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The effect of the mobile phase additives on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

The study of the effect of mobile phases on sensitivity in the analysis of peptides and proteins by high-performance liquid chromatography (HPLC)–electrospray mass spectrometry (ESI-MS) has been the aim of this review. Reversed-phase chromatography (RPLC) is the chromatographic mode most suitable for coupling with ESI-MS since mobile phases containing organic modifiers are used. The analysis of proteins and peptides by RPLC mostly involves the use of trifluoroacetic acid (TFA) as an ion-pairing agent despite its being a strong suppressor of the MS signal. Different studies reporting the effects of using other ion-pairing agents (other perfluorinated acids, acetic acid, formic acid, etc.) and buffers (ammonium acetate, ammonium formate, ammonium bicarbonate, morpholine, etc.) in RPLC–ESI-MS of proteins and peptides did not yield a single strong candidate that could generally replace TFA. The enhancement in sensitivity with other reagents observed in some cases strongly depended on the analyte, the experimental conditions used, and the mass spectrometer and, usually, it did not compensate for the loss in separation resolution related to TFA. The examples of direct coupling of affinity, size-exclusion, or ion-exchange chromatography (IEC) to ESI-MS are very limited because of incompatibilities related to the use of mobile phases containing high salt concentrations. To overcome this problem, an intermediate desalting step is needed. Multidimensional chromatography, microdialysis, and ion-capture modules can be used to couple these chromatographic modes with ESI-MS. Multidimensional chromatography with RPLC as a second dimension has most often been used. Although most examples involve the trap and analysis in the second dimension of a certain part of the first separation, some comprehensive analyses of the entire sample in the second dimension have also appeared.
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1. Introduction

In order to understand the role biomolecules play in biochemical processes, it is important to develop methods for their identification. Since in most cases there is a clear limitation of the sample amount, these methods have to enable the sensitive detection of these molecules. Mass spectrometry (MS) is the detection technique most widely used for this purpose. Nevertheless, this technique requires the use of more or less clean samples in order to obtain suitable detection limits. Matrix components coeluting with a target analyte may affect its ionisation and thus provide no valid results [1].

In order to overcome this problem, a separation technique enabling the clean up of a sample or the preconcentration of analytes prior to detection is needed. High-performance liquid chromatography (HPLC) has been widely coupled to electrospray ionisation mass spectrometry (ESI-MS) for the analysis of proteins and peptides. Nevertheless, the performance of HPLC–ESI-MS can be compromised if no adequate mobile phases are chosen. In fact, the eluent choice in ESI-MS is severally restricted to volatile compounds while mobile phases typically used for the separation of proteins and peptides by HPLC may not be readily volatile and, therefore, not

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compatible with the electrospray process [2]. A suitable eluent for electrospray ionisation should contain an organic modifier (methanol or acetonitrile) and a volatile buffer whose concentration could also be critical; concentrations that are too high may result in the suppression of the analyte signal, while concentrations that are too low may lead to poor peak shape and efficiency.

This review is mainly centred on HPLC–ESI-MS for the analysis of peptides and proteins although certain relevant examples comprising the analysis of other organic molecules have also been included. The effect of the mobile phases additives used in every chromatographic mode on the sensitivity in HPLC–ESI-MS and the problems faced in each case to match LC with ESI-MS have been reviewed.

2. Reversed-phase chromatography

Reversed-phase chromatography (RPLC) constitutes a powerful tool for the separation of very heterogeneous samples and has been widely used in combination with mass spectrometry detection. RPLC is the chromatographic mode which best matches ESI-MS since the mobile phases used present low ionic strengths and contain organic modifiers. The chemical composition of these mobile phases plays an important role both in the chromatographic separation and in the mass spectrometer performance.

Mobile phases used in RPLC for the analysis of proteins and peptides contain an additive which is working as an ion-pairing agent. This component increases the hydrophobicity of molecules by forming ionic pairs with their charged groups. As a consequence, interaction of the molecules with the hydrophobic stationary phase is possible and, therefore, so is their separation. Moreover, these additives usually yield very high or low pHs and promote protein unfolding and denaturation. Thus, molecules are present in the same random coil conformation, eluting in sharper and more symmetrical peaks [3–5].

Table 1 shows the ion-pairing agents most used in the separation of proteins and peptides by RPLC. As can be seen, these additives are added to the mobile phase in low concentrations. The ion-pairing agent most widely used for the separation of proteins and peptides is trifluoroacetic acid (TFA) although reagents such as acetic acid or phosphate buffer have also been employed. As well as ion-pairing agents, other additives such as sodium dodecyl sulphate (SDS), urea, and mercaptoethanol can also be added to the mobile phase to improve separation. Unfortunately, many of these compounds result in deleterious detection by ESI-MS.

In fact, typical RPLC mobile phases containing phosphate buffer or TFA result in low sensitivity in the detection of proteins and peptides by ESI-MS. Salts and other non-volatile compounds are not suitable for ESI-MS since they can deposit on the ion source. This would result in capillary obstruction affecting the sensitivity and the accuracy of the quantitative analysis. Nevertheless, volatility is not the only limitation. A widely used volatile reagent such as TFA is also not suitable for sensitive mass spectrometry detection. In fact, TFA forms very strong ion pairs with analytes that can not be broken apart in the conditions used in the electrospray ionisation in-

Table 1
Ion-pairing agents used in the analysis of proteins and peptides by RPLC [5–10]

Eluent	Concentration in mobile phase	Characteristics
Perfluorinated carboxylic acids		
Trifluoroacetic acid	0.05–0.5%	Very volatile
Heptafluorobutytic acid	10–50 mM	Volatile
Others: nonafluoropentanoic acid, tridecafluoroheptanoic acid, pentadecafluorooctanoic acid, etc.	10–50 mM	Retention controlled by the alkyl chain-length; volatile
Other acids		
Acetic acid	0.5–1 M	Volatile
Formic acid	20–60%	Volatile; lower resolution and recovery than TFA
Hydrochloric acid	5 mM	Volatile and corrosive
Salts		
Phosphate buffer (pH 2–9)	10–20 mM	Not volatile
Triethylammonium phosphate (pH 4)	125 mM	Not volatile
Formate (ammonium, trialkylammonium or pyridinium salt)	20–200 mM	Volatile
Acetate (ammonium, trialkylammonium or pyridinium salt)	20–200 mM	Volatile
Perchlorate	100 mM	Not volatile
Ammonium bicarbonate (pH 7–11)	50–100 mM	Volatile
Ammonium sulfate (pH 4)	125 mM	Not volatile
Alkylsulfonates/alkylsulfates (e.g., heptanesulfonate)	10–15 mM	Retention controlled by the alkyl chain-length; not volatile
Teraalkylammonium salts (e.g., tetrabutylammonium salts)	10 mM	Retention controlled by the alkyl chain-length; not volatile
Alkylamines (e.g., triethylamine, dodecylamine, pyridine) (acetate, formate or phosphate)	10 mM	Volatile
Bases		
Morpholine	10 mM	Volatile

terface preventing the ionisation of analytes. Moreover, TFA also results in spray instability and signal reduction due to the high conductivity and high surface tension of the eluent [3]. Consequently, ion-pairing agents allowing for a compromise between a sensitive mass detection and an efficient chromatographic separation are required for suitable RPLC–ESI-MS performance.

Numerous attempts have been made to achieve this compromise. One approach has been the reduction of the detrimental effects derived from the use of TFA by the addition of an organic sheath liquid. In fact, the use of an organic auxiliary liquid has been demonstrated to help the generation of ions in the electrospray process [11–13]. The most recent trial base on the use of an auxiliary liquid was developed by Apffel et al. [3]. They proposed the post-column addition of a highly concentrated weak acid (RCOOH) which would compete with TFA (strong acid) to pair with the analyte. As the weak acid is present in a high concentration, the competition between TFA and the weak acid would be driven towards the deprotonation of the weak acid:

$$CF_3COO^- + RCOOH \rightleftharpoons CF_3COOH \uparrow + RCOO^-$$

Consequently, the protonated TFA could be evaporated from the droplet and the analyte would form ion pairs with the new anion ($RCOO^-$). As these ion pairs would be weaker than those previously formed with the TFA anion, the presence of the analyte in its protonated form would be promoted. Apffel and his colleagues tried different organic acids (propionic acid, valeric acid, formic acid, acetic acid, and butyric acid) added post-column at concentrations ranging from 0 to 20% in an organic carrier solvent. With the exception of formic acid, the rest of the additives resulted in enhanced sensitivity, with propionic acid observed to perform best. They also tried different organic carrier solvents (2-propanol, acetonitrile, methanol, ethanol, butanol-acetonitrile, 2-methoxyethanol, 2-methoxyethanol-2-propanol, and 2-methoxyethanol-butanol) since this could also affect the stability and intensity of the signal. The post-column addition of propionic acid-2-propanol (75:25, v/v) in a 1:2 proportion in the HPLC mobile phase resulted in an overall 10- to 100-fold improvement in the signal-to-noise ratio. Fig. 1 shows the effect of the addition of propionic acid-2-propanol in the analysis of different amounts of a tryptic digest of lysozyme by RPLC–ESI-MS using TFA. In all cases, significant increases in the MS signal were observed when the auxiliary acid was added. Similar improvements were also detected with other ion-pairing agents such as heptafluorobutyric acid (HFBA) and hydrochloric acid. Nevertheless, these interesting results were only valid for small proteins and peptides but not with larger proteins in whose case it is not possible to break up enough ion pairs formed with TFA to efficiently recover the signal.

Different studies were focused on the discovery of alternatives to TFA which do not interfere with the ion-formation process while still yielding acceptable chromatographic behaviour. The first studies were performed with thermospray (TSP) RPLC–MS since problems similar to those observed in ESI were found. In fact, TSP performance is also highly dependent on mobile phase composition requiring the presence of volatile buffers. Voyksner and Haney [14] evaluated the effect of different volatile buffers (ammonium carbonate, ammonium bicarbonate, ammonium acetate, ammonium formate, and triethylamine) on the analysis of organic molecules. They found better results when using ammonium acetate and ammonium formate, whereas the strong bases (carbonate, bicarbonate, and triethylamine) yielded the lowest sensitivities. The authors supported these results on the basis of a competition mechanism between analytes and buffer for protons and attributed the low signal observed when using basic buffers to their stronger affinity for protons than the analytes. They also studied the influence of the concentration on sensitivity, with the buffer ammonium acetate yielding the highest signal. By increasing its concentration to 0.08 M, an increase in the MS response was observed. Higher concentrations of buffer did not result in a significant increase in sensitivity. These results were similar to those obtained by Duchateau et al. [15] who, some years later, confirmed the applicability of ammonium formate, ammonium acetate, and ammonium bicarbonate for the analysis of three decomposition products of an α-dipeptide by RPLC–MS. The separation of these compounds by RPLC and UV detection had been described several times using alkylsulphonates as ion-pairing agents. These authors tested the analysis of these compounds by RPLC–MS using TFA, ammonium formate, ammonium acetate, and ammonium bicarbonate as volatile reagents. Even though ammonium formate resulted in the highest signal-to-noise ratio, it yielded very poor resolution. The presence of 8 mM TFA in the mobile phase significantly improved separation without increasing background signal.

The first studies with ESI compared its performance when analysing organic compounds dissolved in different LC eluents containing ion-pairing agents. Miller and Fisher [16] tried different volatile (valeric acid (a weak ion-pairing agent) and perfluoroheptanoic acid (a strong ion-pairing agent)) and non-volatile reagents (heptane sulphonic acid) in the positive mode and in the negative mode (tributylamine (a strong, volatile ion-pairing agent), tetrabutylammonium hydroxide (a strong, non-volatile ion-pairing agent) as ion-pairing agents for the analysis of organic compounds using ESI-MS. As expected, volatile ion-pairing agents (valeric acid, perfluoroheptanoic acid, and tributylamine) yielded higher responses than non-volatile agents (heptane sulphonic acid and tetrabutylammonium hydroxide). In addition, the signal observed was, in some cases, related to the volatility of the analyte itself, the most volatile producing the highest signals. Temesi and Law [17] also studied the effect of TFA, formic acid, and ammonium acetate on the MS response observing the best results with formic acid and the worst with TFA. Furthermore, analyte responses decreased with an increased concentration of the ion-pairing agent (in the range 1–100 mM) and significant variations of the signal were detected depending on whether the organic modifier

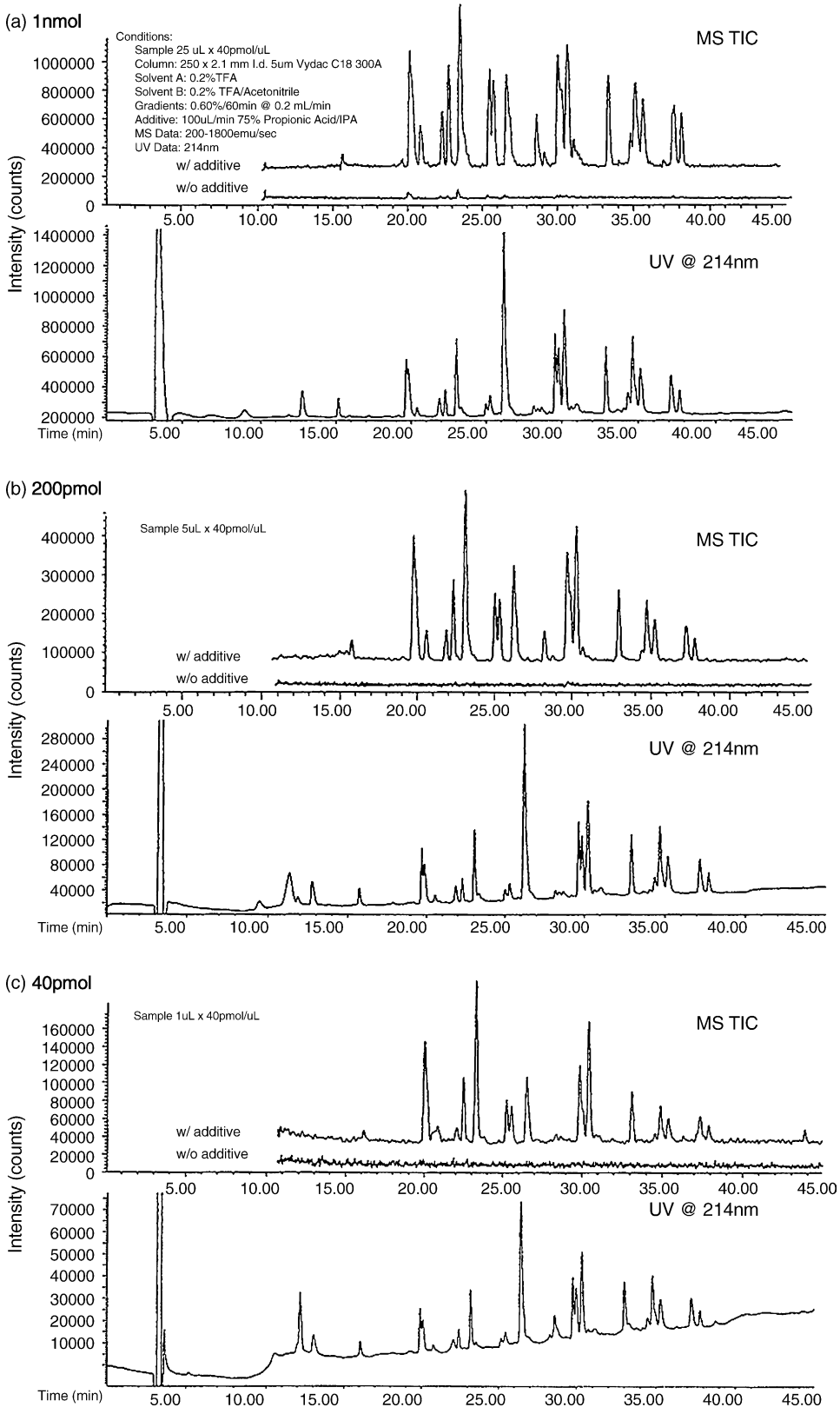

Fig. 1. Peptide maps of tryptic digests of different amounts of lysozyme by RPLC–ESI-MS (with and without postcolumn addition of propionic acid-2-propanol) and RPLC–UV. With permission from ref. [3].

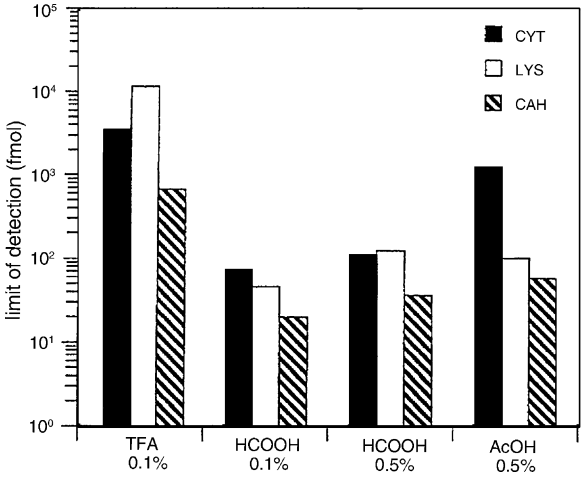

Fig. 2. Influence of the mobile phase additive on the detectability of proteins (cytochrome c (CYT), lysozyme (LYS), and carbonic anhydrase (CAH)) by RPLC-ESI-MS. With permission from ref. [19].

was methanol or acetonitrile. In this respect, Vanhoutte et al. [18] discovered an important dependence of sensitivity on the percentage of organic modifier used in the mobile phase when using a nano-electrospray interface. This dependence was also related to the nature and concentration of the buffer. Thus, sensitivity was significantly reduced when less than 40% methanol was present in the mobile phase using a 0.1 M ammonium acetate buffer, while this percentage decreased to 20% when the buffer was removed. As well as methanol and acetonitrile, trifluoroethanol or hexafluoroisopropanol, known for their tendency to stabilize the secondary structure of peptides, have also been used as organic modifiers [19]. Moreover, non-polar solvents such as chloroform in combination with polar solvents (methanol/water) and acidified with acetic acid or TFA have successfully been applied for the analysis of hydrophobic peptides [19].

From the experience of previous work focussing on the analysis of small peptides and other organic molecules (e.g. drugs), formic acid and acetic acid were seen to be the most promising candidates for the replacement of TFA in the analysis of proteins and peptides by RPLC–ESI-MS. Huber and Premstaller [20] compared the performance of acetic acid, formic acid, and TFA for the analysis of proteins with molecular masses ranging from 14,000 to 80,000. Fig. 2 shows the influence of the mobile phase additive on the detectability of these proteins. They found a 35- to 160-fold improvement in protein detectability when using 0.1% formic acid in comparison to 0.1% TFA. This improvement was significantly greater than that obtained by Liu et al. [21] who observed a three-fold increase in MS sensitivity with 2% formic acid in comparison to 0.1% TFA, although in this case a different method and instrumentation were used. Even so, in both cases the improvement in sensitivity obtained was in detriment of the separation efficiency, with increases ranging from 32 to 104% in peak width at half height when substituting TFA for acetic or formic acid. This loss in efficiency did not constitute a problem when working with standard proteins or with very clean samples, nor with peptides in which case RPLC with acetic acid or formic acid resulted in minimal chromatography penalty. Nevertheless, there should be a better balance between separation performance and sensitivity detection when more complex samples are used.

An example is observed in the work of Corradini et al. [22] who tried TFA, formic acid, and acetic acid for the analysis of a more complex kind of protein (membrane proteins binding chlorophylls isolated from spinach leaves) by RPLC–ESI-MS. Despite the fact that formic and acetic acid showed less signal suppression than TFA in ESI-MS, these acids gave poorer resolution. In this particular case, the decrease in resolution was so critical that the authors suggested the use of a lower percentage of TFA (0.05%) as the most suitable alternative for the analysis of these proteins. The use of low TFA concentrations minimised signal suppression in comparison with 0.1% TFA. Moreover, part of the decrease in sensitivity observed in comparison with other ion-pairing agents such as formic acid was regained through the increase in sample concentration entering the mass spectrometer and derived from the sharper peaks encountered when using TFA [3].

Other approaches have been the use of mobile phases containing mixtures of different ion-pairing agents [23,24] and the use of other perfluorinated carboxylic acids with longer *n*-alkyl chains than TFA [25,26].

The idea of using mixtures of TFA with other volatile ion-pairing agents in the mobile phase was first applied in TSP–MS by Duchateau et al. [15] who suggested the use of a mixture of TFA with formic acid for sensitive and high resolution RPLC–MS. Some years later other authors applied the same idea for the detection of proteins by RPLC–ESI-MS. Clarke et al. [23] selected a mixture of 0.5% acetic acid and 0.02% TFA to reach a balance between sensitivity and resolution in the detection of amyloid-β polypeptides while Chong et al. [24] used mobile phases containing 0.1% TFA and formic acid in concentrations ranging from 0.2 to 0.3% for the analysis of protein fractions from human breast cancer whole cells using a non-porous RPLC column coupled to ESI-MS.

Regarding the use of other perfluorinated carboxylic acids other than TFA, Elfakir and co-workers [9,25,26] compared the effect of using tridecafluoroheptanoic acid (TDFHA), HFBA, nonafluoropentanoic acid (NFPA), and pentadecafluorooctanoic acid (PDFOA) as ion-pairing agents related to TFA for the analysis of amino acids by RPLC. They studied the effect of the concentration and length of the side alkyl chain of the perfluorinated acid on the MS response and chromatographic selectivity, respectively. As expected, the best separation of amino acids was obtained with the perfluorinated reagent with the longest chain (PDFOA) using a classical silica column. Nevertheless, this agent did not yield the best separation when the silica column was replaced by a graphitic carbon column. Moreover, they also observed increasing signal intensities at higher ion-pairing concentrations [25]. These results contrasted with those observed by

other authors [27,28] and with those observed by the same authors when using other experimental conditions [26]. In these cases, the presence of a significant concentration of ion-pairing agent in the mobile phase resulted in analyte signal suppression.

The same perfluorinated acids (TFA, HFBA, TDFHA, NFPA, and PDFOA) were applied two years later by the same group for the analysis of 23 small peptides (with masses ranging from 133 to 613) by RPLC–ESI-MS [29]. They observed TFA and HFBA (the agents containing the shortest side chains) were not efficient enough to separate most polar peptides. PDFOA (the agent containing the longest side chain) resulted in a very high retention time but not the best selectivity. Although, in principal, both NFPA and TDFHA yielded good peptide separations, the higher volatility of NFPA, its lower re-equilibration time, and its general higher selectivity lead to the choice of this reagent for the LC–MS analysis of small peptides.

More recent papers describe the results obtained when comparing the performance of ion-pairing agents of a different nature in the analysis of peptides and proteins by RPLC–ESI-MS. TFA, HFBA, acetic acid, and formic acid have been compared in the analysis of peptide mixtures [30]. TFA gave the best resolution while HFBA was the ion-pairing agent yielding the worst separation, and acetic and formic acid provided a better balance. Regarding detection, formic acid yielded the strongest (100 times higher than TFA) signal by ESI-MS. They also compared the separation obtained when using methanol and acetonitrile as organic modifiers. In general, better resolution, selectivity, symmetry, and efficiency were observed with acetonitrile.

One recent work [31] studied the effect of different reagents (TFA, formic acid, ammonium formate, and phosphate buffer) at constant low pH (2.3–3.2) in the separation of basic peptides. The worst separations were observed with formic acid. This was attributed to reduced column capacity derived from an increased mutual repulsion of charge peptides in the stationary phase surface when low ionic strength buffers such as formic acid were used. As inorganic salts are non-volatile and TFA is a signal suppressor, the authors suggested the use of ammonium formate since its ionic strength was high enough to reduce column overloading.

García et al. [32] tried a wide range of reagents in the positive mode at pHs ranging from 2 to 11.5 for their performance in the separation of standard proteins by RPLC–ESI-MS. The compounds used were: TFA, formic acid, acetic acid, HFBA, ammonium acetate (pH 3 and 6), ammonium formate (pH 3 and 6), ammonium bicarbonate, ammonium hydroxide, and morpholine. Formic acid (0.2%) was the additive yielding the highest responses (in FIA experiments) followed by acetic acid (0.3%), ammonium formate (10 mM, pH 3), and ammonium bicarbonate (50 mM, pH 9), while the worst results were observed with TFA, ammonium formate (pH 6), and ammonium acetate (pH 6). The effect of the variation of the concentration on the MS signal with the ion-pairing agents giving the highest responses (formic acid, acetic acid, ammonium formate (pH 3), and ammonium bicarbonate (pH 9)) was also investigated using FIA experiments (Fig. 3). In general, responses increased initially and decreased when very high concentrations were used with the exception of ammonium bicarbonate in which case no such decrease was observed. These selected ion-pairing agents were tested for the separation of standard proteins in comparison with TFA. Ammonium formate (pH 3) and ammonium bicarbonate (pH 9) did not result in any separation of the protein tested. Although the separation quality was worse than that observed with TFA, formic acid and acetic acid were the only agents enabling that separation. Nevertheless, problems related to a poor recovery of proteins in the separation column when using formic acid and the significant signal suppression observed when TFA was employed, made neither of them suitable for the sensitive detection of the proteins by RPLC–ESI-MS. The use of 0.3% acetic acid as an additive in RPLC–ESI-MS provided a suitable balance between mass response and separation of proteins in LC–MS. Furthermore, the comparison of calibration curves obtained by FIA with those observed when the separation took place showed an increase in the sensitivity when the column was connected [32].

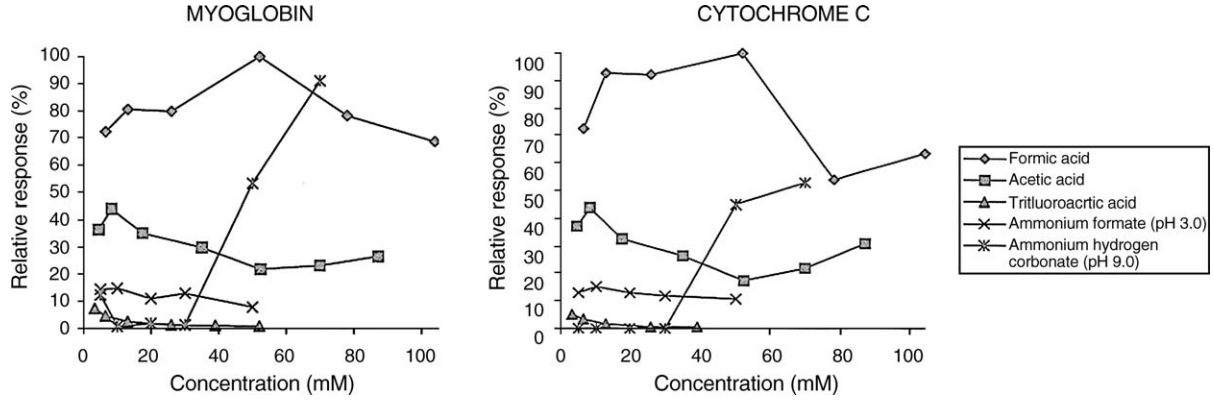

Fig. 3. Variation of the signal (expressed in percentage related to the maximum signal) obtained by ESI-MS for 2 μM solutions of myoglobin and cytochrome c with different buffers. With permission from ref. [32].

As can be seen, and despite the efforts to the contrary, TFA continues to be the ion-pairing agent most widely used with no solid alternative having been discovered, especially when complex separations are required. In fact, the use of other additives proposed as alternatives to TFA resulted in ruined separations when complex samples are used as in the case of proteomic and mapping studies. The only change proposed in these cases to alleviate the suppression effects of TFA has been the reduction of its concentration to 0.05%, although accompanied by a small decrease in chromatographic performance [33–35]. More recently, Naidong [36] reported that the addition of acetic acid to a mobile phase containing TFA resulted in reduced ionisation suppression while chromatographic peak shape was preserved.

3. Hydrophilic-interaction chromatography

Liquid chromatography has been used for the reduction of matrix interferences in mass spectrometry. Nevertheless, very polar compounds are poorly retained on a reversed-phase column and this weak analyte retention may result in detrimental matrix effects. Moreover, the use of very high aqueous mobile phases, such as those used in RPLC, can compromise the spray formation and be critical for sensitivity. One approach to achieve better sensitivity and column retention could be the use of hydrophilic-interaction chromatography. This chromatographic mode uses polar mobile phases containing low water and high organic modifier proportions, more favourable for the electrospray process. Unlike normal phase chromatography, hydrophilic-interaction chromatography uses water-miscible organic solvents such as acetonitrile instead of water-immiscible ones such as hexane or chloroform [36]. This chromatographic mode has been applied to the analysis of polar analytes in LC–MS and could be an alternative to RPLC for the separation of some kinds of peptides.

4. Chromatographic modes involving the use of salts

The application of ESI-MS as a detection technique has centred on the chromatographic modes using volatile buffers such as RPLC. Ionic HPLC systems (hydrophobic-interaction, affinity, ion-exchange, and size-exclusion chromatography (SEC)) have had much less success due to the incompatibility of ESI-MS with the non-volatile buffers commonly used in these chromatographic modes. In fact, the use of mobile phases containing inorganic salts affects the electrospray stability and provokes the formation of adduct peaks. Adduct formation makes molecular ions of the sample disperse among many different species at different mass-to-charge ratios (m/z) resulting in decreased sensitivity and complicated mass spectra. Nevertheless, when the preservation of biological activity and native structure is required, chromatographic modes involving the use of salts are preferred and, consequently, the interest in coupling these chromatographic modes with ESI-MS is clear.

4.1. Hydrophobic-interaction chromatography (HIC)

Hydrophobic-interaction chromatography and RPLC are related techniques, but stationary phases are less hydrophobic and, therefore, operate under less denaturing conditions. One of the main differences with RPLC are the elution solvents used. HIC gradients begin with a very high ionic-strength solvent (high concentration salt solutions) and end with low concentration salt solutions or with the addition of organic additives (e.g. ethylene glycol or isopropanol) while in RPLC, elution gradients start with aqueous buffers and end with an organic solvent (e.g. acetonitrile). Although there are no examples of the use of HIC coupled on-line with ESI-MS, a few examples can be found for the off-line combination with MALDI [37,38].

4.2. Affinity chromatography

Affinity chromatography, especially frontal affinity chromatography (FAC), has been coupled on-line with ESI-MS as a screening technique, as a purification technique, and as a preconcentration technique for the analysis of proteins and peptides [39]. FAC consists of the continuous infusion of analytes (potential ligands) through a column containing an immobilized biological receptor (e.g. a protein or a cell fragment). The elution time for each analyte is related to its binding strength. Thus, the strongest the binding of an analyte in the column results in the longest retention time. The coupling of FAC with ESI-MS allows for the monitoring of eluted analytes. For that purpose, the elution buffer used has to enable the elution of retained compounds and provide a suitable sensitivity in ESI-MS.

FAC–ESI-MS has been used to screen oligosaccharide, peptide, and enzyme inhibitor libraries against an antibody, a lectin, and protease ligands [40–43]. In all cases, the FAC–MS system consisted of three syringes in parallel containing the test sample, the elution buffer, and the makeup solution that were connected to a pump by a switching valve connected to the inlet of the affinity column. The outlet of the affinity column was connected to a tee for the addition of makeup buffer that flowed directly to the ESI-MS. The elution buffer and makeup solution used were compatible with the electrospray process and consisted of 2 mM ammonium acetate (pH 6.6) and 10% (v/v) of the elution buffer in acetonitrile, respectively.

Ogata et al. [44,45] designed a lab-on-valve apparatus for coupling FAC and ESI-MS which enabled bead regeneration, column repacking, and repetitive measurements in minutes. This system was applied to screen the binding of immobilized streptavidin to biotin containing conjugates, and the binding of a peptide mixture to certain proteins (human and *Trypanosoma brucei* tPex5). In this case, the elution buffer

consisted of 4.5 mM 2,2-difluoroethylamine, 2.3 mM acetic acid (pH 7.0) [45].

Another mode of affinity chromatography that has been coupled with ESI-MS for the analysis of proteins and peptides is immobilized metal-ion affinity chromatography (IMAC). This technique is based on the selective complexation of electron-donating groups, such as phosphate, cysteine, tryptophan, and histidine, with an appropriate immobilized metal ion (e.g. Fe (III)). However, optimal conditions for the elution of proteins from an IMAC column are not compatible with ESI-MS and result in very low detection limits and poor separations. In this respect, Nuwaysir and Stults [46] developed an on-line IMAC (Fe (III))–ESI-MS method for the determination of phosphopeptides and phosphoproteins. They observed that the limiting factors in sensitivity of IMAC–ESI-MS derived from the use of eluents incompatible with ESI-MS were less important than sample losses derived from sample handling in off-line experiments.

Different alternatives have been proposed to couple IMAC and ESI-MS: off-line IMAC followed by solid-phase extraction (SPE)–capillary electrophoresis (CE)–ESI-MS [47], off-line IMAC followed by RPLC–ESI-MS [48,49], on-line IMAC–CE–ESI-MS [50], off-line IMAC–RPLC followed by ESI-MS [51], and on-line IMAC–RPLC–ESI-MS [52–55]. Of these approaches, on-line multidimensional chromatography–ESI-MS is the most popular option and will be reviewed below.

4.3. Ion-exchange chromatography (IEC)

Ion-exchange chromatography has widely been used for the separation of proteins and peptides since both of them contain amino acids or carbohydrates with groups that can be positively or negatively charged. In ion-exchange separations of proteins and peptides, the composition of the mobile phase plays a decisive role. Usually, protein to be isolated is bound to an ion-exchanger and then sequentially separated by linear or step variations of the mobile phase composition (salt concentration or pH) resulting in decreasing interactions between the protein and the stationary phase. It is possible to distinguish four types of ion-exchangers: weak anion, weak cation, strong anion, and strong cation. The terms weak and strong are related to the degree of ionisation of the ion-exchanger as a function of pH. Strong ion-exchangers are fully ionised over a broader pH range (2–12) while weak ion-exchangers are charged at pHs ranging from 9.5 to 5.5.

When applying IEC to the separation of peptides and proteins, cation-exchange is the main choice. At pHs lower than 3, negative charges of carboxyl groups are neutralized, which protonates the N-terminus making them ready to be fractionated by cation-exchange chromatography. In the anion-exchange mode, the opposite is required. Thus, carboxylic groups have to be negatively charged while the N-terminus are neutralized. This situation involves the use of mobile phases with pHs greater than 12 which are not compatible with most chromatographic columns based on silica [56].

Direct IEC–ESI-MS is possible using as eluents organic modifiers such as acetonitrile, and volatile reagents such as ammonium formate or formic acid [57,58]. Loughlin et al. [58] used IEC–ESI-MS to separate highly polar peptides (glutathione and its related products) employing chromatographic conditions compatible with mass detection. These conditions consisted of a gradient elution with phase A being 0.1% (v/v) formic acid in water/acetonitrile (1/1) and phase B being 0.2% (v/v) formic acid in water/acetonitrile (1/1) [58].

Despite these examples, in most cases the elution in IEC involves the use of high salt concentrations that suppress ionisation and result in deposits of non-volatile salts at the ion source inlet. Consequently, the connection of this chromatographic mode with ESI-MS has mostly been carried out by the prior removal of salts, primarily through off-line methods. Alternative on-line desalting methods have also been developed. In some cases, these methods involved multidimensional chromatography using a reversed-phase column, the incorporation of an ion-capture module, or on-line dialysis [21,59–66]. These possibilities will be discussed below.

Salt gradient is the usual elution mode in IEC. Nevertheless, there is an alternative elution mode in IEC that does not require high salt concentrations in the mobile phase and is based on the change of the eluent pH. This mode, called chromatofocusing, is, in principle, more compatible with ESI-MS detection and could be very promising for the direct analysis of proteins and peptides by IEC–ESI-MS. For that purpose, weak ion exchangers are needed and two buffers are used: an equilibration buffer defining the upper pH of the separation gradient, and a focusing buffer defining the lower pH of the gradient. The pH gradient formed inside the ion-exchange column enables the elution of bound proteins in order of decreasing isoelectric points. An on-line coupling of chromatofocusing and mass spectrometry has been carried out for the determination of nucleoside triphosphates. For that purpose, mobile phases consisting of ammonium acetate in acetonitrile at pH 6 (buffer A) and pH 10.5 (buffer B) were employed [67]. Other examples of coupling of chromatofocusing and ESI-MS involving the use of an intermediate RPLC separation will be discussed below [68].

4.4. Size-exclusion chromatography (SEC)

Size-exclusion chromatography is a technique enabling the separation of analytes according to their hydrodynamic volumes. Stationary phases used consist of porous materials with size-defined cavities. Molecules smaller than the pore size enter the cavity and travel through the chromatographic column. Compared to other chromatographic modes, SEC provides limited resolving power. Nevertheless, this chromatographic mode is used extensively due to the fact that it demonstrates a universal separation principle: size can be applied to the analysis of all biological molecules. Moreover, SEC is also known as a biologically friendly chromatographic mode since elution conditions, in general, do not promote

protein denaturation. In fact, mobile phases used in SEC normally consist of buffers at pHs ranging from 6.5 to 8. In some cases, a small proportion of an organic modifier (acetonitrile or methanol) is also added. In order to take advantage of SEC, a universal detection system such as mass spectrometry is required. Nevertheless, the connection of SEC to ESI-MS is restricted due to the use of high concentrations of non-volatile buffers (e.g. KH_2PO_4, NaCl, Na_2SO_4, etc.) to overcome interactions between the stationary phase and the protein, as well as protein–protein interactions [37,69].

SEC has been coupled off-line to MS for the analysis of biopolymers. There are very few examples of methods based on the direct coupling of SEC with MS with the exception of the detection of inorganic elements in biological samples by inductively coupled plasma mass spectrometry [70]. Moreover, the limited number of examples of on-line SEC–MS have been applied to the analysis of small molecules (small peptides or polysaccharides such as heparin) using ESI [71–73]. In most of these cases, volatile mobile phases containing acetonitrile and TFA (for peptides) or methanol and ammonium bicarbonate (for heparin) were employed. Prokai and Simonsick coupled SEC and ESI-MS and demonstrated that the use of tetrahydrofuran containing 10^{-5} to 10^{-4} M of a sodium salt as a mobile phase provided a stable electrospray [74]. Naylor and co-workers [75–77] developed different on-line SEC–ESI-MS methods for the detection of protein–protein, protein–DNA, and protein–metal interactions without requiring any desalting or buffer-exchange protocol. Mobile phases containing volatile salts such as ammonium acetate and ammonium bicarbonate were employed in these studies. Fig. 4 shows the spectra and deconvolution obtained from the SEC–ESI–MS analysis of the inhibitory effect of disulfiram on the rmALDH (mitochondrial aldehyde dehydrogenase) enzyme using 5 mM ammonium acetate in water as a mobile phase.

Lecchi and Abramson [78,79] interfaced SEC with an innovative MS technique called chemical reaction interface mass spectrometry (CRIMS) for the analysis of biopolymers. This system did not provide information about the molecular weight of intact molecules but about the elemental and isotopic composition of any organic molecule after its decomposition by a chemical reaction with a gas (e.g. SO_2) into low-molecular-weight products. These authors tested different buffer solutions containing volatile salts since volatile buffers previously used in SEC–ESI-MS (e.g., TFA) resulted in unacceptable chromatographic separations derived from the interactions between proteins and the column. They observed that ammonium acetate was the buffer that worked the best for polysaccharides (dextrans, heparin) and nucleic acid polymers (RNA, oligonucleotides), while for proteins (thyroglobulin, catalase, collagen, transferrin, albumin, carbonic anhydrase, lysozime, and insulin) the best separation was obtained with triethylammonium formate (pH 3) in water/tetrahydrofuran (2/1).

The coupling of SEC to RPLC and ESI-MS is reviewed below [80–82].

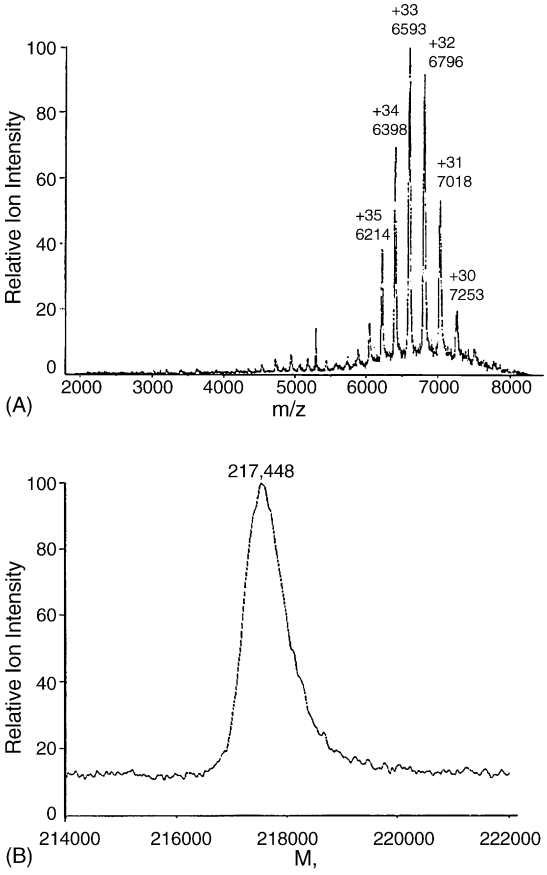

Fig. 4. Spectra and deconvolution obtained from the SEC–ESI-MS analysis of the inhibitory effect of disulfiram on the rmALDH (mitochondrial aldehyde dehydrogenase) enzyme. With permission from ref. [75].

5. On-line desalting methods

5.1. Multidimensional chromatography

Multidimensional HPLC separations are enjoying great acceptance for the analysis of proteins and peptides. This technology, first described by Giddings [83], enables a significant increase in system peak capacity and has widely been used for the separation of very complex samples when a single chromatographic mode does not provide for suitable resolution and results in fractions enriched with similar components. Multidimensional chromatography also enables the on-line coupling of chromatographic methods using high salt concentrations with ESI-MS. In fact, most multidimensional separations used first dimensional separations needing high salt concentrations while RPLC is the final dimension due to its desalting ability. Thus, the sample arrives at the mass detector in its most desirable form for electrospray ionisation.

Multidimensional separations mainly consist of the trapping of the effluent from the first separation column (that involving the use of salts) on a reversed-phase column while salts and other unwanted compounds are washed off the col-

umn and directed to waste [38]. Nevertheless, in principle, salt containing mobile phases are not compatible with RPLC since the solubility of salts and its miscibility are highly related to the presence of organic modifiers. Thus, optimising buffer conditions will be needed in every case.

Other limitations can arise when testing the coupling of chromatographic modes with different flow rates, as is true of SEC and RPLC. Separations by SEC are carried out at low flow rates, whereas separations by RPLC employ higher flow rates. In order to overcome this problem, a preconcentration device between the two systems could be used [23]. An SEC–RPLC–ESI-MS system was applied to the analysis of peptides using ammonium acetate–acetonitrile–water as elution solvent in SEC and a gradient water–acetonitrile–acetic acid/TFA for the separation in RPLC.

Tuyten et al. [52] applied IMAC–RPLC–ESI-MS to the analysis of nucleotides. They developed a column-switching set-up preventing the on-line coupling of the IMAC column (precolumn) with the analytical column (RP column) thus avoiding contamination of the RPLC–ESI-MS system with possible metal leaking from the IMAC column. In order to do so, they introduced the concept of an elution plug. The elution of the sample from the IMAC column to the RPLC column was performed by injecting only 20 μL of a suitable solvent from the autosampler. Since conditions enabling the appropriate clean up of a sample by IMAC could ruin the analytical separation by RPLC, they studied the influence of different eluents (ammonium hydroxide, ammonium acetate, and ammonium phosphate in different concentrations and pHs and water) on the performance of IMAC and RPLC. In all cases, a methanol makeup flow was added through a tee in order to improve electrospray performance and sensitivity. Although ammonium phosphate (pH 4.7) yielded better results from a chromatographic point-of-view, an ammonium phosphate buffer (pH 8.0) was chosen since only this yielded satisfactory elution from the IMAC column. Nevertheless, the sensitivity of the system was still unsatisfactory partly due to the use of an ammonium phosphate buffer in the mobile phase in the RPLC separation which resulted in significant background and possible ion suppression [52].

Apffel et al. [53] described a multidimensional affinity chromatography–RPLC–ESI-MS method for peptide mapping of digested glycoproteins. Two immobilized lectin affinity columns were used to fractionate glycopeptides according to glycosilation type (N-linked glycopeptides in a Concanavalin A column, and O-linked glycopeptides in a column with different immobilized lectins) while non-glycolysated peptides were retained in a C18 column. Finally, the isolated fractions were sequentially separated in an analytical reversed-phase column and detected by ESI-MS. Samples were loaded and washed off the lectin column with salt containing mobile phases and the mobile phase used in RPLC was the typical one consisting of water–acetonitrile–TFA although in this case, as previously described, the postcolumn addition of propionic acid-isopropanol resulted in reduced signal suppression due to TFA.

An on-line coupling of affinity chromatography to ESI-MS has also been developed for the purification and identification of transferrin isoforms from diluted serum using an immunoaffinity column with immobilized anti-human transferrin [54]. The connection of the affinity column with the mass spectrometer was performed by a C4 column.

Canarelli et al. [55] developed a multidimensional chromatographic separation/reactor with ESI-MS for the on-line verification of the identity and integrity of (recombinant) proteins. The protein was first captured in an affinity column, automatically transferred to an immobilized trypsin column for digestion, and then the resulting peptides were separated by RPLC and detected by ESI-MS. The carrier buffer in the affinity separation consisted of a phosphate buffer (pH 7.0) containing sodium chloride and the elution buffer was a solution containing glycine, guanidine, and dithiothreitol (pH 3.0). The digestion buffer was ammonium bicarbonate (pH 8.5) containing cysteine. The outline of the digesting column was connected to a reversed-phase column and the separation of peptides was successfully accomplished with water–acetonitrile–formic acid.

Chromatofocusing has also been used for the separation of biomolecules as first dimension followed by RPLC–ESI-MS. Chong et al. [68] applied this technique to the separation of proteins from human breast epithelial whole-cell lysates in an off-line combination with RPLC–ESI-MS.

In addition to these multidimensional configurations in which only part of the effluent from the first dimension is directed to the second dimension (heart cutting systems), there are also some examples of comprehensive systems developed for the analysis of peptides and proteins by ESI-MS. This alternative configuration enables the entire eluent from the first dimension to be subjected to the second dimension.

While there are numerous examples of methods based on the first approach, the number of comprehensive LC–LC systems is quite limited. Moreover, most examples of comprehensive systems do not use MS detection but optical detection techniques [84]. Opiteck et al. [59] applied a comprehensive system previously used with UV detection to the analysis of proteins by ESI-MS. The system used a cation-exchange column as the first dimension and a reversed-phase column as the second dimension. The columns were connected through an eight-port switching valve with two loops. As one loop was filling with eluent from the ion-exchange column, the other loop was pumping out through the reversed-phase column. The ion-exchange separation was carried out by gradient elution and mobile phases consisting of sodium/ammonium formate, urea, and acetonitrile. The reversed-phase separation used a typical water–acetonitrile gradient containing 0.05% TFA.

Another approach has been the use of a unique column for the first dimension and different columns in the second dimension. Opiteck et al. [80,82] designed a comprehensive system consisting of one size-exclusion column connected to two parallel reversed-phase columns. The analyte eluting from the size-exclusion dimension was transferred alter-

natively to one of the two reversed-phase columns. While one reversed-phase column was being loaded with the effluent from the SEC column, the second RPLC column (already loaded) was running the separation [80,82]. The mobile phases used in the RPLC separation consisted of water–acetonitrile–TFA while SEC separations were carried out with a salt solution [80] or with a solvent containing water–TFA [82]. Wagner et al. [60] developed a method for protein mapping by coupling an ion-exchange column with two parallel reversed-phase columns. Using this configuration they also coupled a restricted-access material (RAM) with ion-exchange functionalities in the first dimension with four reversed-phase columns for analysing proteins and peptides from a human hemofiltrate [61]. They used a phosphate buffer gradient for the elution in IEC and a water–acetonitrile–TFA gradient for the separation in RPLC.

Link et al. [62] have combined multidimensional IEC and RPLC with tandem mass spectrometry to identify proteins in biological samples using a single column containing both stationary phases. Complex peptides were loaded into the column in an acidic pH and, in an iterative process, a fraction of the peptide was displaced from the ion-exchange part to the reversed-phase part by a salt-step gradient. This displaced fraction was separated in the reversed-phase part and directed to the ESI-MS. After re-equilibration, another fraction of peptides was displaced from the ion-exchange material by the use of an increased salt concentration and separated by RPLC. This technology was optimised and automated two years later by Yates and co-workers [63,64] who called it multidimensional protein identification technology (MudPIT). This device used a unique microcapillary column containing two kinds of chromatographic packing materials, a strong cation-exchange and a reversed-phase. Mobile phases for IEC and RPLC were compatible with mass detection. The IEC separation was accomplished by gradient elution with mobile phases consisting of 5% ACN–0.02% HFBA and containing ammonium acetate. Mobile phases used for the gradient elution of peptides from the reversed-phase column consisted of water–acetonitrile–HFBA. They compared the performance of this system when using acetic acid instead of HFBA observing that the use of HFBA enabled the identification of a higher number of proteins and peptides, especially low-abundance peptides.

5.2. Microdialysis

Microdialysis has also been used as a desalting system. This technique incorporates a dialysis membrane into a microprobe. The integration of microdialysis on-line with other analytical techniques such as HPLC or CE has resulted in increased sample throughput and experimental efficiency [85,86].

The first applications of microdialysis as an on-line desalting device were developed for direct ESI-MS and no chromatographic separation took place [87–89]. Wu et al. [90] described an on-line microdialysis system for the desalting

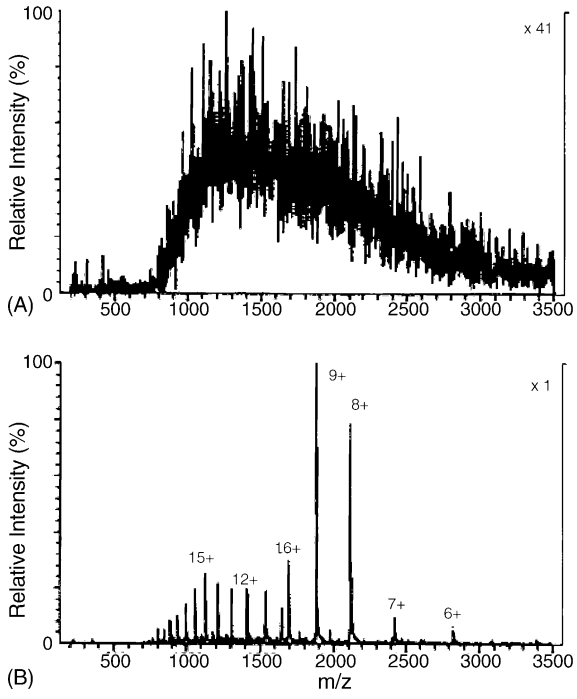

Fig. 5. ESI-MS spectra of apomyoglobin in 10 mM ammonium acetate and 250 mM sodium chloride obtained from direct infusion (A) and after on-line microdialysis (B). With permission from ref. [90].

of samples for ESI-MS that was applied to the analysis of proteins and peptides. Fig. 5 shows the effect of microdialysis on the mass spectrum of apomyoglobin. The direct infusion of the protein in 10 mM ammonium acetate and 250 mM sodium chloride produced a no interpretable spectrum due to sodium adduction. A significant improvement in spectrum quality was observed when samples were microdialyzed before detection. Moreover, the signal-to-noise ratio increased by a factor of more than 40 after desalting. The same group used this technique for the analysis of oligonucleotides and DNA [91,92]. They also proposed a dual-microdialysis configuration for a fast clean up of complex biological samples such as whole-cell lysate for ESI-MS. In the first stage, the system enabled the removal of high-molecular-weight components while low-molecular-weight components such as salts were removed in the second stage [93]. A miniaturized version of microdialysis has also been constructed and applied to the rapid clean up of biological samples for ESI-MS [94].

The applications of microdialysis to desalt effluents from chromatographic separations have been more recent. Liu and Verma [66] developed a cation-exchange HPLC–on-line microdialysis–ESI-MS system that was applied to the analysis of peptides and proteins. Cation-exchange chromatography was carried out with non-volatile mobile phases (phase A consisted of a Tris–HCl buffer and phase B was the same buffer containing 500 mM NaCl). The effluent was desalted by microdialysis against an ammonium acetate buffer. Moreover, they observed that the sensitivity of the method increased at lower dialysis-buffer concentrations, at

higher dialysis temperature, and when adding a sheath liquid consisting of 2% acetic acid in methanol [66].

Canarelli et al. [95] also developed a microdialysis system allowing the on-line coupling of ESI-MS with LC methods requiring the use of salts. They applied this system to the separation of proteins by affinity chromatography-, IEC-, and SEC–ESI-MS. In all separations, salt solutions were employed for the elution of proteins (glycine buffer in affinity chromatography, Tris–HCl buffer and a gradient of NaCl in IEC, and a phosphate buffer containing NaCl in SEC). The effluents were split at 10 μL/min and dialysed against a 5 mM acetic-acid solution. To enhance protein ionisation, a 1:1 (v/v) mixture of (A) 10% formic acid in water and (B) 2% formic acid in water–acetonitrile (1:1) was used as a makeup solvent.

5.3. Ion capture

Another approach for on-line desalting in mass spectrometry detection is the use of ion-capture modules. These devices usually consist of chromatographic materials and constitute an alternative to full high-performance liquid chromatography systems [96]. Emmett and Caprioli [97] developed a concentration desalting device integrated in a capillary needle and containing C18 chromatographic packing material. The sample, consisting of peptides and proteins, was desalted on-line and directly sprayed from this capillary needle [97]. Benson et al. [77] developed a rapid in-line desalting (RILED) system consisting of a small gel cartridge that was coupled to ESI-MS. The system was applied to the analysis of protein complexes and protein–metal interactions established in salt solutions. The results were compared with those obtained by SEC observing that RILED provided a much faster high-throughput desalting than conventional SEC. Ion-exchange resins have also been used as desalting matrices for the analysis of biomolecules prior to ESI-MS [98].

6. Conclusions

There is a great interest for coupling chromatographic techniques and ESI-MS, with RPLC being the chromatographic mode which best matches with ESI-MS and the one most widely coupled. While much effort has been focused on instrumental improvements, not much has been done regarding the intrinsic incompatibilities of both techniques. Trifluoroacetic acid continues to be the ion-pairing agent most often used for the separation of peptides and proteins by RPLC–ESI-MS despite the fact that its use results in limited sensitivity. The efforts aimed at looking for other reagents that work better in ESI-MS and that enable a suitable separation have not yielded any solid alternative. Moreover, the results observed are not conclusive and, in some cases, can even be contradictory because the experimental and instrumentation conditions as well as the sample itself can dramatically affect the results. Chromatographic modes involving the use of mobile phases containing salts present even greater limitations when coupling with ESI-MS. Although there are some examples enabling the direct coupling of these chromatographic modes with ESI-MS using volatile mobile phases, most couplings involve the use of an intermediate desalting step. The desalting procedure can comprise a full high-performance liquid chromatography separation in which RPLC is the second dimension. Other approaches include microdialysis and the use of ion-capture devices.

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