Chapter 2

Potential of capillary isotachophoresis coupled to mass spectrometry of peptides using spacer molecules

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Abstract

Capillary isotachophoresis (cITP) is an electrophoretic separation technique that is able to concentrate analytes during separation, in consequence improving the sample concentration detection limits. In health sciences, the importance of peptides is widely recognised, since changes in body fluid peptide profiles often reflect disturbed homeostasis or disease. The analysis of peptides is complicated, since peptides typically occur in very low concentrations. In conventional cITP coupled to mass spectrometry (MS), trace peptides are focused in adjacent bands. This causes several adjacent peptides to enter the MS all at once, resulting in multiple peptide mass spectra and signal discrimination due to ion suppression. In this paper, we explore the use of spacer molecules to overcome this complication of cITP-MS. Carrier ampholytes (CA), which are normally used to generate pH gradients in (C)IEF, can act as spacer compounds in cITP. In this way the peptide bands are separated from each other, making MS analysis of pure peptides possible. As an example the analysis of a set of 6 model peptides as well as a tryptic digest of bovine serum albumine (BSA) is reported. Finally, some recommendations for spacer compounds in cITP are done.

1. Introduction

Qualitative and quantitative information about the concentration of trace compounds such as peptides, for example in plasma, is highly important for health sciences, since they often are markers for diseases [1]. Counting all proteins and peptides of all variations and origins, the plasma proteome is believed to contain as much as 1 million molecule species. Their physiological range spans from 35-40 mg/mL in the case of the protein serum albumin and 0-5 pg/mL in the case of the peptide interleukin 6 [2]. The role of peptides in important biological processes such as inflammatory response, tumour biology and endocrine processes has been established [3], so there is a great demand for knowledge about human peptides and their functions. Progress is mainly limited due to the lack of techniques that enable a comprehensive peptide analysis [4], especially at very low concentrations where many relevant peptides occur (pg/mL). Nowadays, (nano)LC and CZE coupled to MS are the two most common techniques used for peptide analysis. However, both approaches have limitations regarding their dynamic range and limit of detection (LOD).

A benefit of CE-based systems is the simplicity of the system, i.e. a disturbed CE-system is repaired by just replacing the capillary, which is cheap and not time-consuming. On the contrary, troubleshooting in a disturbed nano-LC system can be complex [5], time-consuming and maintenance costs can become high.

CZE coupled to electrospray ionisation MS (ESI-MS) offers high separation power, high mass sensitivity and structural information about the peptides, but due to the low loadability of CZE this technique suffers from high sample concentration LOD values. For this reason and for the fact that CZE is just as chromatography in essence a diluting separation technique, very low-abundant peptides are not detected. This can be partly overcome by using appropriate sample pretreatment, making the overall analytical process more complex.

However, cITP offers a way to increase the analyte concentrations during separation. Thanks to a discontinuous electrolyte system consisting of a leading electrolyte (LE) and a terminating electrolyte (TE), in cITP the ultimate migration velocity of the analyte ions is the same in the whole capillary. The choice for leading and terminating electrolytes depends on the mobilities of the analytes that are to be studied; i.e. a suitable 'isotachophoretic window' should be created. In the sample plug, which is injected between LE and TE, analytes are arranged according to their electrophoretic mobilities. As a result, the peptides are separated from each other. The capillary can be completely filled with sample, implying

an improvement in detectability via extended loadability.

According to Kohlrausch's regulation function (Equation 1), the concentration of ion a (the analyte) is dependent on the concentration of the preceding ion l (the leading ion).

1) $$C_a = C_l \frac{\mu_a(\mu_l + \mu_r)}{\mu_l(\mu_a + \mu_r)}$$

In this equation, C_a and C_l are the molar analyte and LE concentrations, respectively, and μ_a, μ_r and μ_l are the electrophoretic mobilities of the analyte *a*, counter ion *r* and the leading ion *l*, respectively. The effect of this process is concentration of sample compounds with concentrations below the LE concentration, while on the other hand sample compounds present at concentrations higher than LE will be diluted. As a consequence, the dyamic range of cITP-MS is increased compared to LC- or CZE-MS, enabling detection of low as well as the high abundant compounds in one single analysis. As stated above, the concentrations of the peptidome span a dynamic range of 10 orders of magnitude [2]. The concentration factor that can be achieved using cITP can be as high as 10^6 times [6]. In addition to the concentrating effect, the diffusion of analytes is counter-acted, since the analyte bands are self-sharpening as they have each their own electric field strengths. And finally, cITP can aid in sample clean-up by choosing suitable TE and LE so that interfering matrix compounds having higher or lower electrophoretic mobilities than the peptides investigated can be excluded from the separation. In literature a clear explanation of ITP is given by Holloway and Trautschold [7] and Petr *et al* [8].

The detection method of choice for peptides (and proteins) nowadays is MS [9]. Using MS, mass information as well as structural information about peptides can be obtained, enabling quick identification of peptides and discovery of new peptide species. A drawback of MS is the fact that, when more compounds are present at the same time during ESI, ion suppression compromises the linear response of the analytes, which makes reliable quantitation problematic [10].

Coupling cITP with MS is not as straightforward as for example CZE-MS or LC-MS, since in the isotachophoretic process the analytes are focused in directly adjacent zones instead of physically separated zones. This creates mixed zones at the boundaries between the analyte zones [11], which was already noted in the same time frame as the invention of ESI in 1989 [12]. In these mixed zones, ion suppression takes place and as a consequence quantitative determinations are affected, especially in the case of low analyte concentrations, when the zones are prone to collapse and mix fully [13]. This is a severe drawback of cITP-

MS for trace analysis with small sample volumes. According to Equation 1, the very low-abundant peptides are strongly concentrated, resulting in very narrow bands. These narrow bands will enter the MS at virtually the same time. As a consequence, the separation is lost and therefore ion suppression between the analytes may occur during ESI, making reliable quantitation cumbersome [13].

For these reasons, the direct coupling of cITP with MS has not often been utilised yet and instead cITP-MS has mostly been applied in combination with CZE [14], via directly coupled cITP-CZE [15,16,17] or via transient isotachophoresis coupled to CZE (tITP-CZE) [18,19,20, 21]. Direct comparison of analyses of the plasma levels of the peptides angiotensin II and gonadorelin with tITP-CZE-UV and LC-UV has already shown that tITP-CZE-UV is superior to LC-UV in terms of concentration LOD [22]. In [23], preparative tITP-CZE and strong cation exchange solid phase extraction (SCX-SPE) as first dimension in a twodimensional shotgun proteomics set-up were compared. The second dimension was nano-reversed phase liquid chromatography coupled to MS in both cases. Analysis of glioblastoma-derived cancer stem cells demonstrated that tITP-CZE achieved superior performance in total peptide, distinct peptide and distinct protein identifications. Moreover, its analytical reproducibility was good. The authors conclude that with the help of tITP-CZE multidimensional proteomics platforms can mine deeper into the proteome and the technology is expected to serve as a critical component in the overall toolset required for biomarker discovery in proteomics.

From a CZE perspective, the combination with cITP means an improvement, but from the cITP perspective the combination with CZE means sacrificing two of its major benefits, i.e. the self-correcting ability of the analyte zones and the high loadability. It is desirable for higher resolution as well as the LOD that the isotachophoretic process is maintained until the sample is infused into the MS, because, CZE starts as soon as the zone-sharpening isotachophoretic process is aborted, implying that zone broadening by diffusion inherent to CZE occurs. To be able to perform cITP coupled directly to MS detection, we explored the use of spacer molecules. These will be positioned between the different isotachophoretic peptide zones, preventing that several peptide bands enter the MS at the same time. To demonstrate this approach, experiments with carrier ampholytes (CA) as spacer compounds were carried out.

CA are mixtures of many amphoteric molecule species that have slightly different isoelectric points. Originally, they were designed to generate pH-gradients in IEF and consisted of N- methylated or -ethylated oligoamines that have reacted with acrylic acid [24]. The

commercially available CA used in this research consisted of the products of a copolymerisation reaction of amines, amino acids and dipeptides in epichlorohydrin [25]. Since CA are complex mixtures of molecules, a wide range of electrophoretic mobilities is present. In a cITP separation of peptides with CA present, both should behave electrophoretically in a comparable way, in order to let the CA end up as spacers between the peptides. Since hundreds of CA species are present, the chance that bands are being positioned between the peptides is expected to be high. For cITP-MS of proteins, it has already been shown that CA act as spacer molecules between the protein zones [26], while for cITP-UV the use of CA as spacer compounds has been shown for the analysis of lipoproteins [27]. Inorganic acids, organic acids and amino acids were used as spacer molecules in cITP-UV analysis of humic acids [28], while 2-chloropropionic, glyoxylic and levulinic acids were used as spacers in cITP of ribonucleotides [29]. Moreover, a procedure has been described to choose suitable amino acids and dipeptides as spacers for cITP of various anionic species via a calculation that takes isotachophoretic steady-state parameters based on the ionic mobilities and pK values of the separated compounds and migration order into account [30].

However, CA are known to disturb MS detection. For example, in cIEF 0.2% CA added to the sample causes a loss in peptide MS signal of 15-45% [31]. In cITP, this effect is expected to be less present or even absent because the peptides and CA are better separated from each other and, more importantly, according to Kohlrausch' regulation function the CA concentration will never exceed the LE concentration. As a result, when a typical LE concentration (5-10 mM) is used, the maximum CA concentration that is reached during ITP should have a limited influence on the ionisation efficiency of the co-introduced peptides. Adding a higher amount of CA will not affect their concentration in the various ITP bands, only their bands will be broader.

In this study, different from the approaches described in literature so far, we focus on the direct coupling of cITP to MS with the help of CA as spacer molecules. This is demonstrated with peptides as model compounds. Furthermore, the potential of this approach for bio-analysis is investigated with the cITP-MS analysis of a digest of BSA.

2. Materials and methods

2.1 Equipment

cITP was carried out in untreated fused silica capillaries using a programmable PrinCE capillary electrophoresis system (Prince Technologies, Emmen, The Netherlands). Fused silica

capillaries (ID 75 μm, OD 320 μm) were purchased from Bester (Bester B.V., Amstelveen, The Netherlands). Elutions from solid phase extractions were freeze-dried in a Zibus Vaco I freeze-dryer (Zirbus Technology, Bad Grund, Germany) under vacuum that was applied with an Alcatel 2008A vacuum pump (Alcatel Vacuum Products, Hingham, MA, USA). Mass spectrometric experiments were carried out on a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using a CE-MS electrospray interface supplied by Agilent Technologies (Amstelveen, The Netherlands).

2.2 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water, ACN was obtained from Biosolve (Valkenswaard, The Netherlands), formic acid from Acros Organics (Bridgewater, NJ, USA), ammonium acetate, ε-aminocaproic acid, BSA and trypsin from Sigma (St. Louis, MO, USA), acetic acid and sodium hydroxide from JT Baker (Philipsburg, NJ, USA) and the model peptides from Genscript Corporation (Piscataway, NJ, USA). The CA mixture (Pharmalyte 3-10) was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

2.3 Capillary electrophoresis

Prior to every cITP experiment, the separation capillary (length 100 cm) was conditioned by subsequently flushing for 15 min with 1 M NaOH, 15 min with 0.1 M NaOH and 15 min with LE. The LE consisted of 5 mM ammonium acetate and the TE of 10 mM ε-aminocaproic acid. LE, TE and sample were adjusted to pH 3 with acetic acid [18,19]. Hence, the leading ion was NH_4^+ and protonated ε-aminocaproic acid functioned as the terminating ion. Hydrodynamic injection was applied to fill the capillary with the electrolytes and sample.

Prior to loading a sample for cITP analysis, CA were added to the sample. A typical cITP procedure consisted of filling the capillary with LE, loading a sample plug and finally injecting a TE plug. The sample plug as well as the TE plug comprised roughly 5 % of the capillary volume, corresponding with around 0.22 μL. Since the BSA peptides occurred in low concentrations in the sample, a larger amount of sample was injected in order to obtain broader, better detectable BSA peptide zones: 25% of the capillary was filled (1.1 μL). To avoid the sample to enter the MS before separation (by electro-osmotic flow and the pulling

effect of the electrospray) was completed, a higher backpressure than in the experiments with model peptides was used. The counter pressure was generated by a hydrostatic pressure (standard difference in height of inlet and outlet of 35 cm (inlet lower) combined with a negative pressure (±11 mbar in the case of experiments with model peptides and -20 mbar in the case of BSA digest) at the capillary inlet. While applying counter pressure a separation voltage of 24 kV was applied. After 60 min of counter pressure, the sample was allowed to enter the MS by applying 50 mbar pressure at the capillary inlet, while maintaining the ITP voltage.

cIEF-experiments were carried out with 10 mM NaOH as anolyte and 1% FA as catholyte in the same capillaries as were used for cITP. After flushing the capillary with the anolyte, a sample plug consisting of the model peptides and 2% of CA was injected hydrodynamically, followed by catholyte. Then, the analytes and CA were allowed to focus for 30 min before the sample was transferred to the MS using a hydrodynamic pressure of 50 mbar generated by the injector, while maintaining the IEF voltage.

CZE was carried out with 1% acetic acid as background electrolyte. The capillary was filled for ±1% with sample, after which high voltage and 50 mbar hydrodynamic pressure were applied for separation and transfer to the MS.

2.4 Mass spectrometry

The coupling of cITP to the MS was based on a pre-fabricated CE coupling sheath-liquid assisted grounded spray source delivered by Agilent Technologies (Agilent Technologies, Amstelveen, The Netherlands). The sheath liquid consisted of a 50/50 mixture of ACN and 0.2% formic acid and the sheath liquid flow rate was set at 3 µL/min.

Identification of peptides was based on matching of the known masses of the target peptides with the mass information obtained with the MS. To decrease noise levels, all ion traces were processed with Gaussian smoothing (value 4.03) using the software delivered with the MicrOTOF MS by Bruker Daltonics (Bruker Daltonics MicrOTOF DataAnalysis version 3.3). Mass spectra were processed with background subtraction (using an MS spectrum taken from the TE without any other added component) to remove some neutral contaminants in the TE that were detected throughout the whole sample.

2.5 BSA digestion and sample clean-up

BSA was digested using sequence grade trypsin. To improve digestion, the protein

was pretreated with *S*-methyl methanethiosulfonate (MMTS) and tris(2-carboxyethyl) phosphine (TCEP) to break and reduce disulfide bonds, respectively. The digested proteins were purified by SPE using TopTip© pipette tips (Glygen Corp., Maryland, USA) filled with a small amount of Poros RP-2 SPE material. In brief, the material was wetted with ACN, then equilibrated with 0.1% formic acid after which the sample (acidified to pH 3 with formic acid) was applied. Then, the bound analytes were washed with 0.1% formic acid, after which elution was achieved with a 60/40 mixture of ACN and 0.1% formic acid. Next, the eluent was freeze-dried and resuspended in 10 mM TE. The proteomic tool Peptide Mass on www.expasy.ch was used to forecast masses of peptides after digestion.

3. Results and discussion

3.1 Proof-of-principle using model peptides

First, an experiment was done to confirm that the chosen LE and TE were suitable for the cITP of peptides. This was done by using two LE concentrations, 5 mM and 20 mM, in a cITP separation of the 3 model peptides. When using 20 mM, the peptide zones were higher and narrower than with 5 mM LE (Fig. 2.1), which is in agreement with ITP theory. Therefore, the combination of protonated ε-aminocaproic acid as terminating ion and NH_4^+ as leading ion appeared to be suitable for cITP of peptides. Moreover, a direct comparison of CZE with ITP was conducted, which showed considerably higher peptide zones when using the two-buffer system consisting of the leader ammonium acetate and the terminating ion protonated ε-aminocaproic acid (data not shown), while the initial concentrations of the injected samples were equal. On top of this, a series of experiments has been carried out in which the sample loading was varied. These experiments resulted in isotachopherograms with peptide zones of different widths, but of equal concentration (zone heigths) for the different sample loadings (data not shown). According to the ion concentration regulating principle of Kohlrausch (Equation 1) an increase of analyte amount results in broader zones, but not in higher concentrations. Moreover, the current profile was typical for ITP, starting at ± 12.5 μA and decreasing to reach a stable plateau at ± 5 μA after 30 min. Based on the results of these experiments, it can be concluded that cITP takes place.

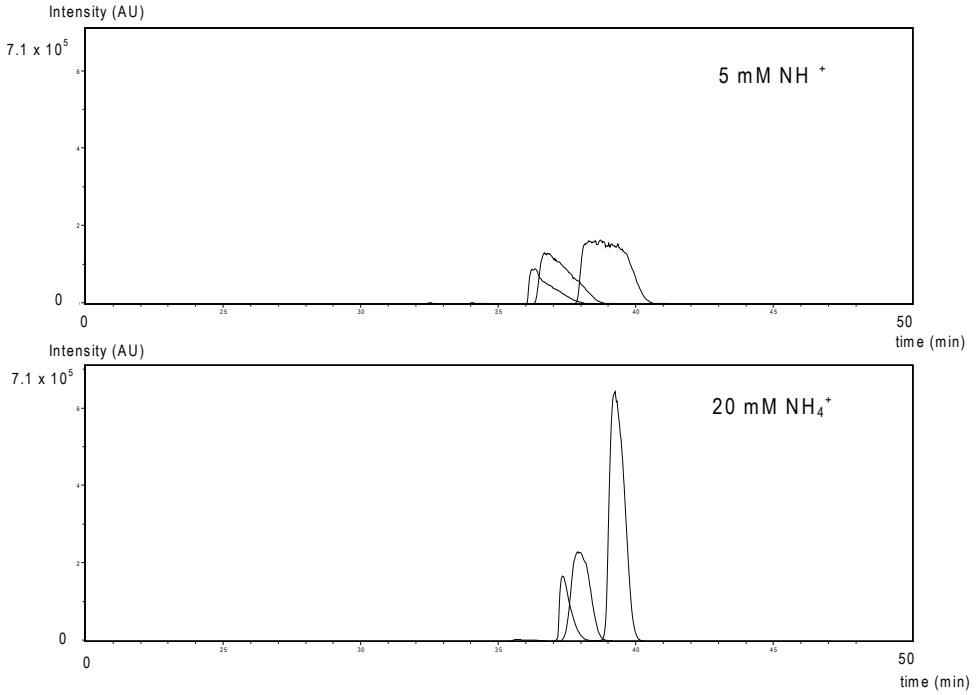

Figure 2.1 Comparison of cITP separations of, in migration order, bradykinin (1-5), bradykinin (1-6) and angiotensin 2 (3-8) with 5 mM and 20 mM NH_4^+ as LE.

Next, the influence of CA on the isotachophoretic behaviour of a set of 6 model peptides (Table 2.1) was examined by comparing the isotachopherograms as obtained with different concentrations of CA (Fig. 2.2). The concentration of the model peptides was 62.5 µg/mL, corresponding to a molar concentration range of 48-110 µM and 10-25 pmol per injected peptide. As expected, the addition of CA to an academic mixture of model peptides improved the separation of the peptides (Fig. 2.2).

Table 2.1 The analysed model peptides, their physicochemical properties and migration orders in cIEF, CZE and cITP.

PEPTIDE DETAILS				**MIGRATION ORDER**		
Name	**Sequence**	**$[M + H]^+$**	**p*I***	**cIEF**	**CZE**	**cITP**
Thymopentin	RKDVY	680.76	8.88	4	1	1
Bradykinin	RPPGFSPFR	1061.20	11.97	1	2	2
Angiotensin 1	DRVYIHPFHL	1297.48	7.38	5	3	3
Bradykinin (1-5)	RPPGF	573.66	10.00	2/3	4/5	4
Bradykinin (1-6)	RPPGFS	660.80	10.00	2/3	4/5	5
Angiotensin 2 (3-8)	VYIHPF	775.90	7.19	6	6	6

Without CA, thymopentin, bradykinin and angiotensin I were not separated, while in the presence of 1% CA they were. When 10% CA was present, the peptide zones were more separated compared to the presence of 1% carrier ampholyte. With only one exception, the mass spectra of all peptides (Fig. 2.2 A-G) revealed that each zone contained only one peptide. For example, whereas without CA the model peptides bradykinin and thymopentin were not separated at all, after addition of 1 or 10% CA, separated single peptide zones were observed. Two peptides could hardly be separated at all, with or without CA, i.e. bradykinin (1-5) and bradykinin (1-6) (Fig. 2.2 F). These peptides differ by only one amino acid (see Table 2.1) and as a consequence apparently have almost the same electrophoretic mobility. Adding CA did not have a spacing effect between the bradykinin (1-5)/bradykinin (1-6) zone and the angiotensin II 3-8 zone. Apparently the CA mix did not contain compounds with the required electrophoretic mobilities to act as spacer between these peptides. In the mass spectra of the isotachopherogram it can be seen that most CA are migrating ahead of the peptides (Fig. 2.2 A). Between bradykinin and thymopentin, only one CA species is observed (Fig. 2.2 C), demonstrating that the majority of the electrophoretic mobilities present in the CA mixture are not in the range of the electrophoretic mobilities of the peptides. In the absence of CA, some peptide zones are not adjacent, because some contaminants present in the academic mixture or in the buffer were concentrated too. These contaminants were not detected by the MS, possibly because their masses were outside the tuning range of the MS (300-1500 m/z).

It is reasonable to suspect that the addition of CA to the peptide mixture results in IEF rather than ITP, since CA were originally designed for IEF separations. To exclude this possibility, a cIEF experiment was performed, which resulted, as expected, in a separation according to the iso-electric points of the peptides (Table 2.1). The peak order in CZE corresponded with the electrophoretic mobilities of the peptides (Table 2.1), excluding the option of a hybrid IEF-ITP separation too. Moreover, in IEF the principle of Kohlrausch does not apply due to the absence of leading and terminating ion zones. This was supported by a comparison between the isotachopherograms with 1% CA and 10% (Fig. 2.2); when more CA were present, the zones were further apart.

In IEF separations, ion concentrations are not regulated by the LE concentration which in practice often results in peptide or protein precipitation at their pI during focusing because too high concentrations are generated [31], instead of the broader zones with regulated concentrations that would be generated in cITP.

Comparison of the intensities of the peptide isotachopherograms shows a decrease in

intensity and differences in releative zone heights between the peptides when more CA are added. This is due to the nature of the CA mixture, which will be discussed in the next section.

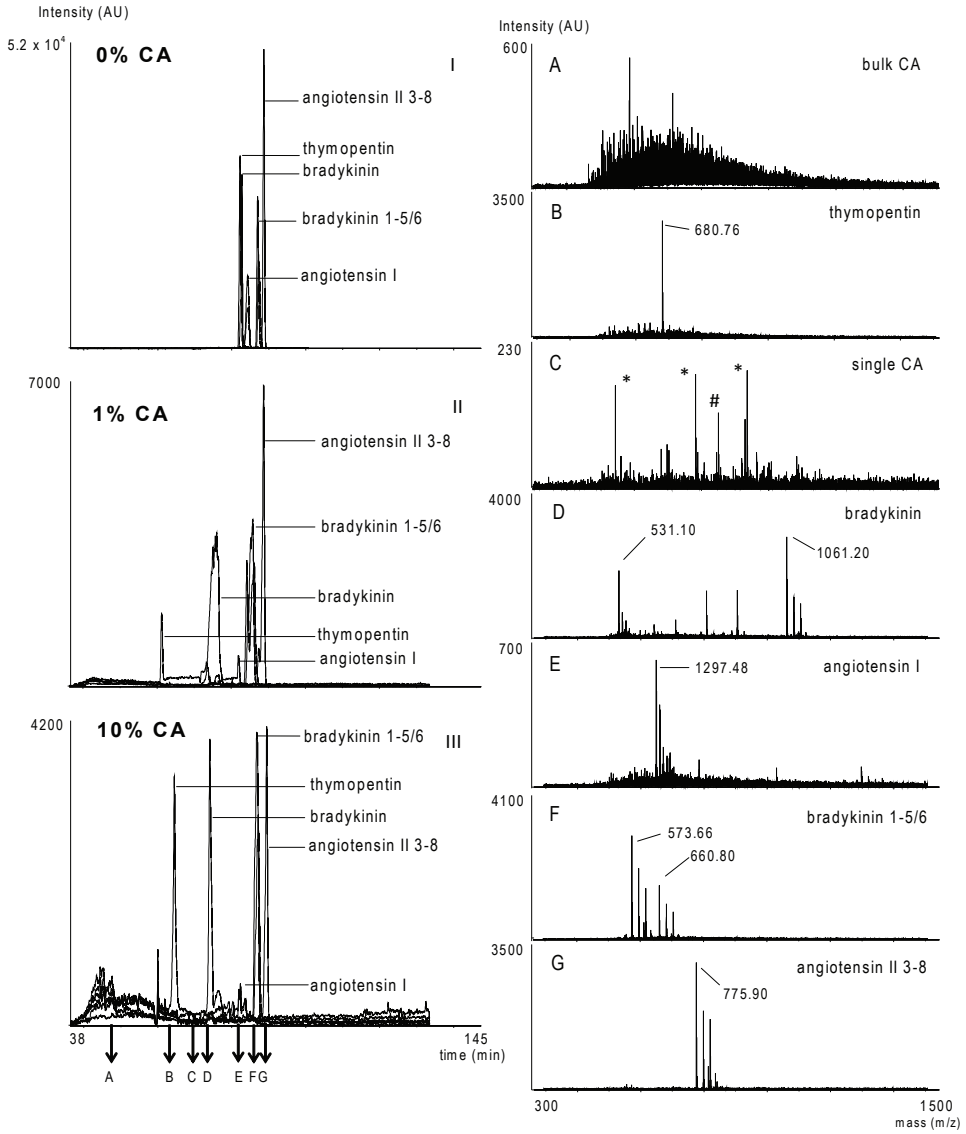

Figure 2.2 Reconstructed ion cITP-MS electropherograms of model peptides and corresponding mass spectra. (I) no CA added, (II) 1% CA added, (III) 10% added to sample. (A-G) MS spectra of zones in III. *) = TE background contamination, #) = CA. Peptide masses are indicated; most masses have 2 sodium adducts. AU means arbitrary units.

3.2 CA as spacers in cIPT-MS of complex biosamples

The initial concentration of BSA before digestion was 4.9 μM. After digestion, RP-SPE and freeze-drying, the peptides were diluted 10 times with TE so that the final concentration was 490 nM in the case of complete recovery. This corresponds to around 500 fmol injected BSA digest.

In Fig. 2.3, the results of the analysis of the BSA peptides without CA are shown. Since the concentration of the peptides is low, they are concentrated in adjacent narrow zones that collapse easily [13] and in consequence enter the MS virtually simultaneously in one plug. In a mass spectrum taken from this plug, it can be observed that many peptides are present indeed.

After adding 1% CA to the sample, the peptides are more separated and spread in the resulting isotachopherogram. In Fig. 2.4 it can be seen that five peptides are fully separated and single-peptide mass spectra could be acquired, of which four were BSA peptides and one was an unknown compound, most probably a trypsin peptide, keratin peptide or a BSA peptide that originated from hydrolysis instead of trypsin digestion. The same five peptides were also detected in the bulk of co-migrating peptides when no CA were added. So far, only these five separated peptides were found. When 10% CA was present during cITP, no peptides could be observed at all (data not shown). It should be noted that since lower backpressure was used during these experiments, migration times and zone lengths in Fig. 2.3 and 2.4 were increased in comparison with Fig. 2.2..

A big disadvantage of the CA mixture used in this study was that the composition of the mixture is not exactly known, as it is a commercial product, meaning that the production and product composition is proprietary and not disclosed. This is actually also true for other commercial CA mixtures. However, a recent series of studies have provided more insight into the composition of commercial CA mixtures [33]. The CA were separated by preparative iso-electric focusing using a Rotofor device and the composition of the obtained fractions was characterised using MS. It appeared that, Pharmalyte pH 2.5-10.5 consists of hundreds of compounds in the mass range of 150-1179. However, it was also discovered that a significant part of the CA in Pharmalyte mixtures were poorly functioning as CA. For Pharmalyte 8-10.5 this was estimated to be as much as 50% of all the detected 'CA'. This was concluded from the fact that no focusing took place, since a continuous distribution along the entire pH gradient in the Rotofor was observed [33]. The CA mixture that was used in our study, Pharmalyte 3-10, also contained as many as hundreds of molecule species. Given the migration behaviour of ampholytic molecules in a pH-gradient with an applied

electric field, the fact that some components were not migrating at all can only be explained by the conclusion that these molecules were neutral. These findings can most likely explain the poor results that were obtained from the BSA digest analysis in our study. Since the sample is confined between the LE and TE zone and due to the very low EOF at this (low) pH, the neutral components of the CA mixture will be inseparable from the peptides by electrophoresis once they are mixed with the sample. These neutral CA cause a high background as well as ion suppression.

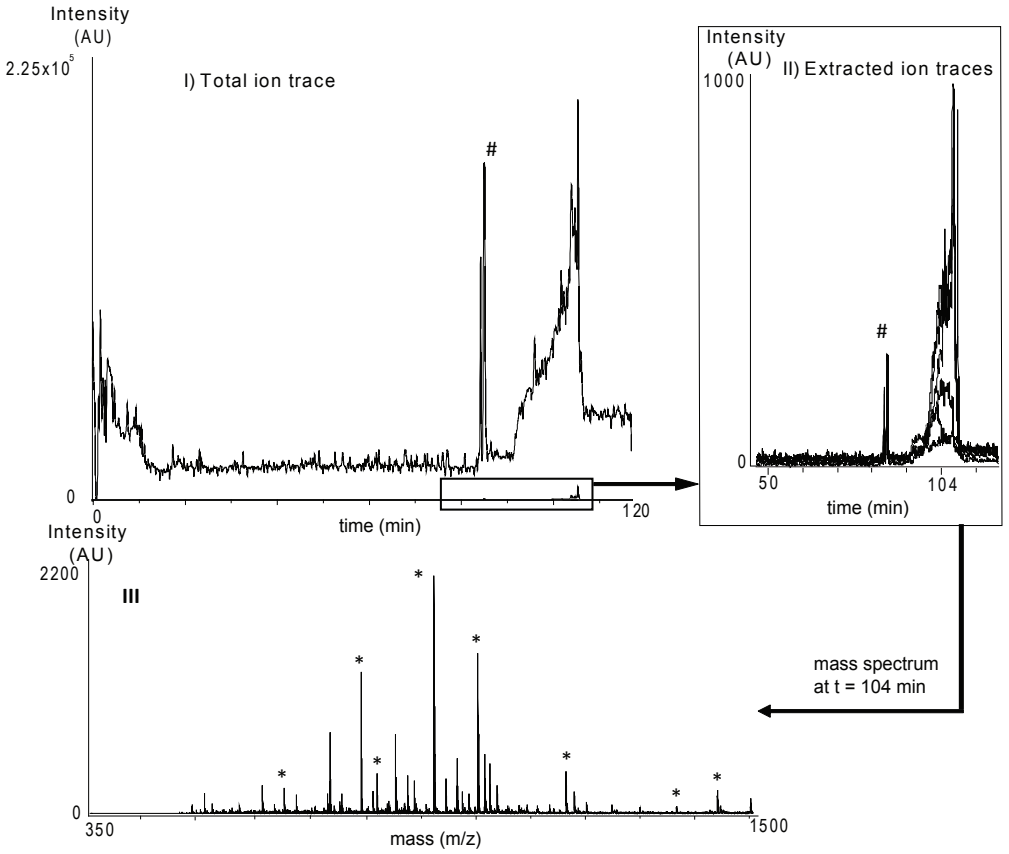

Figure 2.3 cITP-MS of BSA peptides without CA: (I) total ion count isotachopherogram, (II) reconstructed ion isotachopherograms of selected peptides, (III) mass spectrum of the highest peptide signal at 104 min; masses due to BSA peptide are marked (*), (#) indicates an artefact.

In the analysis of academic mixtures of model peptides they already caused a severe signal decrease, from 5.2 x 10^4 AU for angiotensin II 3-8 when no CA were added, to 4.2 x 10^3 AU when 10% of CA were added (Fig. 2.2). This negative influence of impurities in the CA

mixture was not equal for every zone, since the relative zone height differed between 0, 1 and 10% added CA, as can be observed in Fig. 2.2.

To infuse the sample into the MS, some hydrodynamic pressure had to be used, due to the aforementioned very low EOF, which inevitably caused some zone broadening. When academic mixtures with relatively high analyte concentrations were injected, broad sotachophoretic peptide zones were obtained, that are less susceptible for disturbing effects

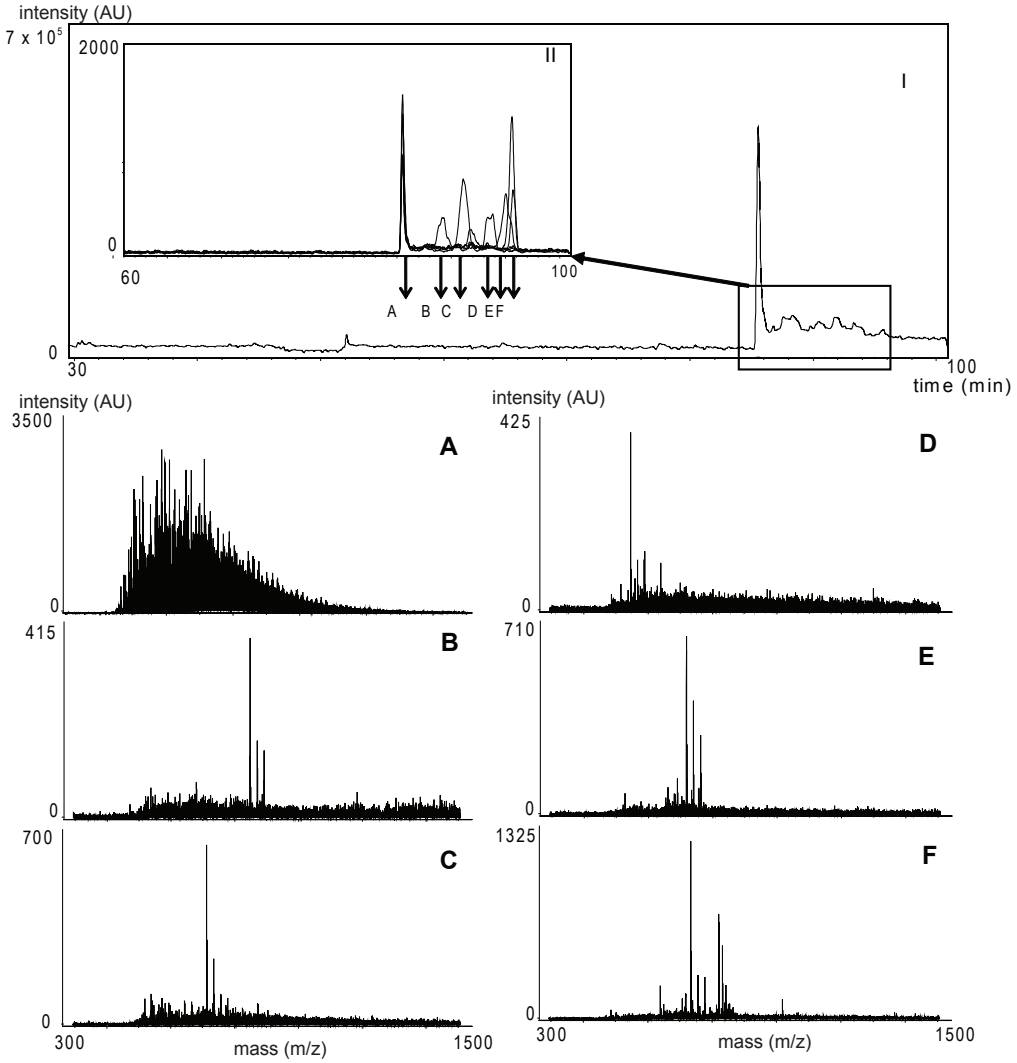

Figure 2.4 cITP -MS of BSA peptides containing 1% CA: I) total ion count isotachopherogram, II) reconstructed ion isotachopherograms of selected peptides, (A) mass spectrum of CA, mass spectra of BSA peptides (B) LSQKFPK, (C) SEIAHR, (D) VASLR, mass spectra of (E) unknown molecule species and (F) mass BSA peptide FGERALK and an unknown molecule species.

due to zone broadening. However, when the peptide zones are narrower, as was the case with the BSA digest, a little zone broadening already causes a severe decrease of signal. In combination with a high background this results in a poor bioanalytical performance.

We have demonstrated that it is possible to physically separate peptide analyte zones by mixing the sample with spacer molecules, making a consecutive zone broadening step such as CZE unnecesary. However, due to the severe contamination in the CA mixture, the overall performance of the developed cITP-MS method was not satisfactory yet, and just a few peptides were detected in a complex biosample. To obtain better results, more suitable spacer compounds should be developed. This spacer compound mixture should meet two main criteria. First, such a mixture should contain a large and continuous gradient of electrophoretic mobilities that are close to peptide electrophoretic mobilities in order have a maximal chance of spacers between the peptide zones. Secondly, the spacer compounds should not interfere with the ionisation of the peptides during ESI to avoid ion suppression of the peptide signals. This can be achieved by choosing compounds that are more polar and hydrophilic than peptides. Polar compounds have a lower surface activity and therefore tend to be on the inner side of the spray droplets during ESI and therefore they ionise worse and cause less ion suppression [34].

4. Concluding remarks

In this paper, we explored the use of spacer compounds to couple cITP directly to MS. We have shown that it is possible to physically separate and detect peptide analyte zones in cITP-MS after when spacer molecules are added to the sample before injection. With this approach, we were able to detect sub-micromolar peptide concentrations in a complex sample, in single-analyte mass spectra. However, especially in the analysis of the more complex sample with lower peptide concentrations we encountered a severe drawback of CA as spacer molecules: CA mixtures appeared to be ill-defined mixtures with a lot of disturbing contaminations present. To be able to detect sub-nanomolar peptide concentrations, i.e. for bio-analysis, there is a demand for more suitable spacer compounds. Future research will be dedicated to the development of alternative spacer compounds.

5. References

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