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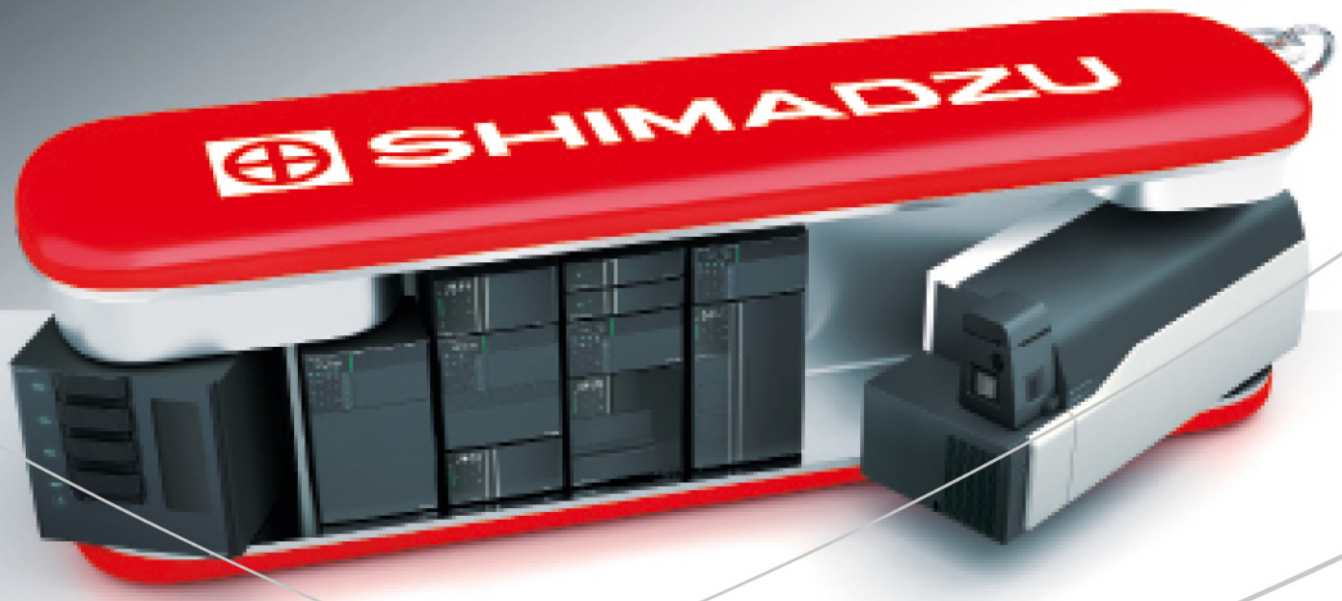

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Received: 11 June 2023 | Revised: 8 August 2023 | Accepted: 9 August 2023

DOI: 10.1002/jssc.202300425

JOURNAL OF SEPARATION SCIENCE

SHORT COMMUNICATION

Controlling the positive ion electrospray ionization of poly(ethylene glycols) when using ultra-high-performance supercritical fluid chromatography-mass spectrometry

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Poly(ethylene glycols) are complex polymers often added to pharmaceutical formulations to improve drug solubility and delivery. One of the main challenges when using chromatographic techniques coupled to mass spectrometry is the unselective ionization of poly(ethylene glycols) oligomers. Additionally, when the chain length is large enough, multiple charged species are formed, further complicating the mass spectra and processing. This study uses the advanced oligomer separation provided by supercritical fluid chromatography with a mass spectrometry approach that selectively ionizes poly(ethylene glycols) as ammoniated molecules to simplify data analysis and facilitate batch-to-batch comparisons. Several visual representations of the response of the ionization events based on the polymer molecular weight and the repeating unit were used to elucidate trends in ionization. Evaluation of the influence of the oligomer length and end-group on the electrospray ionization of the polymer allowed the development of a process to enable selective ionization for these complex polymers.

KEYWORDS
electrospray ionization, mass spectrometry, poly(ethylene glycol), polymers, supercritical fluid chromatography

1 | INTRODUCTION

Poly(ethylene glycols) (PEGs) are frequently used in formulations due to their safety, cost, and availability as pure materials with narrow molecular weight distributions [1]. Their addition to pharmaceutical formulations as excipients improve drug delivery, for example, through drug encapsulation in micelles, aid dissolution, or may be reacted with drug molecules (PEGylation) via a covalent bond (prodrug) to enhance drug protection against metabolism [2–4]. Different end groups are selected to modify drug lipophilicity and improve biological targeting. Figure 1 shows the structure of the PEGs discussed in this paper. PEGs are typically low-dispersed materials, characterized by their polydispersity [1]. Polydispersity refers to the nonuniformity of the polymer molecular weight band related to the different chain lengths of the oligomers [5]

Article Related Abbreviations: AmAc, ammonium acetate; mPEG, monomethoxy PEG; PEG, poly(ethylene glycol).

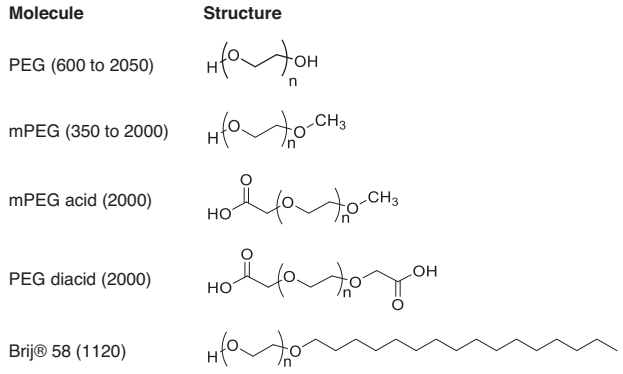

FIGURE 1 Chemical structures of the different poly(ethylene glycols) (PEGs) used in this study.

and appropriate quantitation approaches are required [6]. Uncontrolled polydispersity may lead to reduced effectiveness of drug delivery, resulting in the required therapeutic dose not being reached and compromising the physicochemical characteristics and integrity of formulations [7, 8].

The standard approach for polymer analysis is size exclusion chromatography [9]; however, coupling to mass spectrometry (MS) is required to obtain information about the polymer molecular structure and/or end groups [10, 11]. The complex mass spectra generated are challenging to interpret, especially when analyzing polymeric mixtures. Reversed phase liquid chromatography (LC) and hydrophilic Iinteraction liquid chromatography (HILIC) can provide end-group separation; however, only a small degree of oligomer separation has been reported for linear PEGs [12–14] and sometimes 2D-LC is required for improved characterization [15, 16]. Coupling LC with MS can aid polymer characterization and quantification, but the use of aerosol detectors is more prevalent in quality control environments as an alternative to UV detectors to detect polymeric materials that lack a chromophore [6, 13, 17]. Further, enhanced methods of analysis are still required to fully understand and control the quality of the final drug product when using these materials and ensure drug efficacy [18].

Supercritical fluid chromatography (SFC) is an excellent complementary approach to LC for the analysis of polymeric materials due to improved oligomer resolution [19, 20]. The SFC mobile phase consists of dense carbon dioxide ($scCO_2$) that varies from the supercritical state at initial conditions to a subcritical fluid when a co-solvent is added [21]. The co-solvent is typically methanol with a volatile salt or acid to aid elution and improve chromatographic peak shape. When coupling to MS, a flow splitter delivers a make-up solvent to improve spray stability in an atmospheric pressure ionization source. Gidden et al. showed the dependence of the charge state to the ions generated by polyethers on the structure of the repeating unit and the molecular weight when using positive ion electrospray ionization (ESI) [22]. Poulton et al. proposed the first modern packed-column SFC-ELSD-MS method that separated PEGs with methoxy and acidic end groups [18]; however, the selection of the mobile phase, make-up solvent, and the corresponding ions produced was not fully detailed.

Building on the work by Poulton et al. [18], this work sought to develop new SFC-MS methods to characterize PEGs with different end groups. The use of MS addresses the lack of a chromophore within the PEG structures by facilitating the PEG chain detection by ionization, resulting in a reliable and robust approach that measures differences between PEG batches and supports impurity profiling [6]. Positive ion ESI enables large PEG polymers to form multiple charged species during the ionization process, ensuring that the ions of interest are observed within the *m/z* range of quadrupole mass analyzers. However, one of the challenges is the uncontrolled ionization of PEGs, where different ionic species are formed in the ion source, that is, different ratios of protonated, ammoniated, and sodiated molecules that adds complexity to the data. Further, there is a direct link between the ion formation and the chain length of the oligomer. Also, as PEG oligomers increase in mass, the ionization process changes and is further complicated by the production of multiply charged ions for the longer chain length PEGs (Figure 2). The combination of the improved oligomer resolution provided by SFC and the control of the ionization event in the MS source simplifies data analysis, aids interpretation, and delivers improved detection to allow compound characterization and quantitation.

2 | MATERIALS AND METHODS

Acetonitrile (ACN), methanol, and water (LC-MS grade) were purchased from ThermoFisher Scientific. PEG 600, 1000, 1450, and 2050; monomethoxy-PEG (mPEG) 350, 550, 750, and 2000; Brij 58, LC-MS-grade ammonium acetate (AmAc); LC-MS-grade formic acid; and LC-MS-grade ammonium formate were purchased from Sigma-Aldrich. Monomethoxy-α,ω-(2-carboxymethyl) PEG 2000 (mPEG acid 2000) and α,ω-bis(2-carboxymethyl) PEG 2000 (PEG diacid 2000) were purchased from Laysan Bio, Inc.. Food-grade carbon dioxide was purchased from BOC Special Gases.

SFC analysis was performed using a Waters Acquity UPC^2 system (Waters Corp.). Separation was performed using a Waters Acquity Torus Diol packed column (1.7 μm × 3 mm i.d. × 100 mm) at 70°C with an active back pressure regulator (ABPR) set to 150 bar using a linear gradient of 1%–40% over 10 min of an optimized co-solvent

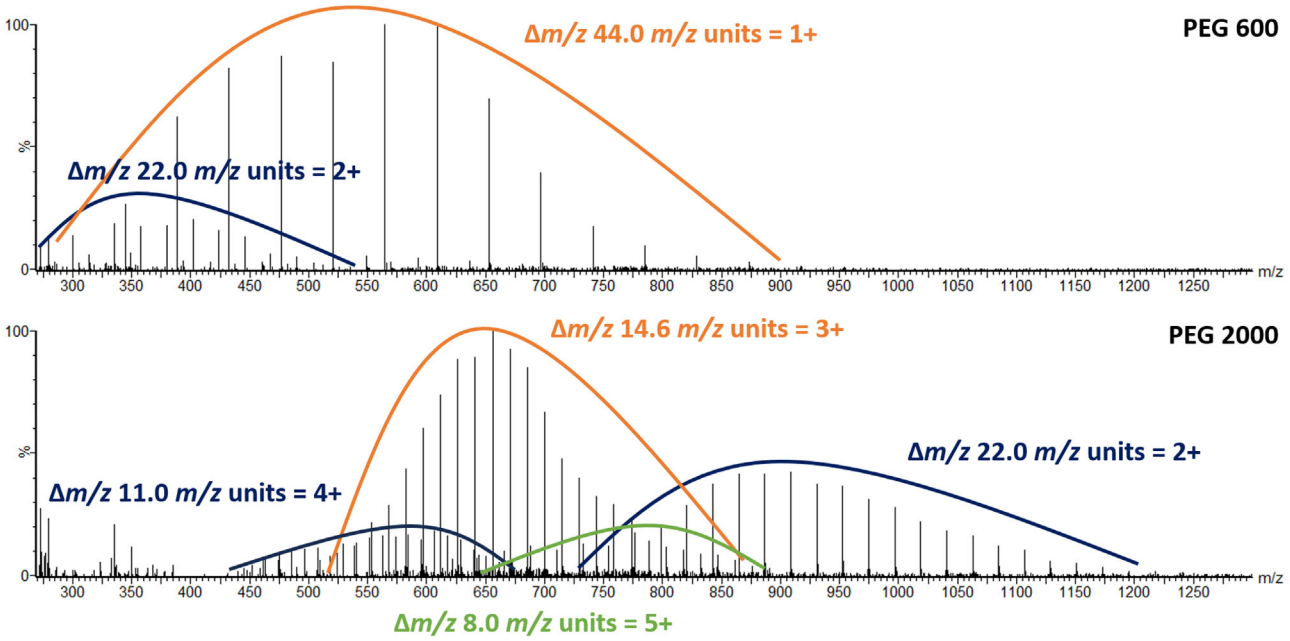

FIGURE 2 The mass spectra of poly(ethylene glycols) (PEGs) highlighting the overlapping ions from different PEG oligomers at different polymer molecular weights.

composition (see Section 3.1) of 15 mM AmAc in 94% methanol/6% water (% v/v) at a flow rate of 1.3 mL/min.

MS was performed using a ZSpray ESI source in positive mode and a Waters Xevo SQD 2 (Waters Corp.). The chromatographic system was interfaced with the mass spectrometer using a splitter, and a make-up solvent flow of 50 μM AmAc was delivered at 0.45 mL/min using an isocratic solvent manager. The capillary voltage was set at 2 kV, source temperature 150°C, optimized cone voltage of 20 V (see Section 3.1), optimized desolvation temperature 350°C (see Section 3.1), desolvation gas flow 600 L/h, and cone gas flow 50 L/h. Continuum data were collected using full scan analysis over the range of *m/z* 100–1500.

Data acquisition was undertaken using MassLynx v.4.1 SCN 855 and processed using Microsoft Office 365 and MZmine 2.52.

3 | RESULTS AND DISCUSSION

3.1 | Achieving selective ionization

Previous work showed that SFC delivers oligomeric separation across various PEG molecules [18]. When considering the mobile phase additives, the presence of ammonium ions—either from hydroxide or acetate salts used as additives in the co-solvent—was crucial to achieving good oligomer separation due to the interactions of the PEG chain with the ammonium ion and the diol groups of the stationary phase. Ammonium formate was considered, but unselective ionization was observed due to a facile formation of protonated species due to a more acidic environment within the ionization source. AmAc was preferred for this work due to the lower volatility of the salt compared to ammonium hydroxide solutions that might result in larger stability of solutions, despite possible bacterial growth in the AmAc solution in a methanolic environment [23].

While the use of AmAc alone as the additive of the methanolic co-solvent provided separation for simple model PEG oligomers, further optimization of the mobile phase was required when more complex PEGs were investigated, for example, mPEG acid and PEG diacid. The addition of small percentages of water to the methanolic co-solvent resulted in an improved chromatographic peak shape of PEG with acidic end groups (Figure 3A,B) with the addition of increased retention from the solvation of the stationary phase (HILIC-like mechanism [24, 25]) and a larger hydrodynamic size of the analytes (size-related mechanism [18]). Increasing the concentration of AmAc in the aqueous methanolic co-solvent (Figure 3B–D) increased the analyte retention, which was similar to observations by Cazenave-Gassiot et al. [26]; however, a larger tailing of the chromatographic peak was observed associated with a higher acidity favoring the ionic state of PEG with acidic end groups.

For short chain length PEGs ($n \leq 5$), protonation is the favored ionization event, while alkali metal cation adducts are favored for longer chain lengths. However, the ionization was found to be complex due to the presence of

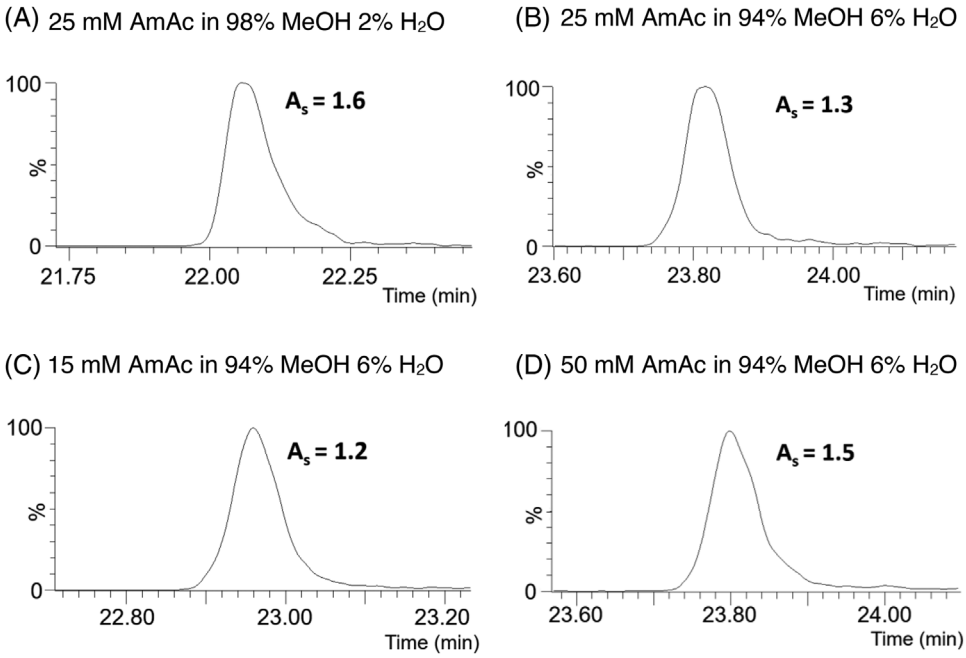

FIGURE 3 Optimization of the mobile phase co-solvent and additive concentration. The chromatographic peak shape corresponds to the reconstructed ion current chromatogram of a representative oligomer of monomethoxy PEG (mPEG) acid ($n = 41$, m/z 650, $[M + 3NH_4]^{3+}$); 100 μg/mL mPEG acid 2000 in ACN + 0.1% formic acid. USP symmetry factor (at 0.05% width of the peak height) is shown for each condition.

proton, sodium, and ammonium ions, the latter from co-solvent additives, which resulted in oligomers forming a mixture of protonated, sodiated, and ammoniated species (Figure 4A). Also, as the oligomer chain length increases, ionization via protonation become negligible, but as multiple charging increases, the complexity of the mass spectral data enhances (Figure 2).

Ratsameepakai et al. addressed ionization control for fatty acid methyl esters with SFC-MS by adding sodium acetate to the make-up flow solvent, thus promoting the formation of sodiated molecules [27]. Attempts in this work to force the formation of sodiated PEG molecules through the use of sodium acetate in the make-up flow solvent failed because of the presence of excess ammonium ions from the additive used in the SFC co-solvent. Hence, ionization control was achieved using AmAc in the make-up flow solvent (Figure 4).

Even with these conditions, the ionization was impacted by instrument source conditions. A lower cone voltage value was preferred to maintain control of the ionization event and stability of the ions formed. A higher cone voltage (Waters specific) is recommended for transmitting higher mass ions and multiply charged species; however, this should be avoided when analyzing high molecular weight PEGs when ionization is achieved through an ammoniation event. At high cone voltage values, the positive ion ESI mass spectra showed ions that could be misinterpreted as protonated molecules; hence, the assumption was that a protonation event was driving the ionization of these species. Further investigation of the impact of cone voltage and desolvation temperature confirmed that these ions were formed through the loss of ammonia from the ammoniated molecules as part of an in-source collision-induced dissociation process. Increasing the cone voltage dissociated the ammoniated molecules $[M + NH_4]^+$ to give the protonated molecule ion $[M + H]^+$, while increasing the desolvation temperature favored the evaporation of ammonia (see Supporting Information).

By controlling this ionization process, batch comparisons could become more accessible since the mass spectral data that underpin the chromatography data are simplified (Figure 4). Further, the simplified mass spectral data facilitated impurity identification within different PEG samples. For example, uncontrolled ionization (Figure 5A) resulted in an ion response divided between 90% ammoniated versus 10% other species. With control of the ionization state (Figure 5B), the MS data were simplified (99% ammoniated species versus 1% others) and the signal improved, resulting in enhanced limits of detection.

3.2 | Understanding ionization current trends

Iavarone and Williams proposed the use of the average charge state of polymers (z_{avg}, Equation 1) to aid in understanding the trends of sodiated events in the mass spectra when PEG was exposed to different solutions [28]. In this

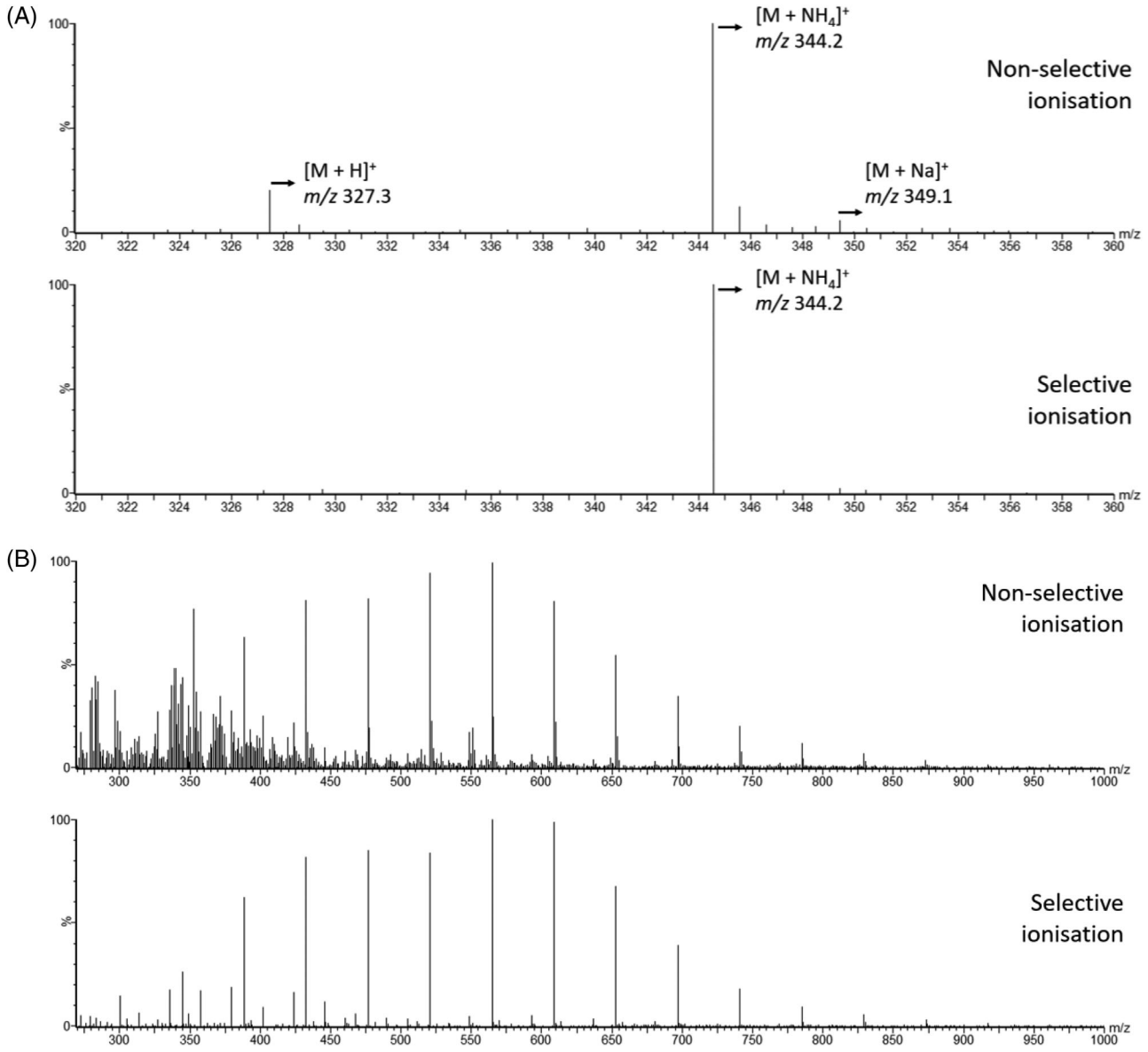

FIGURE 4 Simplification in the mass spectra of poly(ethylene glycol) (PEG) 600 (50 μg/mL in acetonitrile [ACN]) when selective ionization is promoted for (A) one oligomer and (B) the polymeric distribution.

work, z_{avg} was used to evaluate the influence of the chain length and the end group on the PEG oligomer ionization based on the relative ion current for different ionization events. The ionization of polymers with different molecular weights and end groups were compared as pie charts by the relative proportion of each ionization charged state (Figure 6). Oligomer ionization with different molecular weights and end groups was compared by the relative proportion of the total ionization charged for each specific chain length of the oligomer, that is, the repeating unit. z_{avg} is calculated using the number of observed analyte charge states in the mass spectrum N, the charge state of the ith oligomer z_i, and the sum of the peak areas of the RICC of all the oligomers in the ith charge state w_i.

$$z_{avg} = \frac{\sum_{i=1}^{N} z_i w_i}{\sum_{i=1}^{N} w_i} \quad (1)$$

For PEGs that differed in molecular weight, a linear fitting of z_{avg} to the reported molecular weight of different alcohol PEGs proved that the ionization response was independent of the charge state and confirmed that multiple charges predominate when increasing the chain length. When comparing pie charts and the z_{avg} (Figure 6), the influence of the polymer molecular weight was evident and in line with the conclusions of

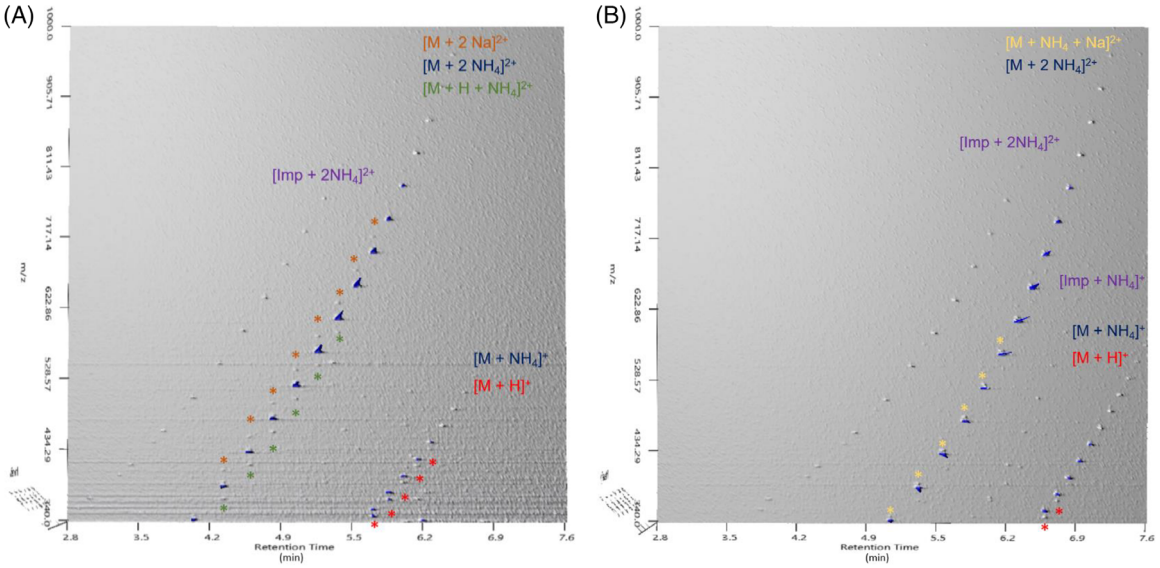

FIGURE 5 Three-dimensional ion map of poly(ethylene glycol) (PEG) 600 (50 μg/mL in acetonitrile [ACN]) with (A) uncontrolled ionization (25 mM ammonium formate in 100% methanol [%v/v]) and (B) controlled ionization (15 mM ammonium acetate in 94% methanol/6% water [%v/v]).

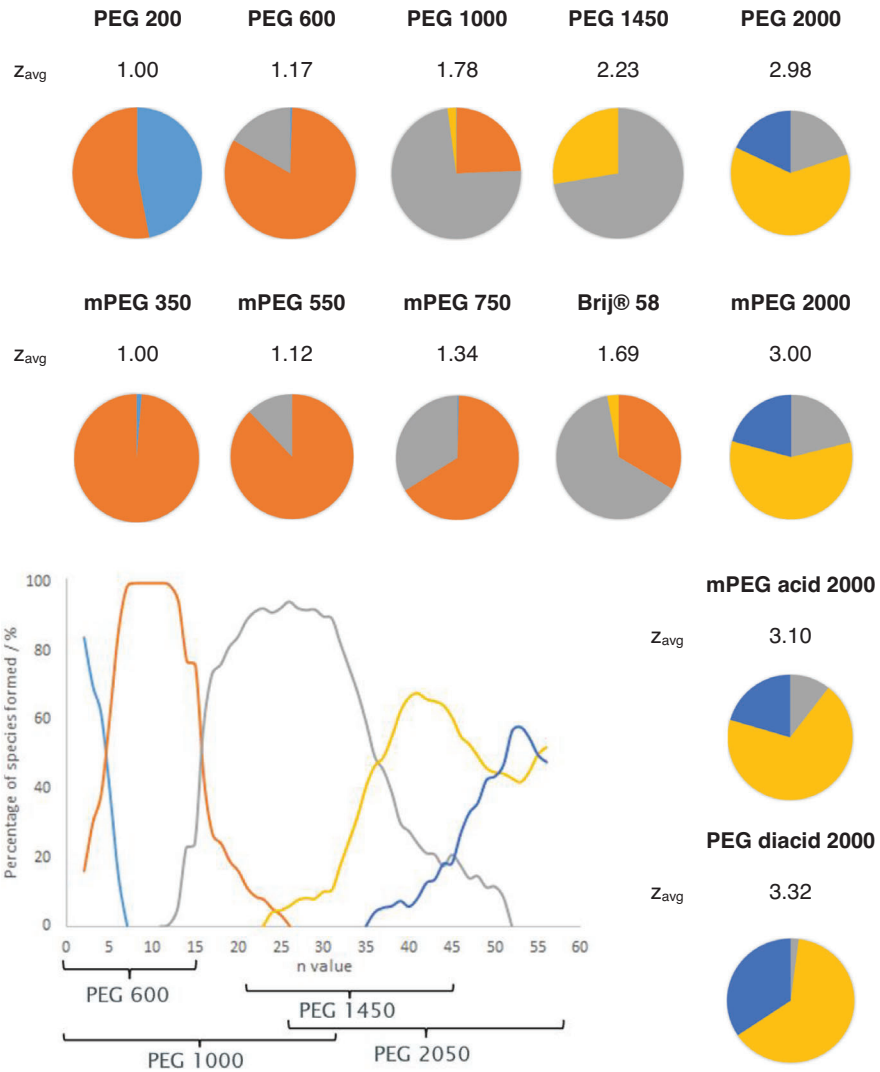

FIGURE 6 Evaluation of the ionization current trends for a variety of poly(ethylene glycols) (PEGs) based on the oligomer chain length, the molecular weight, and the end group. Pie charts were produced from data acquired at a concentration of 50 μg/mL of the corresponding PEG in acetonitrile (ACN). Light blue: $[M + H]^+$, orange $[M + NH_4]^+$, gray $[M + 2NH_4]^{2+}$, yellow $[M + 3NH_4]^{3+}$, dark blue $[M + 4NH_4]^{4+}$.

Gidden et al. [22]. The 2D-graph representation for alcohol PEGs (Figure 6) showed the dependence of the charged state to the oligomer chain length and illustrated that longer PEG chains could accommodate more ammonium adducts due to a minimization of the Coulombic repulsions between ammonium ions chelated to the ethylene oxide units. When comparing PEGs with similar molecular weight but different end groups (pie charts in the last column of Figure 6), slight variations in ionization profiles were associated with differences in electron-donating and electron-withdrawing effects of the end group. The overall ionization event appeared to be governed by the chelation of the alkali metal ions by the lone pairs of electrons in the ethoxylate groups, resulting in the formation of pseudo-crown ether environments during ionization.

4 | CONCLUDING REMARKS

Understanding why different ions are formed during positive ion ESI MS conditions and then controlling this process was found critical to selectively optimize the ionization of PEGs via ammoniated adducts, with the critical aspects being the mobile phase composition (particularly the concentration of ammonium ions), the cone voltage, and the desolvation temperature. The optimized mobile phase conditions were (A) CO_2 and (B) 15 mM AmAc in 94% methanol/6% water (%v/v), and the optimized ionization conditions were cone voltage 20 V and desolvation temperature 350°C. The method shown here provides cleaner mass spectra that aids the characterization and quantitation of these polymers.

An example was presented showing the benefits of selective ionization to provide detailed insights into the influence of the ionization of PEGs based on the oligomer chain length and the polymer end group. This comparison was possible using visual representations of the ionization trends that provided insight into the factors controlling the ionization of PEG species and the preferential formation of ammoniated adduct ions. These results suggest a considerable influence of the PEG chain length on the ionization, while the end groups altered the ionization state to a lesser extent due to an inductive effect that altered the ion cloud.

ACKNOWLEDGMENTS

The authors would like to acknowledge the valuable discussions with Dr. Matthias Baud. The authors appreciate the financial support provided by AstraZeneca and the UK Engineering and Physical Sciences Research Council to support this research project.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Cancho-Gonzalez S, Ferguson P, Herniman JM, Langley GJ. Controlling the positive ion electrospray ionization of poly(ethylene glycols) when using ultra-high-performance supercritical fluid chromatography-mass spectrometry. J Sep Sci. 2023;2300425.
https://doi.org/10.1002/jssc.202300425