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RESEARCH ARTICLE

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Dispersity determination of poly(ethylene glycol)s using supercritical fluid chromatography-mass spectrometry and different mass analysers

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Rationale: Dispersity values are considered critical quality attributes for the quality control of poly(ethylene glycol) formulations due to the direct impact on drug performance. However, when these polymers are analysed using mass spectrometry, the design of the mass analyser can impact the oligomer response and affect the obtained dispersity values, so further understanding is needed.

Methods: The deconvoluted electrospray ionisation mass spectra of poly(ethylene glycol)s obtained using supercritical fluid chromatography (SFC) hyphenated to different mass analysers were compared, and visualisation diagrams were used to understand the differences in the dispersity value calculations. Five calibration approaches based on a surrogate single oligomer that represents the whole distribution, or the whole distribution itself, for response selection, were used to evaluate ionisation efficiency prior to quantitation. The impact of using an internal standard (ISTD) on the expanded uncertainty was also assessed.

Results: Although there were challenges related to the resolution of multiply charged species when low-resolution instruments were used, similar quantitation capabilities were obtained to those when high-resolution mass analysers were used. Evaluation of approaches using a surrogate oligomer or the whole distribution suggested the independence of both approaches and a constant ionisation efficiency across the oligomer chain length. The higher degree of chromatographic resolution of SFC allowed incorporating a monodispersed ISTD to improve the accuracy and precision of the method.

Conclusions: The use of low resolution mass analysers was sufficient to provide accurate and precise dispersity values; however, higher resolution instruments were recommended for characterisation due to the improved mass resolution of ions. The introduction of a monodispersed ISTD improved precision without compromising the calculated dispersity value due to the lack of analyte suppression.

1 | INTRODUCTION

Polymeric excipients are extensively used in pharmaceutical formulations for the delivery of therapies of both small-molecule and biomolecule drugs.[1,2] Their addition provides encapsulation, aids solubility and/or offers protection from chemical degradation of the active drug substance.[3] However, batch and supplier variability within the polymeric material may compromise the effectiveness of the final drug product[4]; thus, accurate and precise measurement of those attributes that impact product performance is required. As such, the pharmaceutical industry identifies the dispersity value (*Đ*) as a critical quality attribute to determine the heterogeneity of the molecular weight distribution of the polymers. *Đ* is calculated using the number average (M_n) and weighted average (M_w) molecular weights. M_n and M_w are obtained from the relative amount N_i of the *i*th oligomer with n_i mer units and M_i molecular weight.[5,6] Equations (1–3) show the calculations for determining M_n, M_w and *Đ* values:

$$\overline{M_n} = \frac{\sum_i N_i M_i}{\sum_i N_i} \quad (1)$$

$$\overline{M_w} = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} \quad (2)$$

$$Đ = \frac{\overline{M_w}}{\overline{M_n}} \quad (3)$$

When accurate and precise *Đ* values are obtained, both the chromatographic technique and the detector must be carefully optimised to become a valid technique that works for the defined analytical target profile. Whereas matrix-assisted laser desorption/ionisation (MALDI) using time-of-flight (ToF) mass spectrometry (MS) is commonly used for polymer analysis, chromatographic approaches are still preferred to minimise ion suppression, being the standard size exclusion chromatography (SEC) with different detectors (e.g., UV–Vis. or light scattering detectors).[7–10] Alternatively, when chromatographic methods that use gradient elution are used, variable response factors of the oligomers are observed due to enhanced ionisation response at higher organic elution volumes.[11] For MS approaches, this results in the possibility of obtaining significant bias in the determined values due to the different ionisation and detection efficiencies over the molecular weight distribution. Quantitative methods that determine *Đ* values using MS require that special attention is given to the response factor of different oligomers.[12]

Previous studies emphasised that different instrumentation set-ups provide intrinsic alterations in *Đ* values, so that an evaluation is required to facilitate data comparison. Knol et al. presented an extensive review of the challenges with polymer quantitation when using liquid chromatography (LC).[11] Trathnigg et al. reported 2% differences for poly(propylene glycol) 450 when measurements from SEC were compared using either a refractive index or light scattering detector, and MALDI ToF MS.[13] When comparing chromatographic techniques, Poulton et al. reported up to a 2.5% difference when analysing poly (ethylene glycol)s (PEGs) with different end groups between data obtained using SEC coupled to a triple detector array and those obtained using supercritical fluid chromatography (SFC) coupled to an evaporative light scattering detector and a mass spectrometer (using electrospray ionisation (ESI) and a Waters QDa detector, a simplified single quadrupole mass analyser).[10] Even though these values were minor, the user should be aware that instrumental differences can impact dispersity calculation when evaluating this type of MS data. Shimada et al. studied the quantitation of PEG oligomers using MALDI and noted that for oligomers with masses >1000 Da, spectral intensities decreased when the oligomer mass increased due to the stability of the ionised PEG molecules during the ionisation process and the desorption of PEGs into the gas phase using MALDI laser.[14] Attempts to minimise instrumental differences include stable isotope dilution assay[15] or the use of internal standardisation.[16,17] Although assay by stable isotope dilution is expensive, the incorporation of internal standards (ISTD) in polymer analysis is rare and observed only in MALDI MS. The difficulty comes from the selection of the ISTD as the use of polymeric materials adds unnecessary chromatographic complexity and often results in peak co-elution that can lead to response enhancement or suppression, whereas the selection of small molecules that elute away from the polymeric distribution and have similar ionisation behaviour is complicated.

The main challenges of using MS to calculate *Đ* values are due to the control of the ions generated and the understanding of the influence of the different chromatographic systems, ionisation sources and mass analysers. The generation of the ions is affected by the sample preparation (especially in MALDI),[18] the mobile phase composition (affecting the ion source desolvation environment)[7,19,20] and the mass spectrometer design of the ion source[21–23] and mass analyser.[24,25] Shimada et al. noted that the ionisation efficiency and ion transmission efficiency are mass dependent, so these two parameters impact the calculation of *Đ* values.[14] For example, MALDI studies of PEGs showed that mass discrimination between samples resulted due to differences in the detector, laser power and cationisation-dependent effects.[14,26] Cancho-Gonzalez et al. showed how selective ionisation of PEGs via the formation of ammoniated species can be obtained using SFC-ESI-MS.[27] In a similar work, Prokai and Simonsick showed that the selective formation of sodiated species of octylphenoxypoly(ethoxy ethanol) using SEC-ESI-MS simplified the monitoring of the elution profiles of the individual oligomers and allowed the formation of a calibration curve using an SEC-like quantitation approach with non-MS detectors.[7]

It should be noted that the determination of a polymer *Đ* value using MS is simpler when only singly charged species are formed as N_i relates to the response of the ions generated for each oligomer. The determination becomes challenging for polymers that form multiply charged species as they require mass spectral deconvolution. Lingaityte showed quantitation of methylene diphenyl diisocyanate polymers using positive ion atmospheric pressure photoionisation MS as they produced singly charged radical cations with an ionisation

efficiency independent of the number of aromatic rings and functional groups.[28] The complex mass spectra obtained when PEGs ionise under positive ion ESI-MS conditions and the formation of multiple-charged envelopes of ions at the same charge states are shown in the literature.[9,10] The work of Cancho-Gonzalez et al. allowed a further understanding of how PEG ionisation varies across the elution profile of the oligomers.[27]

Charge envelopes of ions are interpreted using mathematical algorithms (mass spectra deconvolution tools) that convert the multiply charged ions in the *m/z* scale into a single peak corresponding to the molecular mass of the molecule in a reconstructed mock spectrum in the mass scale.[10,29] Most deconvolution algorithms operate based on the proposal by Mann et al.[30] that resolves a simultaneous set of equations based on Equation (4) by assigning values to the charge state (*n*) of ions formed using adducts with *X* mass. Other mass spectra deconvolution algorithms are based on maximum entropy (MaxEnt), an approach that uses Bayesian statistics to predict the most probable molecular mass spectrum based on pattern similarities after numerous iterations.[31,32] Powley demonstrated the reliability of the algorithm for oligonucleotide quantitation[33]; however, it should be noted that the algorithm is facilitated as the generated ESI mass spectrum of a single oligonucleotide is simpler due to the presence of only one multiply charged envelope. In comparison, deconvolution of the PEG ESI mass spectrum using MaxEnt is more challenging due to the intrinsic complexity of the multiple charges generated, and alternative approaches were proposed to facilitate data processing. For example, Gruendling et al. took advantage of the universal response provided by refractive index detection to confirm the measured ESI response prior to MaxEnt deconvolution of the poly(methyl methacrylate) mass spectra[34]:

$$\mathrm{MW} = n(m/z - X) \tag{4}$$

Before MaxEnt was developed, Waters proposed the Transform algorithm to accelerate the resolution of the simultaneous equations approach initially proposed by Mann et al.[30,35] (as per Equation 4). Recently, Marty et al. developed UniDec (that also uses Bayesian statistics) and compared it to Transform and MaxEnt for the deconvolution of the ESI mass spectrum generated for aquaporin Z (a protein with bound lipids).[36] They found that the quantitative aspect of Transform relies on a user-defined mass window to sum the isolated charge states that might limit their application. A recent investigation allowed deconvolution of a PEG ESI mass spectra using UniDec; however, significant errors were observed when M_n and M_w values (−21% and −30% for PEG 1000 and +16% and +17% for PEG 7000) were calculated, which could be attributed to the lack of selective ionisation of the proposed method.[37]

The aim of this study was to measure the polymer *Đ* values due to their consideration as a critical quality attribute in pharmaceutical drug formulations. An SFC approach was used to address possible issues with ion suppression due to the overlap of the active pharmaceutical ingredient and impurities with the polymeric series that will result in substantial and unavoidable suppression when using ionisation sources that directly introduce the sample into the mass analyser, such as in MALDI. The Transform algorithm was evaluated for ESI mass spectra deconvolution of data generated using SFC-MS from PEG samples. This algorithm was used to estimate the impact of three different mass analysers on the obtained deconvoluted spectra: that is, single-quadrupole, triple-quadrupole and quadrupole ToF (QToF) mass analysers. The influence of each mass analyser on the calculated *Đ* values was then assessed. Additionally, the effect of a monodispersed ISTD was investigated to improve the expanded uncertainty of *Đ* values.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Laboratory-grade PEGs with average molecular weights of 200, 400, 600, 1000, 1450 and 2000 Da; laboratory-grade 18-crown-6; and LC-MS grade ammonium acetate were purchased from Sigma−Aldrich (Gillingham, UK). A grade helium, industrial grade argon and food grade carbon dioxide were purchased from BOC Special Gases (Manchester, UK). Nitrogen was supplied by a nitrogen generator on each instrument: a Whatman N_2 generator when using the single quadrupole mass analyser and a STREAM 120 N_2 generator when using the triplequadrupole and the QToF mass analysers. LC-MS grade methanol was purchased from ThermoFisher Scientific (Loughborough, UK). LC-MS grade Water and LC-MS grade acetonitrile were obtained from Fisher Scientific Ltd (Basingstoke, UK).

2.2 | Sample preparation

The stock solutions of 1000 µg/mL of PEG 600, 1000, 1450 and 2000 and the stock solution of 100 µg/mL of 18-crown-6 were prepared in acetonitrile and stored at 2°C for further analysis. No degradation in solution was observed. For external calibration, a series of six standards within the calibration range of 0.01–500 µg/mL were prepared by serial dilutions from the stock solution. For internal calibration, six working standard solutions in the range of 0.01–500 µg/mL of the corresponding PEG and 0.5 µg/mL of 18-crown-6 (as ISTD) were prepared from stock solutions. Standard samples were injected in triplicates.

3 | INSTRUMENTATION

The chromatographic and MS methods were developed in a previous work to selectively ionise PEGs towards an ammoniated event.[27] The method was developed to selectively form ammoniated adducts $[M + n\mathrm{NH_4}]^{n+}$ of PEG oligomers by optimising the SFC mobile phase and make-up solvent, and the ESI ionisation parameters. Details of the method are provided.

3.1 | Supercritical fluid chromatography

SFC analysis was achieved using a Waters Acquity UPC^2 system (Waters Corp., Milford, USA) with a Waters Acquity UPC^2 Torus Diol (130 Å, 100 × 3.0 mm, 1.7 µm i.d.) at 70°C. The mobile phase was composed of supercritical CO_2 at 150 bar active back pressure regulator and a co-solvent of 15 mM ammonium acetate in 94.0% methanol/6.0% water (% v/v). The separation was produced using a linear gradient of 1%–40% of the mobile phase over 10 min. Ionisation was promoted using a make-up solvent of 50 µm of ammonium acetate in methanol.

3.2 | Mass spectrometry

The chromatographic system was directly coupled to a Waters ZSpray ESI source (Waters Corp.) before the mass analyser. Polymer ionisation was controlled using a make-up solvent of 50 µM ammonium acetate in methanol. Three mass analysers were investigated: a single quadrupole Waters Xevo SQD 2 (Waters Corp.), a triple quadrupole Waters Xevo TQD (Waters Corp.) and a QToF Waters Synapt G2-Si (Waters Corp.).

The common ionisation parameters between all mass analysers were as follows: capillary voltage, +2.5 kV; cone voltage, 20 V; source temperature, 150°C; desolvation temperature, 350°C; desolvation gas flow, 550 L/h; and cone gas flow, 50 L/h. For the single or triple quadrupole mass analysers, continuous data were scanned in full scan mode over an *m/z* range of 270–1500 with a scan time of 0.2 s. QToF continuous data were obtained in full scan mode over an *m/z* range of 270–3000, with a source offset of 40 V and a nebuliser pressure of 7 bar.

Additionally, for one of the single quadrupole mass analyser quantitation approaches tested in Section 5.3, selected ion monitoring (SIM) was performed with the following ions: PEG 600 *m/z*, 564.4 ($[M + NH_4]^+$); PEG 1000, *m/z* 489.5 ($[M + 2NH_4]^{2+}$); PEG 1450, *m/z* 753.5 ($[M + 2NH_4]^{2+}$); and PEG 2000, *m/z* 640.5 ($[M + 3NH_4]^{3+}$). Note that the ions selected to monitor are specific for the polymeric distribution under study, so readers are encouraged to select their own values per the guidelines provided in Section 5.3.

4 | DATA ANALYSIS

4.1 | Software

SFC-MS data were recorded and processed using MassLynx, version 4.1, SCN 855 software. Waters Transform algorithm in MassLynx was used for mass spectral deconvolution. Additional data were processed to present the deconvoluted mass traces and calculate M_n, M_w and $Đ$ values using Microsoft Office 365 (Excel) software.

4.2 | Understanding the Waters Transform algorithm

Transform algorithm is based on the resolution of the simultaneous equations approach initially proposed by Mann et al.[30,35] (as per Equation 4), launched by Waters. The algorithm does not incorporate ammonium ions, and therefore, changes in the coding were required. The MassLynx.ini file located in the C:\MassLynx directory (datafile can be opened using a generic .txt reader) contains a section within the coding called [SpeTransform] that relates to the Transform algorithm, and the most important parameters are discussed. The reader is referred to Data S2–S3–S4 for the MassLynx.ini files used in this work.

- TransformMinMass and TransformMaxMass: allows adjusting the *m/z* range where the algorithm is applied.
- TransformRes: the mass resolution of the mass analyser: 0.5000 Da for the single quadrupole and 0.0125 Da for the QToF.
- TransformThreshold: the background threshold signal neglected by the algorithm.
- TransformWindow: the mass window that is accounted for around the input mass.
- TransformDimers: indicates whether dimers are present.
- TransformAdduct: the accurate mass of the adduct: the accurate mass of the Na ion (22.989768 = Na − e) was changed for the accurate mass of the ammonium ion (18.033826 = N + 4H − e).
- AutoPos, ManualPos and EditPos: these parameters relate to the coefficients and solutions of the simultaneous equations as per Equation (4). AutoPos changed from 38,85,511,259 (sodium ions) to 523,395,750,515 (ammonium ions). ManualPos and EditPos are calculated internally using MassLynx.

4.3 | Waters Transform algorithm data treatment

After the MassLynx.ini files were modified, the theoretical monoisotopic masses of the oligomers were calculated for the oligomers of the PEG chain using Microsoft Office 365 (Excel) software (carbon: 12.000000 Da, hydrogen: 1.007825 Da, oxygen: 15.994915 Da, nitrogen: 14.003074 Da and e: 0.000549 Da). The ion mass was calculated using the input mass of the PEG oligomer and the number of ammonium adducts at the expected range of ion charges (from +1 to +5, depending on the molecular weight of the oligomer, as per Cancho-Gonzalez et al.[27]). The calculated ion masses were introduced as part of the Transform algorithm database.

Initially, the algorithm was executed by identifying a single oligomer in the chromatogram (Figure 1A), obtaining the corresponding mass spectrum (Figure 1B) and applying the Transform deconvolution (Figure 1C). Then, the deconvolution was extended to the whole polymer mass spectrum by first simultaneously repeating the deconvolution to a few oligomers' mass spectra in a stepwise approach (Figure 2A–C) and then to the whole polymer mass spectrum (Figure 2D).

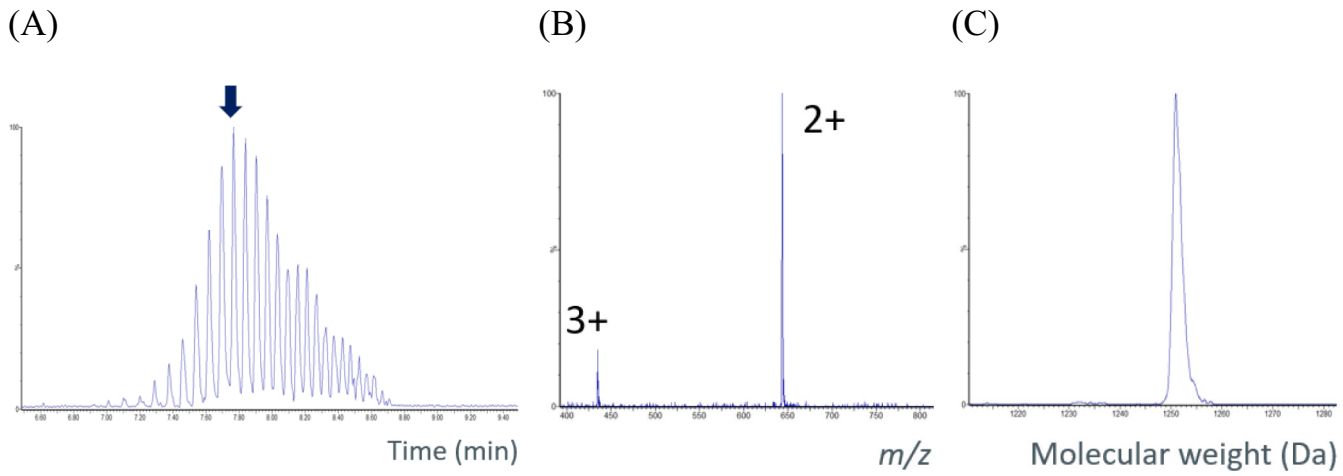


FIGURE 1 Stepwise deconvolution of the mass spectra of one oligomer using Waters Transform. (A) Identification of the oligomer in the chromatogram. (B) Oligomer mass spectrum. (C) Deconvoluted oligomer. [Color figure can be viewed at wileyonlinelibrary.com]

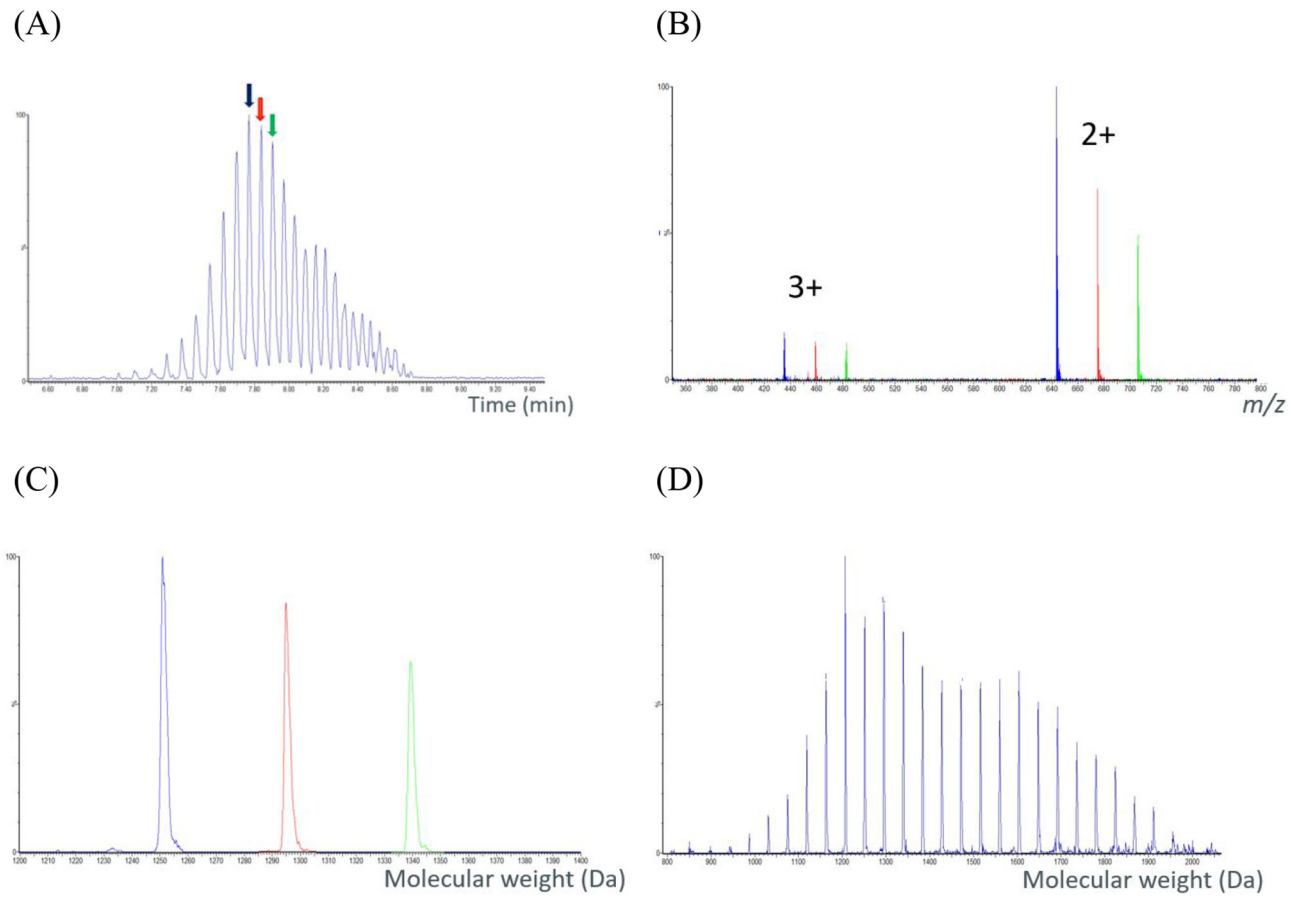


FIGURE 2 Stepwise deconvolution of the mass spectra of PEG 1450 using Waters Transform algorithm. (A) Chromatographic separation of the oligomers with three selected oligomers. (B) The mass spectra of the three different oligomers were combined. (C) Deconvolution of the selected oligomers was possible. (D) The whole polymer distribution was selected, and the mass spectra of all oligomers were deconvoluted simultaneously. [Color figure can be viewed at wileyonlinelibrary.com]

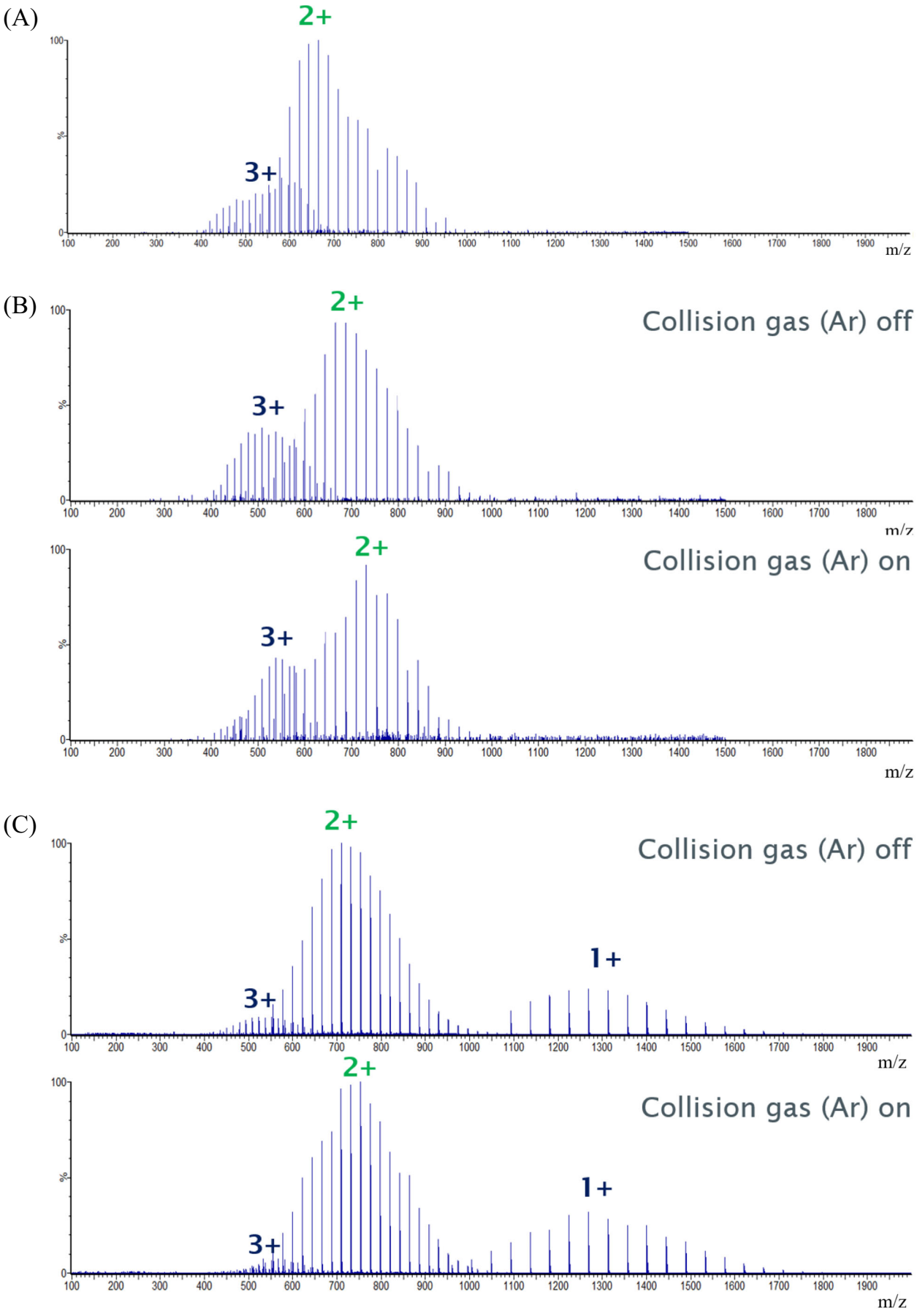


FIGURE 3 Evaluation of the effect of the mass analyser ion transmission and collision gas in the PEG 1450 positive ion electrospray ionisation (ESI) mass spectrum. Numbers indicate the ionisation state of the charge envelopes underneath. (A) Single quadrupole: beam mass analyser without collision gas. (B) Triple quadrupole: beam mass analyser with collision gas. (C) Quadrupole time-of-flight (QToF): pulsed mass analyser with collision gas. [Color figure can be viewed at wileyonlinelibrary.com]

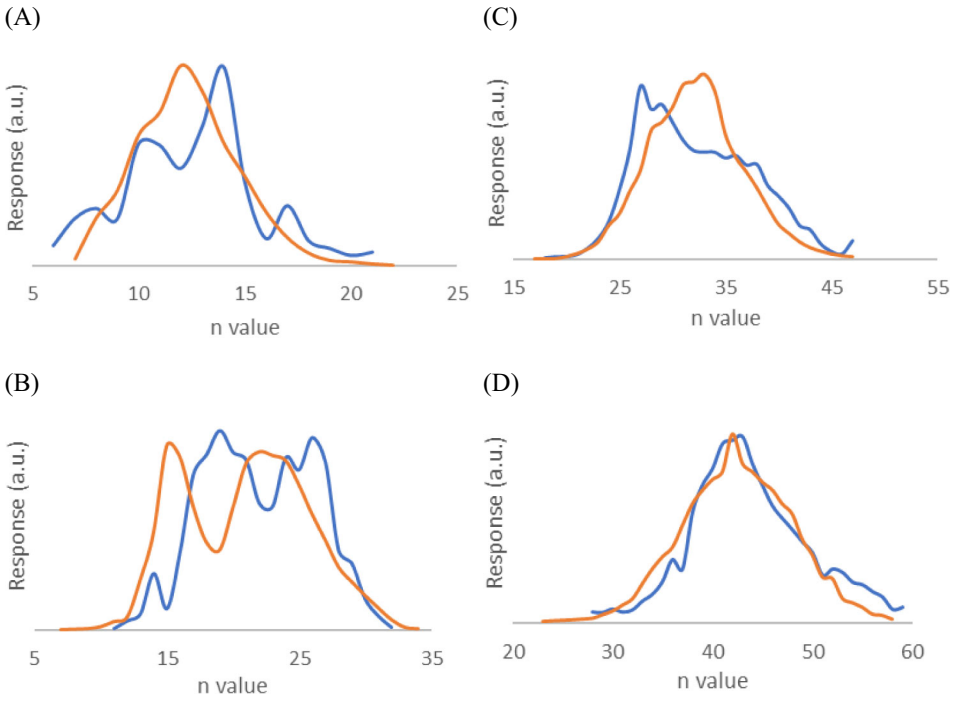


FIGURE 4 Comparison of the supercritical fluid chromatography positive ion electrospray ionisation (ESI) mass spectra deconvolution traces of (A) PEG 600, (B) PEG 1000, (C) PEG 1450 and (D) PEG 2000 when using the single-quadrupole (blue) and quadrupole time-of-flight (QToF) (orange) mass analysers. Note that the response of the single-quadrupole mass analyser was adjusted to align the graphs based on differences in sensitivity and concentration. [Color figure can be viewed at wileyonlinelibrary.com]

5 | RESULTS AND DISCUSSION

5.1 | Effect of the mass analyser on the deconvoluted mass spectra

Initially, the change of the mass analyser on the obtained mass spectrum was assessed to understand the impact on the data obtained. The main parameters that could impact the different acquired mass spectra were the collision gas and the ion transmission. The effect of the collision gas was evaluated in mass analysers using a beam design: the single quadrupole (Figure 3A, no collision gas—shown only as reference spectra) and triple quadrupole (Figure 3B). Even when the collision gas was set to off in the triple quadrupole, the presence of low levels of argon gas in the ion path led to a charge-stripping phenomenon (reducing the average charge state of the polymer distribution[38]) and resulted in a degree of uncontrolled collision-induced dissociation of the ammoniated molecules to protonated species. The ion transmission was assessed by comparing the triple quadrupole and the QToF mass analysers because of the differences in the design of the ion path. The movement from a beam design (Figure 3B, triple quadrupole) to a pulse design (Figure 3C, QToF) resulted in a reduction in the average charge state of the polymer distribution. Note that other factors contributing to charge stripping in the QToF could be the charge reduction that occurs due to the higher kinetic energy of the higher charge state species when travelling through the mass analyser.[38]

Then, the capacity of the mass analyser to resolve the $^{12}C/^{13}C$ isotopic distribution was assessed to evaluate the impact in the deconvoluted response. Both single and triple quadrupole mass analysers have unit mass resolving power resulting in fully resolving the $^{12}C/^{13}C$ isotopic distribution of singly charged species but unable to resolve it for multiply charged species in an equivalent manner. Therefore, only the single quadrupole and QToF mass analysers were assessed. As the ion response differed (even when using the same sample concentration) due to the different instrumental designs, a re-scaling of the response of the deconvoluted traces was performed to allow comparison. Additionally, the *x*-axis was changed from the molecular weight scale to the oligomer repeating unit value scale to facilitate the implementation of the understanding of the polymer ionisation as described by Cancho-Gonzalez et al., therefore the impact of unresolving the $^{12}C/^{13}C$ isotopic distribution in the single quadrupole mass analyser. The redrawn deconvoluted traces of both mass analysers were made to overlap (Figure 4).[27] Their work described the ionisation of PEG oligomers based on the charge state of the ion formed at specific oligomer repeating units. When the single quadrupole and the QToF mass analysers were compared, it can be noted that the lack of resolving power of the single quadrupole affects the deconvoluted response for double or higher charged state species (Figure 4A–C), therefore impacting the representation of the true distribution. Note that the proportion of singly charged species compared to other charges (as per Cancho-Gonzalez et al.[27]) for PEG 600 was approximately three quarters (Figure 4A) that decreased to a

TABLE 1 Effect of the mass analyser on the calculated M_n, M_w and $Đ$ values of different PEGs of different molecular weights.

		Experimental Q (%)	Experimental QToF (%)	Manufacturer (SEC)
PEG 600	M_n	581 ± 4.4	581 ± 1.9	590
	M_w	600 ± 4.4	602 ± 1.6	609
	$Đ$	1.033 ± 0.6	1.034 ± 0.4	1.032
PEG 1000	M_n	991 ± 4.6	982 ± 0.8	987
	M_w	1027 ± 4.4	1015 ± 0.8	1020
	$Đ$	1.036 ± 0.6	1.034 ± 0.3	1.033
PEG 1450	M_n	1437 ± 1.6	1440 ± 0.9	1451
	M_w	1477 ± 1.5	1485 ± 0.7	1491
	$Đ$	1.028 ± 0.3	1.031 ± 0.4	1.028
PEG 2000	M_n	1944 ± 2.7	1954 ± 1.3	1965
	M_w	1979 ± 3.4	1995 ± 1.3	2007
	$Đ$	1.018 ± 0.4	1.020 ± 0.3	1.021

Note: The error corresponds to the expanded uncertainty ($k = 2$, calculated using accuracy and precision components [measured as coefficient of variation]).
Abbreviations: PEG, poly(ethylene glycol); QToF, quadrupole time of flight; SEC, size exclusion chromatography.

quarter for PEG 1000 (Figure 4B), and their amount becomes negligible for PEGs of larger molecular weights (Figure 4C,D).

Alternatively, the total ion current chromatogram[39] or the highly resolved QToF deconvoluted data provided a true representation of the polymeric distribution; however, the single quadrupole data are usable with the caveats regarding the loss of differentiation in the isotopic distribution. If quadrupole mass analysers are used with multiply charged data, then either average chemical mass should be used, or the resolution of the quadrupole should be re-tuned to increase resolution. This results in a significant cost in sensitivity of the measurement; therefore, QToF is recommended for PEGs that mainly form multiple- charged species.

5.2 | Effect of the mass analyser on the calculation of the dispersity values

The validity of the deconvoluted traces obtained for the different mass analysers was evaluated by calculating the M_n, M_w and $Đ$ values. The responses of the deconvoluted traces obtained using the singlequadrupole and the QToF mass analysers (Figure 4) were used to calculate $Đ$ values using Equations (1–3). Although differences in the deconvoluted mass spectra between the two mass analysers were observed, the resultant M_n, M_w and $Đ$ values were in good agreement with the values reported by the manufacturer (Table 1) using SEC (unknown detector).

These values highlighted the validity of this SFC-MS method for the calculation of the critical quality attributes M_n, M_w and $Đ$ of PEG materials. The introduction of a make-up flow solvent in SFC-MS was associated with somehow mitigating the differences in responses of the oligomers along the changes in the chromatographic eluent composition, delivering a more uniform ionisation environment. It is worth noting that the lack of resolving power of the single quadrupole mass analyser (Figure 4) did not significantly impact the calculation of M_n, M_w and $Đ$ values compared to the QToF, as acceptable expanded uncertainty values were achieved.

5.3 | Differences in quantitation approaches

The expanded uncertainty demonstrated the validity of this method to obtain $Đ$ values; however, further investigation was conducted to confirm that the ionisation efficiency was uniform along the oligomeric chains, as literature showed that it might be compromised in QToF mass analysers for multiple-charged species with masses above 10^3 Da.[14,21,23,25] In an attempt to reduce the number of multiple charged species, charge stripping was investigated by increasing the cone voltage; however, unexpected in-source collision-induced dissociation of the ammoniated molecules (data not shown) was observed (as reported by Cancho-Gonzalez et al.[27]). Alternatively, the use of external calibration linear fitting of the response obtained in diverse ways was considered to assess whether all oligomers contributed in the same manner to the deconvoluted response so that their ionisation efficiency was equal and not affected by the mass analyser design. Initially, the response obtained using the single-quadrupole mass analyser was evaluated for simplicity.

The polymer response was calculated using five approaches: three approaches were based on a single oligomer that functioned as a surrogate to represent the whole polymeric distribution, and two approaches were based on the whole distribution itself. The surrogate oligomer was selected based on the oligomer that formed the base peak ion of the polymer mass spectrum as this is the most abundant oligomer of the polymer distribution. For PEG 1450, this ion corresponds to the blue oligomer in Figure 1. The ions produced by this oligomer were treated in three different ways (i–iii) to obtain the response used for the linear fitting for calibration:

TABLE 2 Comparison of the different approaches for external calibration quantitation.

	Mass analyser	Approach	Parameter	PEG 600	PEG 1000	PEG 1450	PEG 2000
Surrogate oligomer	Q	(i) SIM	LDR (μg/mL)	0.01–10	0.5–15	0.5–50	2.5–100
			R^2	0.9999	0.9999	0.9999	0.9999
		(ii) RICC	LDR (μg/mL)	1–75	10–200	50–300	100–500
			R^2	0.9984	0.9993	0.9992	0.9984
		(iii) Deconvoluted mass spectra	LDR (μg/mL)	1–75	10–200	50–300	100–500
			R^2	0.9958	0.9994	0.9983	0.9957
	QToF	(iii) Deconvoluted mass spectra	LDR (μg/mL)	0.5–25	1–50	1–60	5–75
			R^2	0.9997	0.9999	0.9995	0.9995
Whole distribution	Q	(iv) Sum of peak areas of RICC	LDR (μg/mL)	1–75	10–200	50–300	100–500
			R^2	0.9981	0.9979	0.9977	0.9979
		(v) Deconvoluted mass spectra	LDR (μg/mL)	1–75	10–200	50–300	100–500
			R^2	0.9977	0.9981	0.9976	0.9971
	QToF	(v) Deconvoluted mass spectra	LDR (μg/mL)	1–75	1–50	1–60	5–75
			R^2	0.9997	0.9998	0.9993	0.9992

Notes: (i) The response of the base peak ion acquired using SIM acquisition, (ii) reconstructed ion current chromatogram (RICC of the oligomer that produced the base peak ion) or (iii) the response of the deconvoluted oligomer mass spectra, (iv) the sum of the RICCs of all the polymeric ions of the mass spectra and (v) the sum of the oligomer deconvoluted responses. Note that for approaches (i) and (ii) the following ions were selected: for PEG 600, *m/z* 564.6 ($[M + NH_4]^+$); for PEG 1000, *m/z* 489.5 ($[M + 2NH_4]^{2+}$); for PEG 1450, *m/z* 753.5 ($[M + 2NH_4]^{2+}$); and for PEG 2000 *m/z* 640.5 ($[M + 3NH_4]^{3+}$).
Abbreviations: LDR, linear dynamic range; QToF, quadrupole time of flight; RICC, reconstructed ion current chromatogram; SIM, selective ion monitoring.

- Approach i: the peak area of the base peak ion of the surrogate oligomer was obtained using SIM acquisition.
- Approach ii: the peak area of the base peak ion of the surrogate oligomer was obtained using the reconstructed ion current chromatogram (RICC).
- Approach iii: the response of the deconvoluted mass spectrum of the surrogate oligomer.

For the whole distribution, two approaches were considered (iv and v), which were an extension of the described surrogate oligomer approaches:

- Approach iv: the ions corresponding to the polymer distribution were identified, and the peak areas of the RICC of each oligomer ion were summed.
- Approach v: the mass spectrum of each oligomer of the polymer distribution was deconvoluted, and the responses were summed.

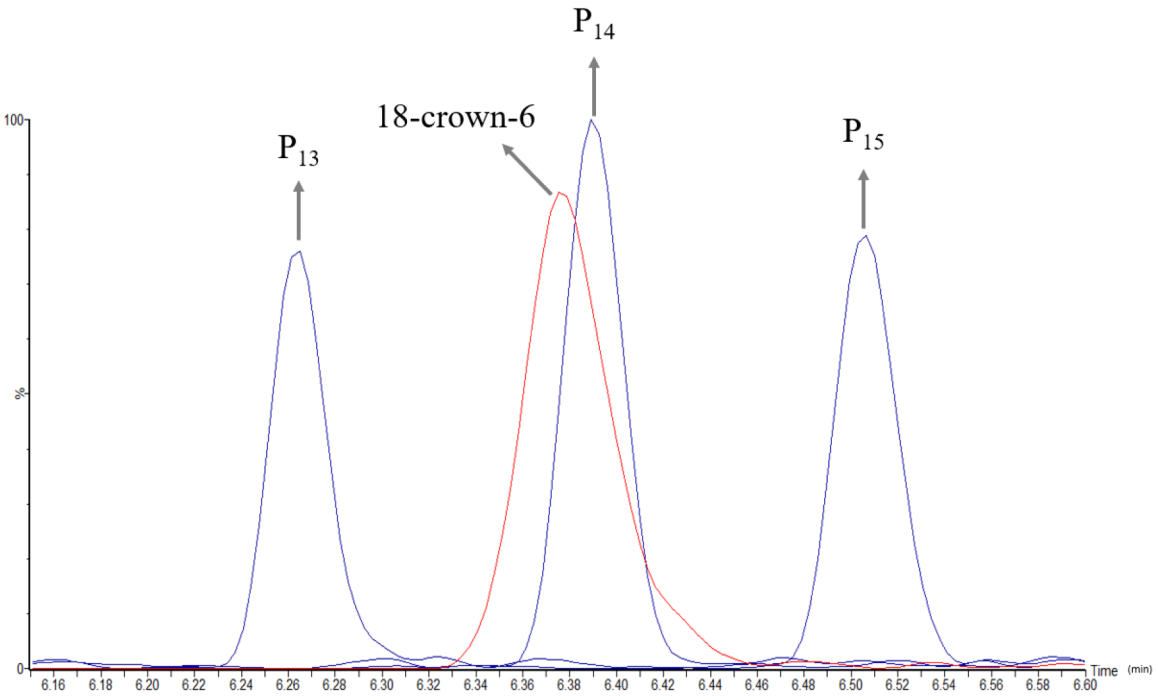
The linearity of all calibration curves in the five approaches was statistically evaluated using lack-of-fit testing and relative residual analysis, and no concerns were observed, so the linear dynamic range (LDR) and the coefficient of determination (R^2) were compared (data are summarised in Table 1). The comparison of the surrogate and whole distribution approaches (Table 2) showed similar values for the LDR for all approaches except when SIM acquisition of the most intense ion was used. Contrary to full *m/z* range data acquisitions, the use of SIM showed the caveat of only recording targeted data for one selected *m/z* value that limited reusing the data for characterisation purposes. When the timing of data processing was compared, those approaches that used the whole polymer distribution took significantly longer than those based on a single surrogate oligomer (0.1 vs. 10 min per mass spectra) due to the larger amount of data required to be processed.

Then, the approach was extended to the QToF mass analyser to assess the differences in the single quadrupole mass analyser. When the quantitation capabilities of both mass analysers were compared (Table 2), the QToF significantly improved sensitivity and precision (coefficient of variation [CV] 5% for QToF vs. CV 10% for the single quadrupole) and a narrower LDR. The improved sensitivity of the QToF data was associated with increased ion transmission due to a larger hole in the sampling cone and the StepWave ion optics technology incorporated in the Waters Synapt G2-Si design. Note that significantly longer data processing was observed for the polymer mass spectra obtained using the QToF (10 vs. 30 min) due to the nature of the higher size of the data file because of the higher-resolution instrument.

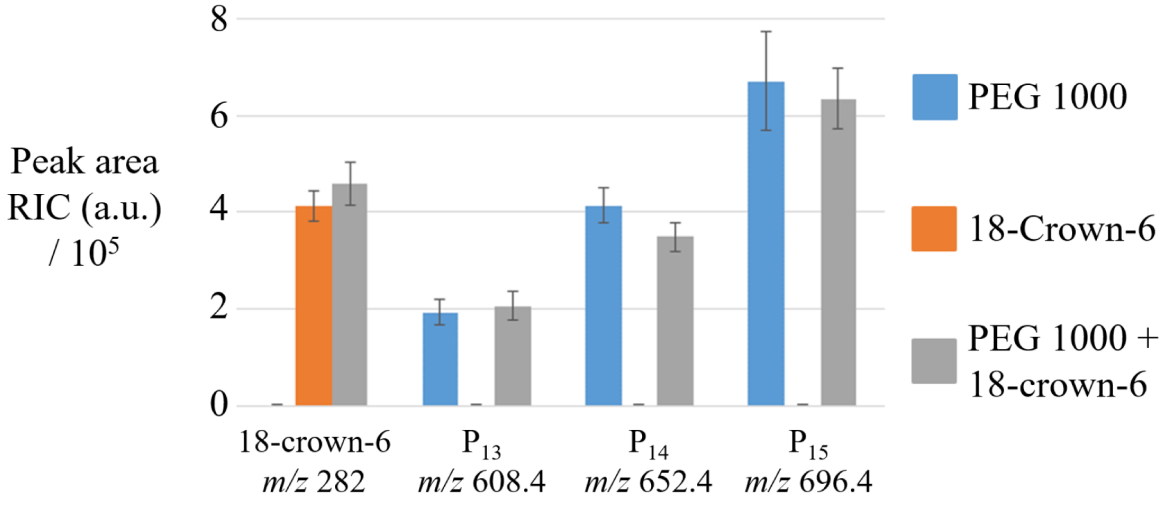
5.4 | Use of an ISTD

An internal calibration approach was considered to improve the expanded uncertainty of the method when using the single quadrupole mass analyser to investigate whether it was possible to replicate the slight improvements in the *Đ* value observed using the QToF instrument (see Section 5.2). 18-Crown-6 was selected due to the structural similarities to PEG except the undesired polymeric characteristics, that is, behaving as a monodispersed ISTD that exhibited only one chromatographic peak. The similarity of the

(A)



(B)



(C)

	Experimental (external calibration)	Experimental (internal calibration)	Manufacturer (size exclusion chromatography)
$\mathbf{M_n}$	991 ± 4.6%	983 ± 0.7%	987
$\mathbf{M_w}$	1027 ± 4.4%	1017 ± 0.6%	1020
Đ	1.036 ± 0.6%	1.034 ± 0.3%	1.033

FIGURE 5 Legend on next page.

FIGURE 5 Effect of the internal standard (0.5 μg/mL of 18-crown-6) on the poly(ethylene glycol) (PEG) response (50 μg/mL of PEG 1000 in acetonitrile) evaluated using the oligomer with 13 (P_{13}), 14 (P_{14}) and 15 (P_{15}) ethylene glycol units. (A) Co-elution between 18-crown-6 and the oligomer P_{14} of PEG 1000. (B) Evaluation of the matrix effect. (C) Effect on the calculation of M_n, M_w and $Đ$ values. The error corresponds to the expanded uncertainty ($k = 2$, calculated using accuracy and precision components [measured as the coefficient of variation]). [Color figure can be viewed at wileyonlinelibrary.com]

structure of crown ethers to PEGs explained the achievement of selective ionisation towards the formation of ammoniated adducts in both molecules.

Evaluation of the chromatographic separation (Figure 5A) showed only co-elution of the PEG oligomer with 14 repeating units (P_{14}, $[P_{14} + NH_4]^+$ with m/z 652.4) with the selected ISTD 18-crown-6 ($[18\text{-crown-}6 + NH_4]^+$ with m/z 282). The presence of co-elution required the evaluation of ion suppression effects and the possible impact on calculated $Đ$ values. The addition of the ISTD to the polymer resulted in (Figure 5B) an ion suppression on the oligomer P_{14} of about −13% with an enhanced response of 18-crown-6 of about +11%; however, their impact of introducing the ISTD on the calculation of M_n, M_w and $Đ$ values (Figure 5C) was minimal. The insignificant matrix effects induced by the ISTD were attributed to the only co-elution of one oligomer with the ISTD and the baseline resolution achieved between the rest of the oligomers and the ISTD provided by SFC.

The comparison of the calibration curves (data in Data S1) determined that the introduction of 18-crown-6 as the ISTD improved the accuracy and precision of the response of the other oligomers (Figure 5B) that resulted in $Đ$ values with expanded uncertainties comparable to those obtained using the QToF (as in Figure 5C).

6 | CONCLUSIONS

The study showed the possibility of using the proposed SFC-MS approach as an analytical target profile method to study the critical quality values M_n, M_w and $Đ$ of PEGs. An SFC-MS approach that allowed full control of the ionisation of PEGs[27] was used, which in turn allowed us to obtain accurate and precise M_n, M_w and $Đ$ values. The research also showed the effect of the design of the mass analyser and the impact on the data. For modern beam instruments such as single quadrupole mass analysers, the fast scan rate (typically 15–20 000 m/z units per second) and low duty cycle (0.1%) delivered an appropriate number of scans across a chromatographic peak which provides a representative profile, with the caveat of the scan rate being m/z range dependent.

The calculation of M_n, M_w and $Đ$ values using both single quadrupole and QToF mass analysers showed acceptable expanded uncertainty that was comparable to more established approaches; however, it is important to note that the isotopic information on the ions measured may be compromised at the fast scan rates of single quadrupole mass analysers and induce errors in the deconvoluted mass spectra that can result in incorrect assignment of polymeric trends. The QToF pulsed ion beam instrumentation with the advantage of higher resolution better differentiated the $^{12}C/^{13}C$ isotopic distribution, facilitated identification of the oligomer m/z value used in the deconvolution and allowed the construction of a deconvoluted mass spectrum trace that was more representative of the polymeric distribution. Although there were challenges when using the single quadrupole mass analyser due to the lower mass resolution of multiply charged species, the similar values for M_n, M_w and $Đ$ suggested that inexpensive single-quadrupole mass analysers can be used to obtain accurate and precise values without the need to invest in expensive instruments; however, polymeric distributions were shown distorted due to the lack of resolution of multiple charged species. Although the single quadrupole mass analyser provided reliable results, the use of a QToF is recommended for calculation of these properties in complex mixtures or PEGs with higher molecular weights as the higher mass resolution aids ion identification.

Evaluation of the proposed quantitation approaches suggested that deconvoluting the most intense surrogate oligomer and acquiring full-scan data was the preferred approach for quantitation due to its faster data processing and allowance of further polymer characterisation if required. Moreover, the introduction of a monodispersed ISTD, such as 18-crown-6, improved the expanded uncertainty of the method that resulted due to the baseline resolution between the ISTD and the majority of the oligomers; however, ion suppression should be evaluated when any partial co-elution is observed, even with a single oligomer.

AUTHOR CONTRIBUTIONS

Sergio Cancho-Gonzalez: Investigation; writing—original draft; methodology; validation; visualization; writing—review and editing; formal analysis; software; data curation. **Paul Ferguson:** Resources; supervision; project administration; writing—review and editing; funding acquisition; conceptualization. **Julie M. Herniman:** Conceptualization; funding acquisition; writing—review and editing; validation; methodology; software; project administration; data curation; supervision; resources; formal analysis. **G. John Langley:** Data curation; supervision; resources; formal analysis; software; project administration; validation; writing—review and editing; conceptualization; funding acquisition.

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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.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.9765.

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