

## PERSPECTIVE

# Revealing the potential of capillary electrophoresis/mass spectrometry: the tipping point

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The hyphenation of capillary electrophoresis and mass spectrometry (CE/MS) remains a minor technique compared with liquid chromatography/mass spectrometry (LC/MS), which represents nowadays the standard instrumentation, regardless of its introduction thirty years ago. However, from a theoretical point of view, CE coupling should be quite favorable especially with electrospray ionization mass spectrometry (ESI-MS). At the time, the sensitivity provided by CE/MS was often limited, due to hyphenation requirements, which at some point appeared to disqualify CE/MS from benefiting from the performance gain driving the evolution of MS instruments. However, this context has been significantly modified in a matter of a few years. The development of innovative CE/MS interfacing systems has enabled an important improvement regarding sensitivity and reinforced robustness in order to provide an instrumentation accessible to the largest scientific community. Because of the unique selectivity delivered by the electrophoretic separation, CE/MS has proved to be particularly relevant for the analysis of biological molecules. The conjunction of these aspects is motivating the interest in CE/MS analysis and shows that CE/MS is mature enough to enrich the toolbox of analytical techniques for the analysis of complex biological samples. Here we discuss the characteristics of the major types of high-sensitivity CE/ESI-MS instrumentation and emphasize the late evolution and future positioning of CE/MS analysis for the characterization of biological molecules like peptides and proteins, through some pertinent applications.

## 1 | INTRODUCTION

It was consequent to the introduction of the electrospray ionization (ESI) source that the implementation of capillary electrophoresis (CE), as a separation technique prior to mass spectrometry (MS) analysis, was investigated alongside liquid chromatography (LC).<sup>1,2</sup> Indeed, similarly to matrix-assisted laser desorption/ionization (MALDI), ESI unlocked the formation of ions from polar molecules especially biological molecules like peptides and proteins. The development of these ionization processes opened a completely unexplored field with the application of MS to the characterization of biological compounds and/or samples. As a matter of fact, we are still experiencing the outcome generated by these discoveries more than 30 years later.<sup>3</sup> The separation of bio-(macro) molecules has been achieved for decades using electrokinetically driven separation like gel electrophoresis and CE.<sup>4</sup> In

CE, the mobility of a compound depends on the charge in solution which is necessarily influenced by the pH of the background electrolyte (BGE), and the hydrodynamic radius of the compound. The difference in mobilities between the different analytes composing the sample then leads to their separation. Because of their chemical structure, the selectivity provided by CE appears to be particularly relevant for the separation of peptides and/or proteins which motivates its use in conjunction with MS for the analysis of biomolecules.

Since the development of the first coupling systems, CE/MS has been successfully hyphenated to ESI and MALDI.<sup>1,5</sup> Also it has been applied for the analysis of a large variety of biological molecules including carbohydrates, glycosylation,<sup>6</sup> peptides,<sup>7</sup> and proteins.<sup>8</sup> CE has the characteristics to be a miniaturized technique. The flow of background electrolyte (BGE) represents only a few tenths nL/min and the reduced internal diameter of the capillary – usually between

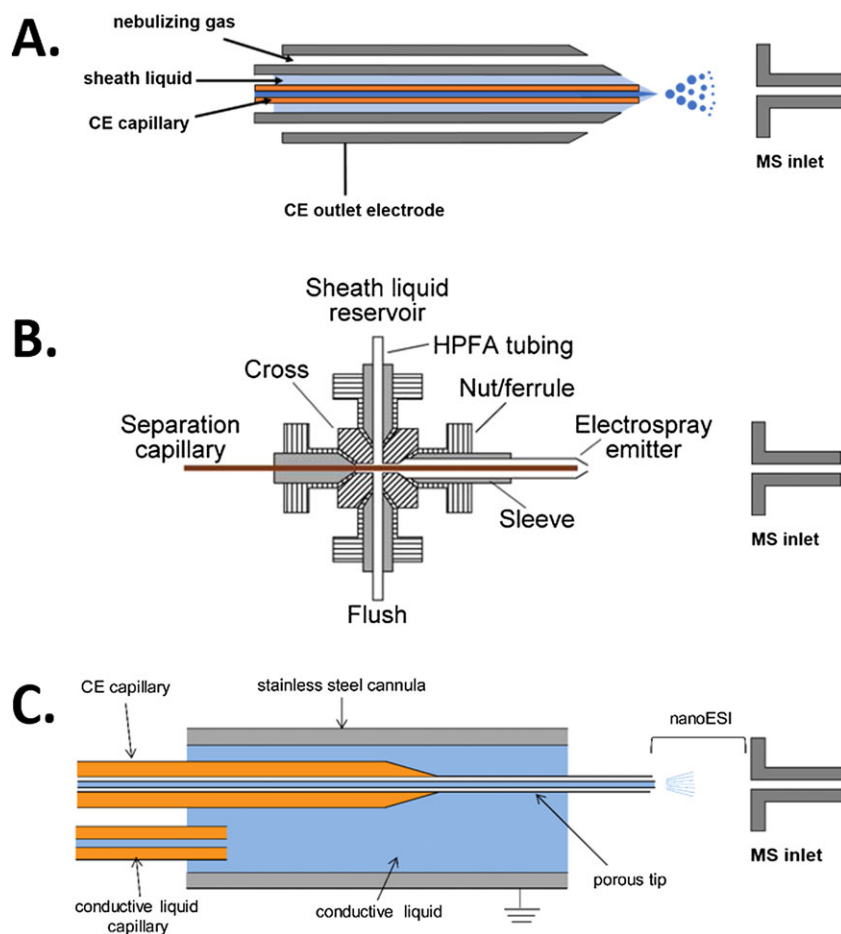
20 and 50  $\mu\text{m}$  – enables the formation of thin droplets at the capillary outlet. Such properties are particularly favourable to the ESI process, providing an optimal ionization efficiency and a good sensitivity.<sup>9</sup> However, the application of CE/MS analysis still remained quite marginal compared with the exponential development of LC/MS hyphenation which represents currently the standard instrumental approach.

In order to successfully couple CE directly to MS instrumentation, it is crucial to be able to maintain the electrical field necessary for the electrophoretic separation while the capillary outlet must be positioned inside the ionization source. Therefore, specific interfaces had to be designed for CE/MS systems and their implementation required some experience which greatly prevented the adoption of the technique within neophyte laboratories. In addition, the instrumental designs of early CE/MS interfaces do not necessarily provide an optimal coupling in term of ionization yields which primarily leads to a significantly limited sensitivity. Nevertheless, hyphenated CE/MS systems have recently benefited from some major instrumental improvements in order for instance to enhance the compatibility between the two techniques. This is particularly the case for CE/ESI-MS hyphenation. In the meantime, the characterization of biological molecules has attracted a growing interest in the analytical chemistry field which required the development of innovative methods. In this context, the characteristics of CE/MS analysis in terms of separation selectivity and characteristics appear particularly relevant. This trend has been emphasized by several recent significant publications demonstrating these aspects.<sup>10</sup>

Here, we discuss the nature and impact on the performance of the latest developments regarding CE/MS hyphenation. Also, we introduce several key applications from the last five years which show the outstanding capabilities of CE/MS analysis for the comprehensive analysis of bio (macro) molecules over the different structural levels in order also to understand the fields where CE/MS could make a significant contribution in the near future.

## 2 | INSTRUMENTAL DEVELOPMENTS IN CE/MS HYPHENATION

Historically, a major part of the CE/MS literature reports the use of sheath-liquid CE/ESI-MS interfacing.<sup>1</sup> For this type of interface, a sheath liquid is constantly injected inside the nebulizer through a coaxial canal, external to the CE capillary (Figure 1A). The background electrolyte (BGE) and the sheath liquid form a junction at the extremity of the ESI nebulizer, and are sprayed in a single process. The sheath liquid is connected to the CE outlet electrode; therefore, the junction formed with the BGE enables the electric field to be maintained. This type of CE/MS interface has the advantage of involving minimal specific instrumentations because the CE/MS nebulizer is designed to be positioned inside a conventional ESI source. However, the ESI process needs to be performed using a grounded nebulizer to prevent any current leak between the electric field of the CE and the ESI source which restricts the panel of MS instruments compatible with sheath-liquid CE/MS. The composition



**FIGURE 1** Schematic representations of the principal design of CE/ESI-MS hyphenation systems. Adapted from Said et al.<sup>37</sup> and Wojcik et al.<sup>56</sup> Copyright (2016) Elsevier and copyright (2010) John Wiley and Sons, Ltd [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

of the sheath liquid requires it to be conductive in order to ensure connectivity between the CE electrodes. However, it does not need to be strictly similar in composition to the BGE, which offers the possibility to use a sheath liquid containing a significant proportion of organic solvent for instance to improve desolvation or a weak acid, in order to improve the protonation of the compounds even if the separation is performed using an alkaline BGE.<sup>11</sup> To provide the electric conductivity, the sheath liquid contains ions. Their quantity is relatively important due to a significant flow rate that tends to limit the sensitivity due to the occurrence of ion suppression effects. These limitations can be partially tackled by careful optimization of the composition of the sheath liquid for each application and increasing the sample concentration.<sup>12</sup> However, the limitations in sensitivity are systematically opposed by the sheath liquid used to prevent CE/MS from profiting to the fullest from the improvements in MS instruments regarding this particular aspect. Different instrumental alternatives were explored, in particular to alleviate the influence of the sheath liquid with the objective to provide an optimal sensitivity. Different research groups, mainly experts in separation sciences and CE, have developed their own type of CE/MS interfaces, certainly motivated by the absence of commercial alternatives to sheath-liquid interfaces.<sup>13</sup> The various CE/MS interfaces resulting from these technical improvements can be divided in two major categories; sheath-flow CE/nanoESI-MS interfaces which tend to reduce to the minimum the flow rate of the sheath liquid and sheathless CE/MS interfaces that do not require a sheath liquid to enable online CE/ESI-MS hyphenation.<sup>14</sup>

Recently, a sheath-flow CE/nanoESI-MS interface was introduced by the group of Norman Dovichi. In this system, the CE capillary outlet is connected to the equivalent of a 'zero dead volume' cross union. On the opposite side of the fluidic connector, a nanoESI glass emitter is positioned in order to spray directly into the inlet of the mass spectrometer at close range. It is important to note that the CE capillary is actually hafted inside the glass emitter. The sheath liquid and the CE outlet electrode are connected to the vertical ports of the cross union (Figure 1B). The flow rate of the sheath liquid is considerably reduced, generally in the range 50–100 nL/min, which is set in motion using the CE instrument through hydrodynamic or electrokinetic injection. This system allows an improvement in the ease of use of sheath-flow CE/nanoESI-MS coupling.<sup>15</sup> The CE capillary and spray emitter are distinct; therefore, the replacement of one or the other can be performed independently with minimal manipulations. Because the CE capillary is simply positioned inside the coupling system using the interface, it preserves the versatility of CE analysis like the use of coated capillaries or different internal diameters. Also this system is compatible with short length capillaries, down to 50 cm, which in many applications is sufficient to achieve the separation due to the excellent efficiency of CE separation, and guarantee a short analysis time. With this type of interface, the glass emitter does not have to be necessarily grounded inside the source to produce a stable ESI and this characteristic has allowed the range of compatible MS instruments to be extended.

The sheath-flow CE/nanoESI-MS coupling has been successfully used for the analysis of complex protein digest mixtures in a shotgun proteomics strategy,<sup>16</sup> the separation followed by top-down MS characterization of intact proteins in bacteria secretome,<sup>17</sup> and the

characterization of protein glycosylation.<sup>18</sup> The results described demonstrate in a general manner an increase in MS signal intensity by 50- to 100-fold compared with conventional sheath-liquid CE/MS which enabled the validation of the concept of reducing the volumes of sheath liquid involved in order to enhance CE/MS sensitivity. The sheath-flow CE/nanoESI-MS interface has been made commercially available recently which should allow more laboratories to be equipped and represent in the near future a formidable opportunity to push forward analytical chemistry research using CE/MS analysis.

Concomitantly to the development of sheath-flow CE/nanoESI-MS interfaces, different instrumental approaches were investigated to maintain the continuity of the electric field inside the CE capillary while ceasing to rely on a sheath liquid to form an electrical contact with the outlet electrode. This category is referred to as sheathless CE/MS interfaces. In the case of the sheathless CE/MS interface developed by Moini and coworkers, the CE capillary walls are etched using fluorohydric acid on the final 3–5 cm. This treatment creates pores in the capillary walls which allow the transfer of charges and small ions.<sup>19</sup> The capillary is then positioned inside a stainless steel cannula that is filled with the BGE via a second capillary connected to the outlet electrode of the CE system (Figure 1C). Due to the porosity of the capillary walls, the electrical contact is maintained with the outlet electrode through the porous part of the capillary. The assembled cannula forms a nanoESI sprayer which can be conveniently positioned inside the source.<sup>20</sup> However, during sheathless CE/MS experiments only the BGE flow exiting the separation capillary is actually transferred to the MS inlet. This system allows complete annihilation of the ion suppression effect generated by the presence of a sheath liquid. As a direct consequence, the analytical conditions commonly used with this type of interface demonstrated a flow rate of BGE ranging from 10 to 135 nL/min, depending on the composition of the BGE and the eventual application of a coating.<sup>21</sup> In addition, the reduced internal diameter of the CE capillary enables the formation of a thin electrospray composed of small droplets, similarly to a nanoESI glass emitter. These two characteristics, conjugated to the absence of sheath liquid, explain the formation of a nanoESI using a sheathless CE/MS interface which provides an optimal ionization efficiency and therefore enhances significantly CE/MS sensitivity. To accomplish this level of performance and a robustness compatible with a large number of analyses, the dimensions of the CE capillary must be relatively standardized and the application of coatings is currently limited which reduces the versatile aspect of CE. Due to the assembly design of the cannula, sheathless CE/MS is compatible with a large majority of ESI-MS instruments.

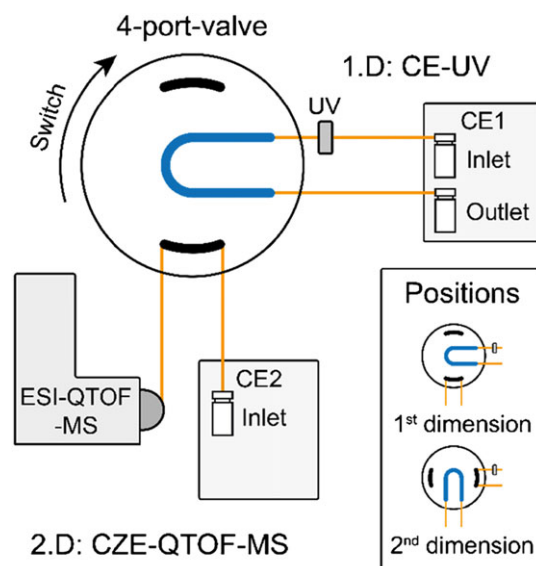
Using this interfacing format, sheathless CE/MS could be used for instance for metabolomic analysis of urine,<sup>22</sup> the analysis of peptide mixtures generated from protein digests in shotgun proteomic approach,<sup>23</sup> the characterization of post-translational modifications,<sup>24</sup> the characterization of intact proteins<sup>25,26</sup> including top-down proteomics applications,<sup>27</sup> and the site-specific characterization of glycosylation.<sup>28</sup> These results show that sheathless CE/MS offers the conservation of an optimal sensitivity between CE and MS techniques. Indeed, a systematic comparison highlighted an improvement in sensitivity of at least 100-fold compared with conventional sheath-liquid CE/MS analysis.<sup>29,30</sup> In a similar way to sheath-flow CE/nanoESI-MS, sheathless CE/MS demonstrated

sensitivities comparable to the current (nano)LC/MS/MS instrumentation. It has been commercially available for a few years which has allowed research groups already using conventional CE/MS to gain access to a highly sensitive system and explains the development of a significant number of applications using this type of interface in recent years.

Because of the volume involved, CE is considered as a miniaturized technique. Thereby, recent instrumental developments report the design of CE/MS hyphenation using a microfluidic chip-based system. The chip incorporates both the CE capillary and the nanoESI sprayer. Similarly to the sheath-flow CE-nanoESI-MS coupling previously described, the electrical field is maintained using a low sheath flow connected to the outlet electrode which is circulating through a second channel carved into the chip as well. The two channels meet and form a junction at the tip of the microfluidic chip close to the spraying part.<sup>31</sup> The sheath flow is limited to a few tenths of nL/min which ensures an excellent sensitivity. In addition, CE separation in microfluidic channels shows an improved efficiency because of lower longitudinal diffusion attributed to the reduced internal diameter of microfluidic channels. Conventional CE instruments are not capable of operating the microfluidic chips so a dedicated instrument is necessary to use this type of CE/MS hyphenation system. However, this system has been commercially available for a few months giving access without any engineering expertise to the specific CE instrument as well as the microchip CE/MS device. This type of CE/MS interface has been recently used to develop particularly interesting applications such as the characterization of carbohydrates and glycosylations,<sup>32</sup> the analysis of haemoglobin and the characterization of different types of intact therapeutic proteins.<sup>31,33,34</sup> This microchip CE/MS system is also commercially available as a standalone instrument including the CE and the interface. Indeed, because the separation capillary is directly fabricated inside the microchip, conventional CE instruments are not compatible. In a similar manner, the types of available capillaries in terms of length or internal diameter for example are limited at the moment. The several types of CE-ESI-MS interfaces available are resulting from different strategies adopted to improve the sensitivity

and robustness achieved in CE/MS experiments. Each type of CE/ESI-MS coupling described exhibits different interests and limitations; therefore, understanding these characteristics is indubitably important in order to select the most appropriate hyphenation system for a given analysis.

The most recent instrumental developments regarding CE/MS are not only confined to interfacing efficiently both techniques. A system enabling bi-dimensional (2D) CE/ESI-MS was described in a recent work by the group of Neusüß. In a fashion close to 2D-liquid chromatography, this 2D-CE/ESI-MS system uses a set of mechanical valves in order to perform two consecutive electrophoretic separations. The outlet of the second capillary is positioned inside the ESI source via a sheath-liquid CE/MS interface. Regardless of the position of each valve, the extremities of the CE capillaries are always connected to the CE electrodes which prevents uncontrolled variations and/or shortage of the electrical fields or current leaks (Figure 2). The system can be assembled using only commercially available elements (valves, connectors).<sup>35</sup> 2D-CE/ESI-MS properties can be used to perform two consecutive electrophoretic separations in order to combine, in a single analysis, two electrophoretic separation modes and improve peak capacity, similar to 2D-LC. 2D-CE/ESI-MS could be used to realize in the first dimension a CIEF separation where the BGE was not compatible with ESI-MS. To enable hyphenation with ESI-MS, CZE using a volatile BGE was performed in the second dimension. Because the volume of each fraction is not important, the low quantities of CIEF electrolyte did not interfere with the ESI-MS analysis.<sup>36</sup> In this configuration, raw separation improvements were secondary whereas the characteristic sought was the possibility to conveniently realize CIEF mobilization followed by high-resolution MS characterization. Because the majority of BGEs conventionally used in CE are not volatile enough to be compatible with ESI-MS, this instrumental approach could be envisaged in the near future to use unorthodox BGEs or implement other electrophoretic separation modes like micellar electrokinetic chromatography (MEKC) in conjunction with ESI-MS.



**FIGURE 2** Schematic representations of the instrumental settings implemented to perform 2D-CE/ESI-MS experiments.<sup>57</sup> Copyright (2018) John Wiley and Sons, Ltd [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

The instrumental developments concerning CE/ESI-MS hyphenation have been important over the last decade, targeting different aspects of the instrumentation. They have allowed improvements in the compatibility between the two techniques in order to provide a better robustness in addition to significant benefits from the outstanding sensitivity provided by late-generation MS instruments. Yet one major opportunity of these technical improvements is in the commercial availability of highly sensitive CE/ESI-MS hyphenation systems which give access for an extended scientific community to this type of innovative instrumentation without requiring an important engineering background.

### 3 | APPLICATIONS AND POTENTIAL OF CE/MS FOR THE ANALYSIS OF BIOMOLECULES

From a theoretical point of view, the selectivity of CE appears particularly relevant for the separation of biomolecules. This aspect is largely emphasized as the technical improvements described previously are in many cases applied to the analysis of biomolecules, like peptides and proteins. In parallel, the interest in the analysis of biological molecules has been constantly growing and innovative methodologies still need to be developed in order to address the challenge rising from their structural complexity.

#### 3.1 | Peptide centric analysis using CE/ESI-MS

Recently, CE/MS(/MS) has been extensively investigated for the analysis of peptide mixtures. In a bottom-up strategy, the analysis of peptide mixtures is generally used after a proteolytic digestion to identify the different proteins constituting a complex sample for example, or to perform the characterization of the primary structure of a purified protein. The last two decades have seen the advent of biopharmaceutical products, especially through the wide adoption of the monoclonal antibodies (mAbs) format. The implication of such complex glycoproteins has rapidly urged for the development of analytical methods able to give a detailed and comprehensive characterization of this type of proteins and CE/MS has been investigated in this context. Results reported have shown, using sheathless CE/MS, the possibility to achieve systematically a 100% sequence coverage in a single analysis for several therapeutic mAbs using a sample quantity corresponding to 16 ng of digested proteins.<sup>7</sup> Thus, the incorporation of the electrophoretic separation demonstrated the successful migration and transfer to the MS source of a wide diversity of peptides regarding their chemical structure in a single experiment, including hydrophilic and significantly large peptides. That characteristic contributed to the improved sequence coverage and proved to be particularly interesting to obtain a consistent and highly detailed characterization of the amino acid sequence of biopharmaceutical proteins in a single experiment.<sup>37</sup> In particular, a similar CE/MS/MS methodology could be used to investigate the similarity between different therapeutic monoclonal antibodies (mAbs) and their respective biosimilar candidates which emphasizes the outstanding level of characterization and robustness of the data generated.<sup>38</sup> Also, CE/MS could be validated through inter-laboratory studies showing the transferability and robustness of this instrumental approach.<sup>39</sup>

High sensitivity CE/ESI-MS coupling such as sheath-flow CE/nanoESI-MS and sheathless CE/MS could be used for highly demanding analyses such as complex mixtures of proteins in shotgun proteomic experiments, previously restricted to (nano)LC/MS/MS, with comparable sensitivities.<sup>40</sup> Indeed, systematic comparisons performed under similar conditions regarding analysis time and the quantity of sample injected show for CE/MS analysis a comparable number of identifications even in this case. In addition, the results reported have highlighted the complementarity of the two instrumental approaches. For the analysis of the same peptide mixture, a significant proportion of the identified peptides were specific to either one of the techniques which is mainly explained by their respective separation selectivity. CE/MS has shown an increased number of identified peptides per protein which allows reinforcement in the confidence of the resulting protein identification.<sup>41</sup> Therefore, the conjunction of these two types of analysis could potentially give additional information resulting in further insights regarding the sample.<sup>42</sup> Complementarily, when the sample quantity injected was drastically reduced, below 100 ng for complex digests, CE/MS experiments led to a higher number of identifications, both for peptides and proteins.<sup>43</sup> This level of performance is attributed to the ionization efficiency of high sensitivity CE/ESI-MS interfaces. From an analytical stand point, this property is interesting for the analysis of precious samples available in small quantities which is also supported by the fact that CE instruments are designed to handle and inject in a robust manner a few nanoliters of sample. Thus, because of these characteristics, the analysis of peptide mixtures using state-of-the-art CE/MS instrumentation could benefit in the near future to clinical applications like early diagnostic and patients' follow-up.<sup>44</sup>

#### 3.2 | Analysis of post-translational modifications

On another aspect, recent applications have demonstrated the relevance of CE/MS analysis for the characterization of large panel of post-translational modifications (PTMs).<sup>45</sup> For instance, the results described in different research articles especially show the possibility to separate, under the influence of the CE electric field, peptides exhibiting PTMs from their intact homologues prior to their transfer to the mass spectrometer. Thus, the occurrence of several types of PTMs like asparagine deamidation modifies either the net charge or the hydrodynamic radius of the molecule which enables their separation using the selectivity of CE, especially on the peptide level. From a MS stand point, this characteristic is also interesting because their simultaneous transfer could lead to the occurrence of competition effects during the ionization process. This effect could then result in lowered sensitivity and biased estimation of the level of modification, if not preventing completely the detection of the PTM. From a biological perspective, identification of PTMs is crucial due to their implication in signalling or regulation processes which places CE/MS as the tool-of-choice to study protein modifications.<sup>46</sup>

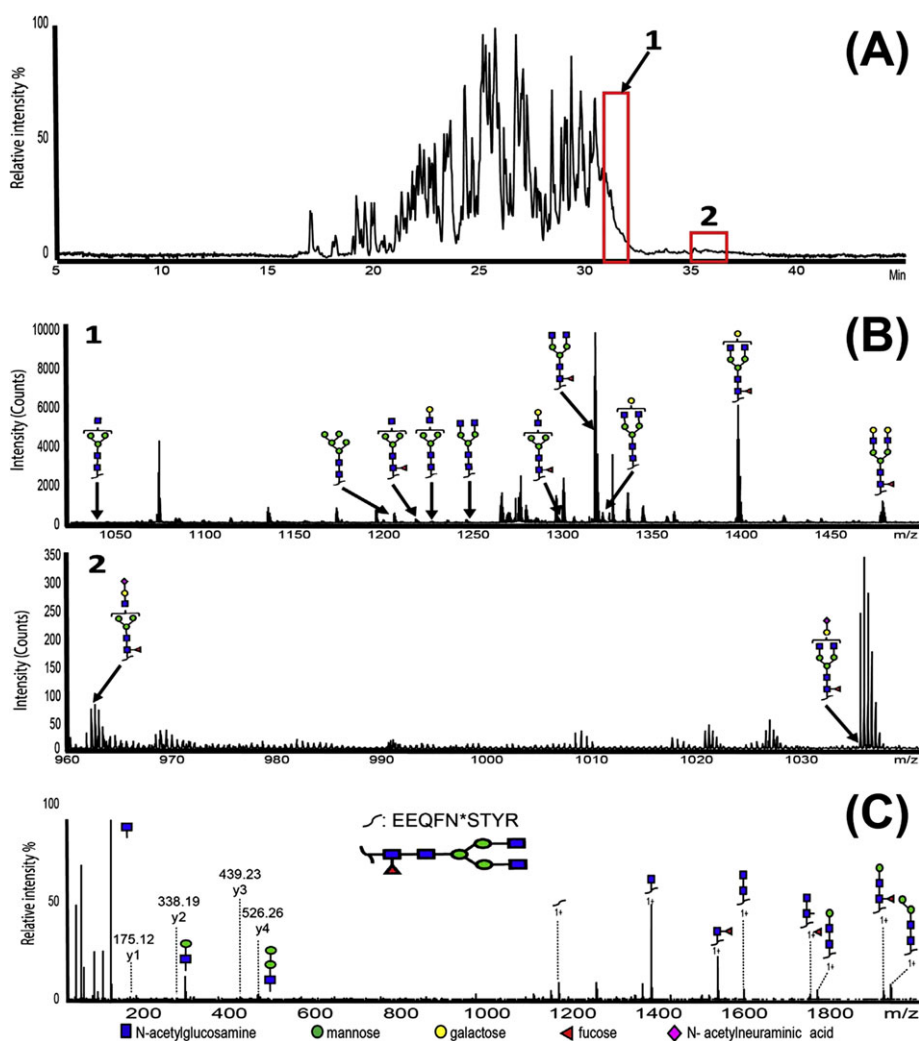
The characterization of PTMs represents a major element in order to accurately define the primary structure of proteins. For biopharmaceutical products, that attribute is therefore considered to be crucial as some PTMs have been identified to influence significantly the pharmacological properties of the protein.<sup>47</sup> Simultaneously to the characterization of the amino acid sequences, CE/MS/MS could be used to achieve the analysis

of asparagine deamidation, methionine oxidation, C-terminal glutamic acid cyclization and aspartic acid isomerization. In each case, the modified peptides could be baseline separated from the intact counterpart which allowed the detection of modification levels as low as 2%.<sup>38</sup> Among the PTM hotspots characterized, systematic and complete separation of homologous peptides containing different isomers of aspartic acid (Asp) allowed the use of CE separation to enrich the level of characterization because the  $m/z$  values for both peptides were logically identical.<sup>24</sup> Finally, CE/MS/MS showed the ability concomitantly to achieve a highly detailed characterization of PTMs including site-specific glycosylation characterization and drug localization in the case of antibody-drug conjugates (ADCs).<sup>37</sup>

CE has long been considered to be an excellent technique for the analysis of carbohydrates and released glycosylation.<sup>48</sup> Indeed, these molecules have the properties to be polar and hydrophilic which makes their analysis facile in aqueous conditions, and the electrokinetic separation has demonstrated the possibility to achieve their separation with high resolution. CE/MS has been extensively used as well for the characterization of glycosylation. In the most efficient approach, glycans were released from the protein using an endoglycosidase enzyme like PNGase F followed by derivatization

using APTS (1-aminopyrene-3,6,8-trisulfonic acid), finally completed with the CE/MS analysis.<sup>49,50</sup> More recently, CE/MS/MS has been applied to the analysis of glycopeptides obtained through proteolytic digestion. Using this instrumentation setup, glycopeptides having a difference of one galactose could be partially separated while glycopeptides exhibiting a difference of one sialic acid demonstrated systematically a baseline separation.<sup>28</sup> That separation selectivity is definitely an excellent asset in order to improve the sensitivity and enable the detection of low-abundance glycosylations. In addition, the MS/MS spectra allowed the identification of the structure of the glycan present on the peptide and to localize its position on the protein by identification of the fragments corresponding to the peptide backbone (Figure 3).<sup>51</sup>

The recent applications suggest that CE/MS performs particularly well for the characterization of peptides. So far it has been extensively used for the characterization of therapeutic proteins; however, because of its properties, it could be an excellent technique in discovery based experiments in order to characterize undescribed proteins purified from biological samples using a *de novo* sequencing approach as an example. Because of the properties of the current CE/MS coupling, it is expected soon that CE/MS could benefit the



**FIGURE 3** (A) Base peak electropherogram corresponding to the analysis by CE/ESI-MS/MS of natalizumab tryptic digest. (B) Mass spectrum of 30.5–31.9 min and 34.9–36.5 min. (C) MS/MS fragmentation spectra of [EEQFN\*STYR] + G0F. Adapted from Giorgetti et al.<sup>51</sup> Copyright (2017) Elsevier BV [Color figure can be viewed at wileyonlinelibrary.com]

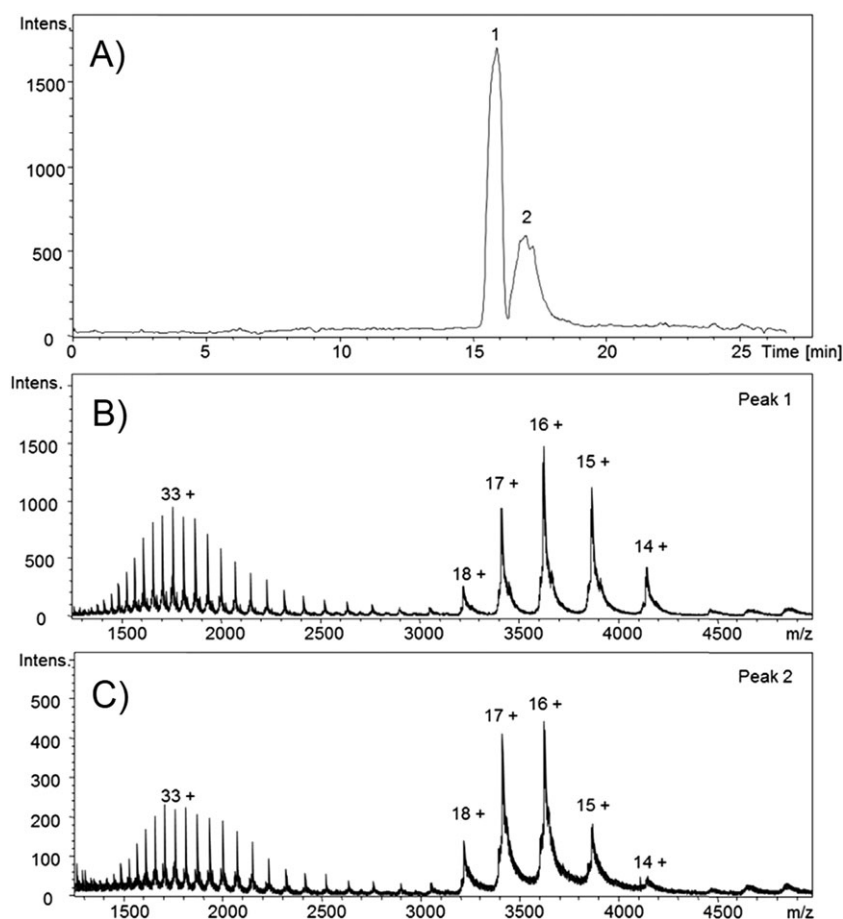
study of PTMs with the opportunity to be applied in a clinical context. Indeed, it could represent a powerful tool to study variations in levels of PTMs depending of the physio-pathological state. In addition, because the number of identified peptides per protein is improved, the implementation of quantitative analysis by CE/MS based on the parallel consideration of several peptides could be exploited and potentially improve the accuracy of the quantification.

### 3.3 | Analysis of intact proteins using CE/MS

Concerning therapeutic proteins, the characterization of intact proteins is particularly valuable. It allows the analysis to be conducted with minimal sample treatment, preventing the introduction of biases, in a state as close to the actual product as possible. Chromatographic separations of intact proteins, especially above 50 kDa, are limited and the charge/hydrodynamic radius separation provided by CE is particularly relevant from this perspective. As a consequence, different CE/MS methods demonstrating the separation and characterization using MS data directly on the intact protein have been reported for various types of biopharmaceutical products.<sup>26,52</sup> In a similar strategy, CE/MS was recently implemented for the characterization of mAbs and ADCs showing in particular the separation of intact charge variants. Indeed, the electrophoretic separation showed, on the intact protein level, the possibility to separate isoforms exhibiting PTMs or also the separation of glycoforms.<sup>34,53</sup>

The conjunction of CE and MS techniques could also be successfully used for the study of protein conformational changes used as therapeutic products. These applications are particularly interesting because CE allowed the separation of the exact same protein adopting different conformations which is attributed to a change in hydrodynamic radius only, demonstrating the exceptional separation efficiency provided by CE even for highly complex macromolecules like proteins. In the case of Aclotine, the MS data between the separated conformers showed dissimilarities concerning the relative abundance of each detected charge state, suggesting that some protonation sites were accessible to the solvent depending on the conformation of the protein (Figure 4). That original work is particularly interesting because it illustrates the powerful analytical methodologies achievable at the moment solely using CE/MS.<sup>54,55</sup>

Indubitably, it is for the analysis of intact proteins that CE/MS can make the most significant contribution and the scientific community is pushing in this direction. In theory, CE separation could be even achieved in native conditions which would open the way to non-covalent complexes separation/study. The analysis would then profit from the excellent ionization efficiency of recent CE/MS coupling. Note the recent development of affinity CE/MS abounds in this sense. Because of the potential of this instrumental approach, further developments are expected for the characterization of 2D-CE/ESI-MS. On this aspect, the analytical method developments are surely profiting from the experience acquired from 2D-LC/ESI-MS promising rapid improvements which



**FIGURE 4** CE/MS of Aclotine® (15 g/L) using a sheath liquid of isopropanol/water (50:50, v/v) containing 14mM ammonium acetate stored at room temperature for 3 weeks (A) BPE (1500–5000  $m/z$ ). (B) and (C) mass spectra of peaks 1 and 2, respectively. Adapted from Marie et al.<sup>54</sup> Copyright (2016) Elsevier BV

should enable accumulation of the selectivity of the two totally different electrophoretic separation modes followed by high-resolution MS characterization.

## 4 | CONCLUSIONS

CE/ESI-MS is attracting remarkable interest in favour of various instrumental developments which have enabled the introduction of highly sensitive CE/MS instrumentation in particular for the characterization of biological molecules like peptides and proteins. The introduction of innovative CE/ESI-MS have allowed improvement in the compatibility between both techniques regarding sensitivity and robustness. The abolition of sensitivity limitations has revealed the true potential of implementing an electrophoretic separation prior to MS analysis, especially for the characterization of PTMs and intact proteins. As such CE/ESI-MS is already differentiating from LC/ESI-MS methods and has the potential to enrich the panel of separation selectivity hyphenated to MS. High-end CE/ESI-MS instrumentations are now widely accessible which represents also a huge asset for a larger adoption of this technique in particular in the mass spectrometry community. The conjunction of these factors is clearly promising for the further development of CE/MS in the near future for a wide range of applications like intact proteins or glycosylation analysis.

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