85	2.78
24	40.11	38.81	3.24
25	37.80	38.53	−1.92
26	41.62	38.38	7.79
27	39.81	38.20	4.05
28	39.76	38.20	3.93
29	37.52	38.11	−1.56
30	42.83	41.46	3.20
31	43.63	40.92	6.23
32	40.48	40.07	1.02
33	36.68	38.08	−3.80
34	37.33	38.01	−1.84
35	39.60	37.58	5.10
36	38.48	37.49	2.57
37	38.29	37.33	2.51
38	57.02	54.24	4.86
39	37.61	37.02	1.58
40	70.53	68.66	2.65
41	42.96	41.10	4.34
42	35.32	33.78	4.36
43	42.07	41.01	2.53
44	42.02	40.83	2.83
45	43.08	40.81	5.27
46	34.06	33.62	1.30
47	34.86	33.62	3.56
48	34.83	33.61	3.50
49	34.50	33.61	2.58
50	41.03	40.55	1.17
51	35.36	34.30	2.99
52	50.96	45.94	9.86
53	58.47	60.02	−2.65
54	41.13	38.91	5.39
55	41.91	39.74	5.18
56	47.60	44.64	6.22
57	40.83	37.88	7.22
58	33.95	33.52	1.26
59	38.41	37.57	2.20
60	38.39	37.44	2.47
61	44.02	42.73	2.93

Table 4 (*Continued*)

Peptide No.	$\mu_{exp} \times 10^5$	$\mu_{theo} \times 10^5$	% Deviation[a]
62	43.31	42.03	2.97
63	44.65	41.94	6.08
64	44.33	41.84	5.62
65	42.29	41.50	1.87
66	48.72	45.55	6.51
67	48.03	45.42	5.44
68	53.68	54.60	−1.70

[a] % Deviation: $[(\mu_{exp} - \mu_{theo})/\mu_{exp}] \times 100$.

conditions, hydrophobia does not influence the peptide mobility. Yet, hydrophobia governs directly the secondary structure, influencing the shape and the effective charge of the peptides. The lack of influence of hydrophobia can be so explained because peptides used are of low and intermediate sizes, with no secondary structure. Then, we can guess that the more the peptide size increases the more the presence of hydrophobic amino acids influences the peptide mobility.

3.3. Comparison between CE–UV and CE–MS

After the determination of the best predictive model, the prediction performance has been compared between CE–UV and CE–MS with this model. Fig. 2 reports the prediction of the peptide mobility by the model $q/M_r^{2/3}$, respectively, in CE–UV (a) and CE–MS (b).

In Tables 3 and 4, these experimental mobilities are compared to the corresponding theoretical mobilities

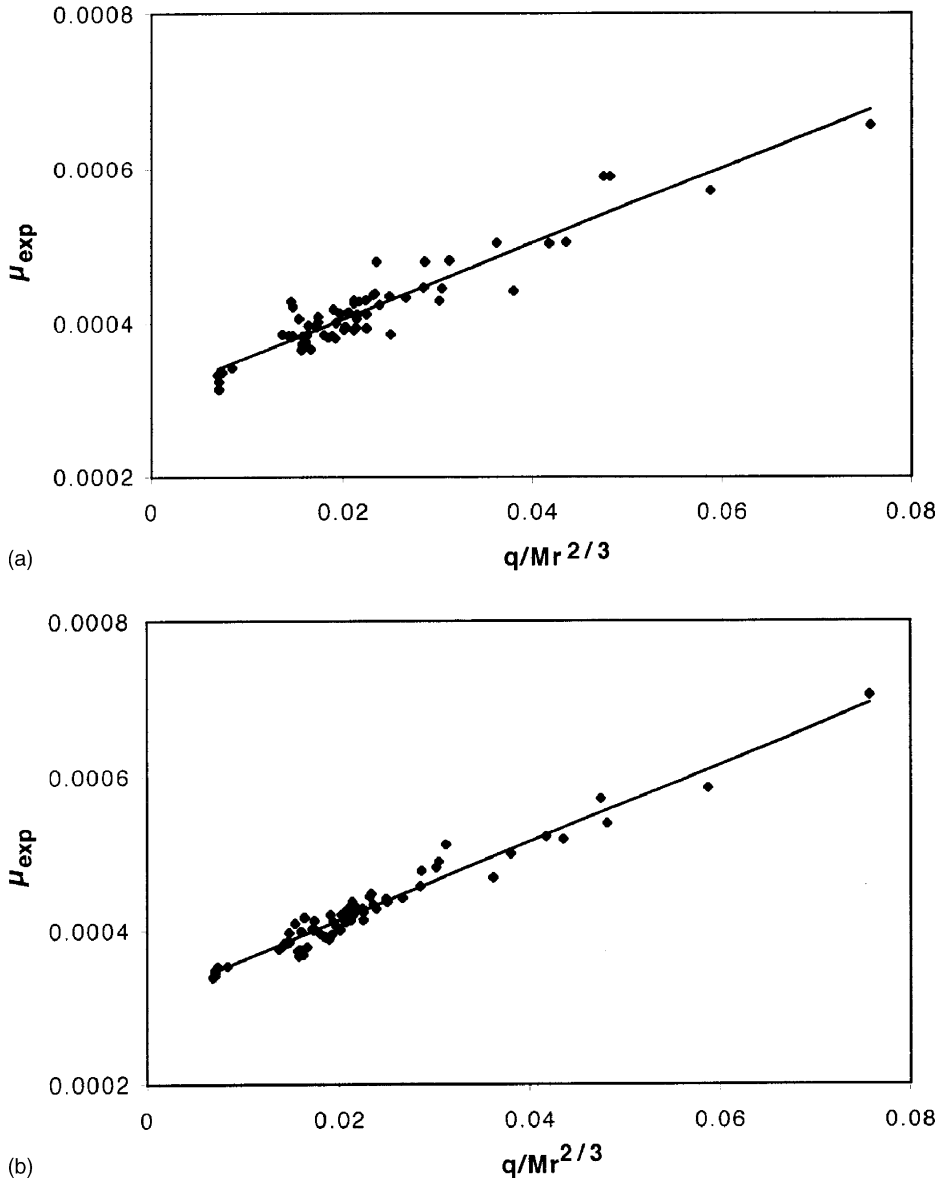

Fig. 2. Correlation of predicted peptide mobilities based on the Offord's model versus experimental, in CE–UV (a) and CE–MS (b) Data are given in Table 3 (a) and 4 (b) and μ_{exp} is expressed in $cm^2/(V\,s)$.

Table 5
Sequence, characteristics and mobility of the peptides used in the mixture

No.	Peptide sequence	N	M_r	$M_r + H^+$	q	HC	$\mu_{exp} \times 10^5$	$\mu_{theo} \times 10^5$	% Deviation[a]
1	PG	2	172.1	173.1	0.74	−0.8	43.72	42.20	3.48
2	AH	2	226.3	227.3	1.74	−1.9	53.66	53.90	−0.44
3	RK	2	302.4	303.4	2.74	−1.2	61.99	61.02	1.56
4	GPA	3	243.3	244.3	0.74	−0.8	43.72	39.68	9.23
5	PLV	3	327.4	328.4	0.74	13.1	37.65	37.65	0.00
6	VYV	3	379.5	380.5	0.74	10.5	37.46	37.20	0.69
7	WVYV	4	565.7	566.7	0.74	24.2	36.08	35.52	1.55
8	TITYDL	6	724.6	725.6	0.59	17.8	33.95	33.73	0.65
9	TVTYKY	6	773.8	774.8	1.74	7.1	44.74	40.53	9.41
10	KNFFKE	6	811.8	812.8	2.72	14.5	50.63	45.94	9.26

N is the number of amino acids residues, M_r the relative molecular mass, $M_r + H^+$ the expected mass by CE–MS analysis, q the charge and HC the hydrophobia coefficient. The experimental mobility (μ_{exp}) was obtained from CE–MS analysis and the theoretical mobility (μ_{theo}) was calculated according to the equation $\mu_{theo} = (31.07 + 507.21(q/M_r^{2/3})) \times 10^{-5}\ cm^2/(V\,s)$.

[a] % Deviation: $[(\mu_{exp} - \mu_{theo})/\mu_{exp}] \times 100$.

($cm^2/(V\,s)$), obtained by the following equations:

$$\mu_{theo} = \left(30.62 + 490.62\frac{q}{M_r^{2/3}}\right) \times 10^{-5} \quad (7)$$

$$\mu_{theo} = \left(31.07 + 507.21\frac{q}{M_r^{2/3}}\right) \times 10^{-5} \quad (8)$$

These equations were obtained from the peptide migration times at 20 cm as injector-to-detector capillary length (UV detection) for Eq. (7) and from the peptide migration times at 100 cm as injector-to-detector capillary length (mass detection) for Eq. (8) (neglecting the residence time in the ESI interface [31]). The objective of this study was to verify the impact of some perturbations linked to the CE–MS technology (use of a sheath–liquid interface [15] and the absence of temperature regulation) on the peptide mobility along the capillary. In this study, the two equations are similar. This result shows a low variation of the peptide mobility, which is confirmed by the comparison of the intensity of

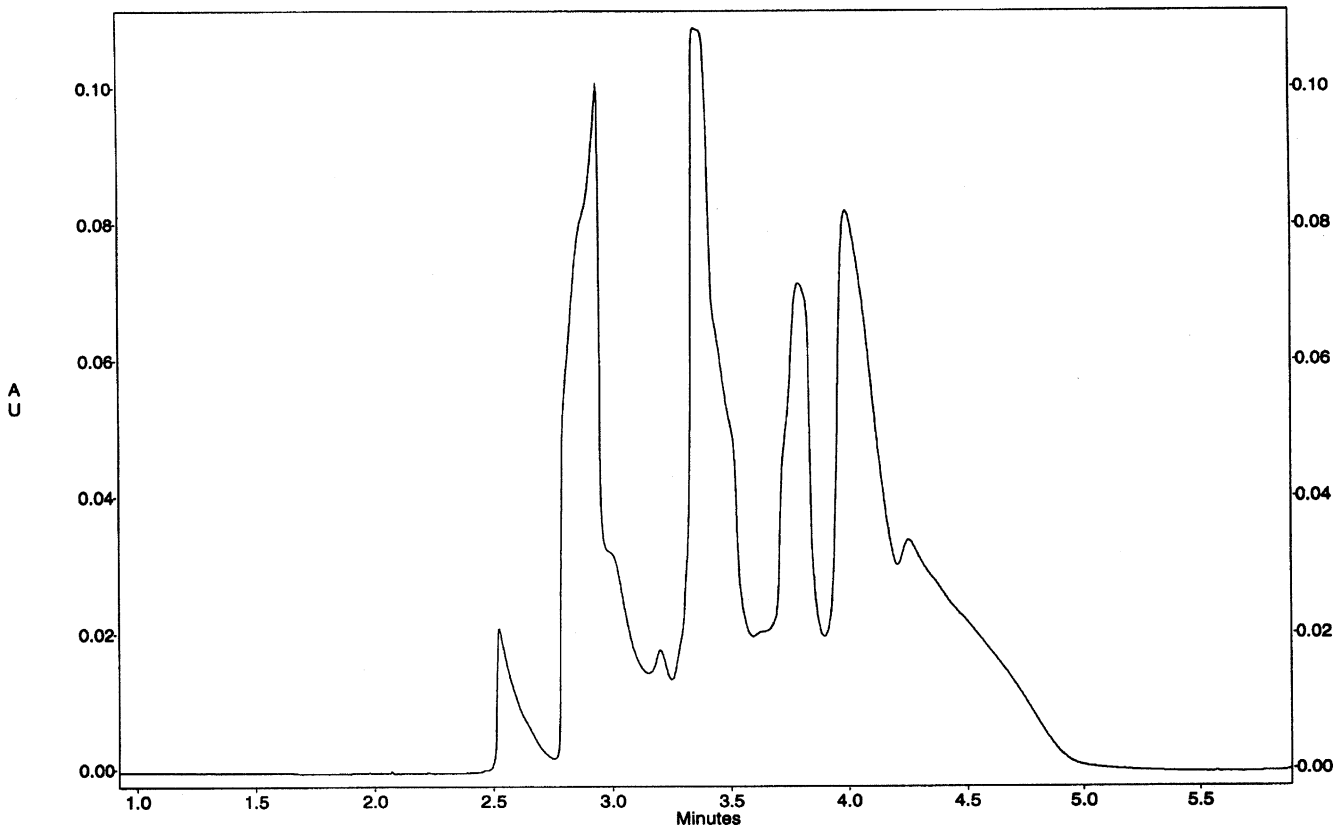

Fig. 3. CE–UV electrophoregram of the mixture of ten peptides listed in Table 5. CE was carried out on a 100 cm × 50 μm i.d. × 365 μm o.d. fused-silica capillary with a voltage of 25 kV (current: 6 μA). Buffer was 50 mM formic acid (pH 2.75).

the experimental mobility for each peptide between CE–UV (Table 3) and CE–MS (Table 4). Most peptides have a similar mobility, which illustrates a constant behavior all the separation process long and the preservation of an equivalent correlation straight line. Then, a predictive model previously determined in CE–UV could be considered representative of the model applicable in CE–MS, in the same electrophoretic conditions.

Nevertheless, Table 2 shows that a better correlation was obtained in CE–MS ($r^2 = 0.96$) compared to CE–UV ($r^2 = 0.89$). This is confirmed by the % deviation observed in Tables 3 and 4 for each peptide. This deviation is slightly greater in UV with an average deviation standard towards theoretical mobility of ±4.67% versus ±2.89% in MS. The better correlation in CE–MS is relatively surprising since there is no temperature regulation of capillary from the exit of CE to the entrance in mass spectrometry (i.e. about 80 cm long). Theoretically, temperature must be controlled along capillary in order to avoid the creation of convection or diffusion currents or even some variations in the buffer viscosity. All these phenomena have some harmful consequences on the separation quality and can disturb peptide mobility. When the capillary is inside the CE, a coolant that flows through the capillary cartridge ensures that control of the temperature. The low current (about 5 μA) delivered during the operation can explain the preservation of the separation quality in CE–MS since a low current leads to a low heat emanation and then, to minimal perturbations. Working with a low current (about 5 μA) is then important for two reasons: it favors a high ionization yield [32] and ensures an identical quality of separation along the capillary. Moreover, some changes in migration order, due to the migration of sheath–liquid inside the capillary and the formation of moving ionic boundaries [15], have not been observed. The influence of that effect, caused by the sheath–liquid interface, was so negligible in our study.

The lower correlation in CE–UV can be attributed to the fast separation time (below 5 min) of all the peptides in that case, which generates a short migration time range (between 2 and 5 min) and, as a consequence, a low resolution. In CE–MS, that migration time range is included between 10 and 25 min, which leads to a better resolution in the peptide separation and an increase in the precision

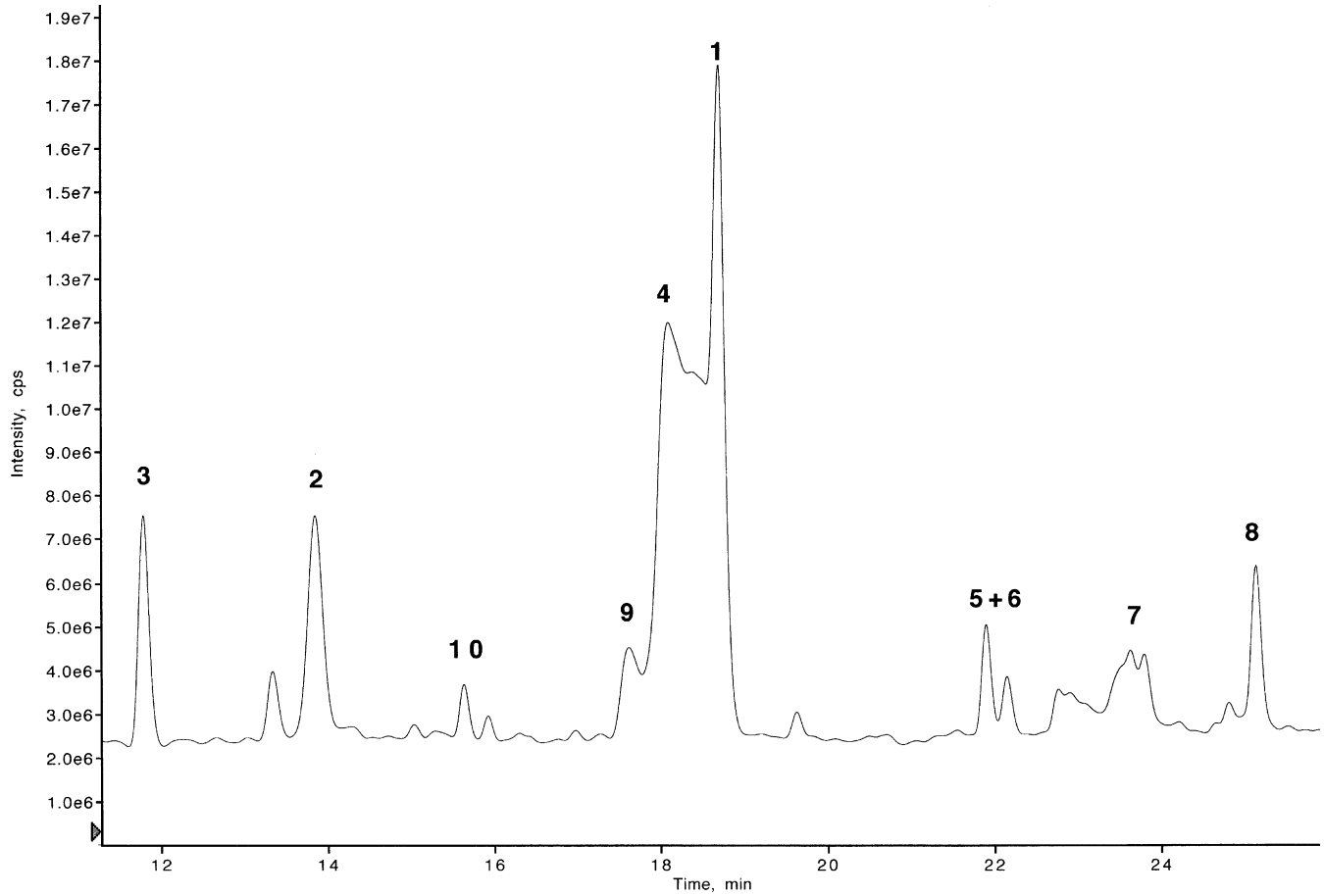

Fig. 4. CE–MS total ion current electrophoregram after the separation of the ten peptides of Table 5 on a 100 cm × 50 μm i.d. × 365 μm o.d.) fused-silica capillary. CE was carried out in 50 mM formic acid (pH 2.75) with a voltage of 25 kV (current: 6 μA). Sheath flow of 6 μl/min was a solution of methanol containing 0.2% formic acid. The numbers refer to the peptide list of Table 5.

of the results. So, although the equations after linear regression are similar, the extrapolation of any identification method from CE–UV to CE–MS requires a new calibration with the mass detection. Then, the peptide characterization will be more accurate. According to the r^2 value of 0.96, the prediction of peptide mobility by CE–MS is even as precise as that obtained by classical CE–UV with an equivalent set of peptides [26]. Moreover, this result seems to show that an amelioration of the correlation is possible in CE–UV and CE–MS by a simple increase of the capillary length before the UV detector. There is not any technical difficulty to increase this length but the analysis time will be then longer for a comparatively low benefit of resolution.

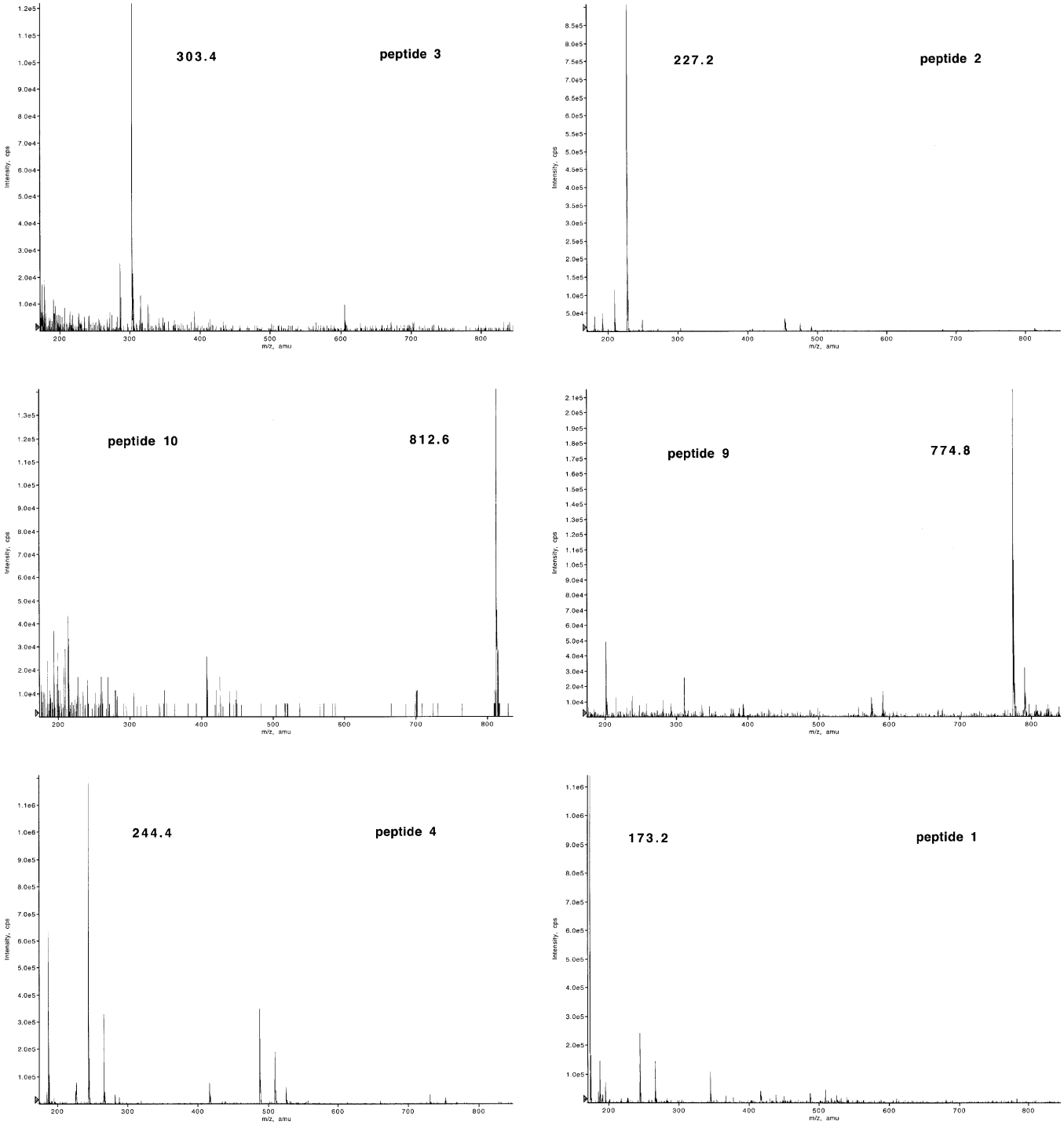

Fig. 5. Positive ion mass spectra of the total ion current peaks obtained in Fig. 4 with a mixture of ten peptides. Peptide number refers to the peptide list of Table 5, associated with its mass to charge ratio (m/z).

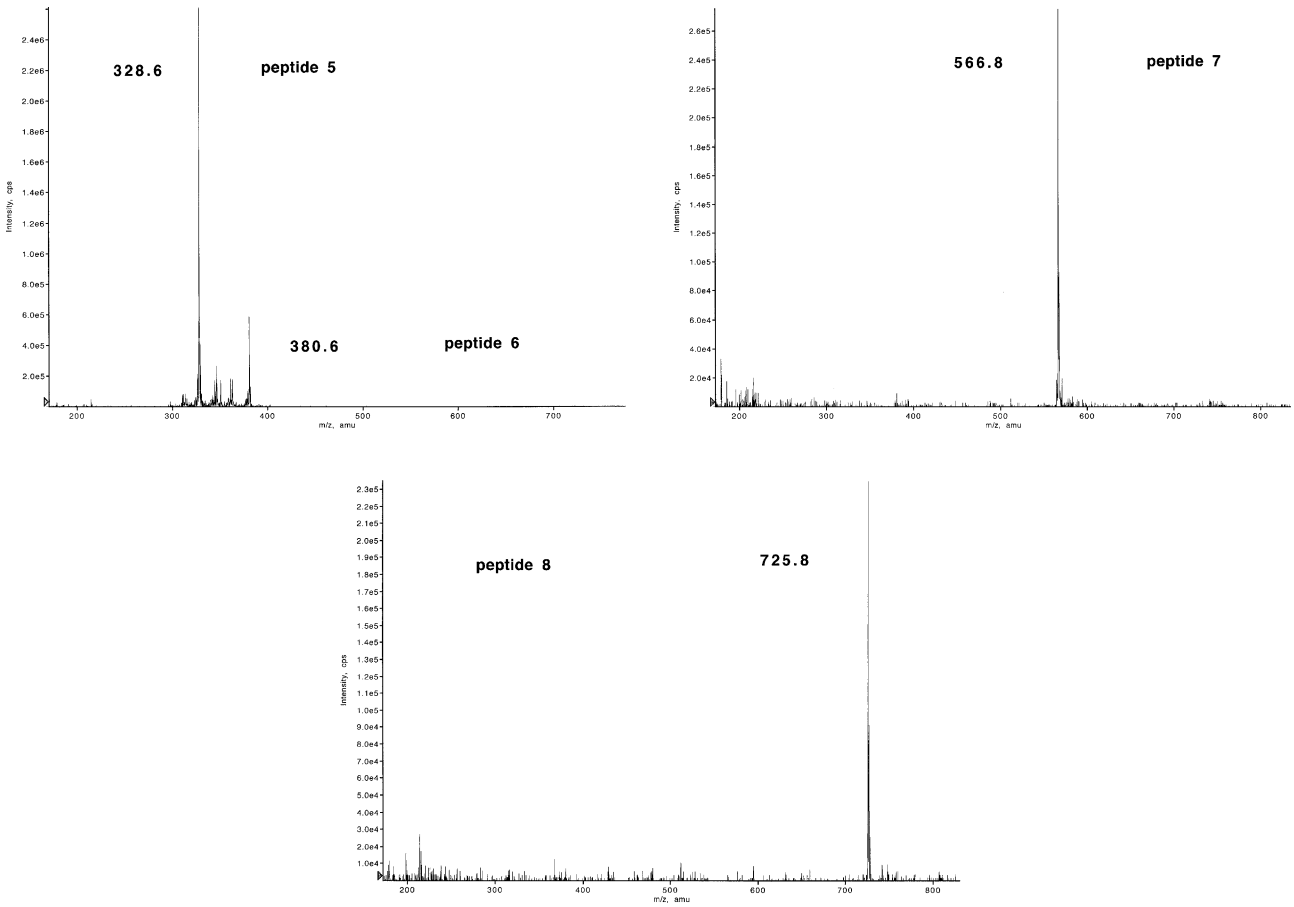

Fig. 5. (*Continued*).

3.4. Validation with a peptide mixture

The simultaneous separation of the ten peptides listed in Table 5 was performed in order to verify the efficiency of the semi-empirical model to predict the mobility of peptides contained in a mixture. In CE–UV, the ten peptides have migrated between 2 and 5 min but the close theoretical mobilities between some peptides and the short separation time have led to a very low resolution in the separation of the ten peptides [33] (Fig. 3). The Fig. 4 shows the electropherogram obtained in CE–MS. As can be seen, even if the resolution is not perfect, the separation of these peptides can be achieved in 25 min and each peptide migrates between 10 and 25 min, in accordance with the results obtained for the establishment of the model. In spite of that not complete separation, the corresponding MS spectra of the ten peptides have been obtained (Fig. 5). It means that it is possible with that method to identify the relative molecular masses of peptides contained in a mixture. Since the system is in the positive ion mode, the detected ions correspond to $M_r + H^+$ (± 0.2 m/z). It can be noticed that the MS signal of each peptide is notably higher than the background noise and that the peptides 5 and 6 stand in the same MS spectrum. The maximum of the MS signal for these two peptides was exactly at the same time of migration. Actually, CE failed to separate these peptides. The low resolution of any peptide separation in CE–MS can be explained by the nature of the buffer, which is less efficient than a nonvolatile buffer to separate some compounds. Besides the nature of the buffer which do not allow a perfect separation, that fact can be explained by the very similar theoretical mobilities of these two peptides, which lead to very close experimental migration times. Some hydrophobic interactions between these peptides, having a similar hydrophobia coefficient, could also partially explain their co-migration.

Besides the efficiency of the coupling to identify the molecular mass of peptides standing in a mixture, it is interesting to mention the good agreement observed between the theoretical and experimental electrophoretic mobilities, given in Table 5. The percentage of standard deviation has not exceeded 4% for seven peptides and the three other peptides (Nos. 4, 9 and 10) had a deviation below 10%. No specific explanation for the higher error of these three peptides could be found. No explicative parameter could be observed from the comparison of their physicochemical characteristics. These peptides exhibited various charge to mass ratios and hydrophobia coefficients. On the other hand, the good prediction obtained with the most hydrophobic

peptides (Nos. 7 and 8) confirms the low, if not negligible, influence of the hydrophobia on the prediction of short peptide mobility. Lastly, we have compared the mobility of peptides with same amino acid composition but different sequence. In fact, two peptides of mixture (peptides Nos. 1 and 10) have the same amino acid composition that, respectively, the peptides Nos. 8 and 52 of Table 4. These peptides, with the same theoretical mobility, have some similar experimental mobility, which means that the sequence does not influence the mobility. The sequence becomes an important parameter only when peptide has any secondary structure, because of its impact on charge distribution.

The model, established from the mobility of standard peptides, is then efficient to predict the behavior of the peptides contained in a mixture. The charge and the relative molecular mass remain the main parameters controlling the mobility, towards some possible peptide-peptide interactions, of hydrophobic or ionic nature. The study, by CE–MS, of the mobility of short peptides standing in a protein hydrolysate appeared then feasible. Such a characterization should allow the identification of unknown peptides contained in complex mixtures.

4. Conclusions

This work shows that models currently developed in CE–UV can be used in CE–MS. The modifications linked to the detection by the mass spectrometry (capillary length, nature of the buffer) require the elaboration of a new calibration step. The increase in the separation distance in CE–MS, creating a better resistance to the applied voltage, improves the separation quality, in the same way as in classical CE–UV. Among the five commonly used models, the best fit of the results has been obtained with the Offord's model, with a r^2 value similar to those determined in classical CE–UV ($r^2 = 0.96$). The effect on the peptide mobility of secondary structure and hydrophobia has been evaluated from the large set of peptides used in that work. In our electrophoretic conditions, an influence of these parameters on the peptide mobility has not been observed. The efficiency of the model to predict peptide mobility has been verified with a mixture of 10 peptides. This result shows that it is possible in CE–MS to characterize mixed peptides, not only by their relative molecular masses, but also by their electrophoretic mobilities. From the knowledge of these two parameters, the peptide charge can be estimated. Using convenient software, the determination of the possible amino acids composition of peptides will be then feasible, from the relative molecular mass and the charge of the peptide. As a future application, this tool could help the identification of unknown peptides standing in a protein hydrolysate (work in progress).

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