Application Note

Biopharma/Pharma

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Reducing the Complexity of Polysorbate Oxidation By-Product Screening by LC/MS/MS

Using an Agilent AdvanceBio Surfactant Profiling HPLC column

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Abstract

Surfactant oxidation in biopharmaceutical formulations is becoming an increasingly visible and significant concern. It is critical to establish a screening method to begin mitigation strategies when surfactant oxidation is observed. This application note presents a novel LC/MS/MS workflow featuring an Agilent AdvanceBio Surfactant Profiling HPLC column.

Introduction

Surfactants, particularly polysorbates, play a crucial role in stabilizing protein-based therapeutics. Unfortunately, surfactants are prone to oxidation, which can be caused by residual peroxides, exposure to light, or leachables such as transition metals, particularly iron.[1] Polysorbate oxidation can lead to the formation of reactive degradation products, which may compromise the stability of the biotherapeutic, leading to challenges in maintaining the efficacy and safety of biotherapeutics and their formulations.

Having a screening method that can quickly and unambiguously identify oxidative degradation markers in formulation buffer is important.[2–8] Establishing a screening method allows scientists to determine mitigation strategies such as reducing iron contamination and oxygen exposure, and helps to ensure the long-term stability of biopharmaceutical products. This application note provides a workflow for identifying polysorbate oxidation markers with an AdvanceBio Surfactant Profiling HPLC column, an Agilent 6546 LC/Q-TOF system, Agilent MassHunter Qualitative Analysis 12.0 software, and PolyMatch[9] software.

Experimental

Materials and sample preparation

All chemicals were purchased from Sigma-Aldrich, and all mobile phases were LC/MS grade. Polysorbate 80 was diluted to a final w/v of 0.02% in mobile phase A. Polysorbate 80 was incubated with 3% (w/v) hydrogen peroxide overnight in a water bath heated to 40 °C.

Instrumentation

An Agilent 1290 Infinity II UHPLC system was coupled to an Agilent 6546 LC/Q-TOF system, including the following:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)

Software

- Agilent MassHunter software
- PolyMatch[9] software

LC/MS/MS analysis and data processing

LC/MS/MS analysis was performed on a 1290 Infinity II UHPLC coupled to a 6546 LC/Q-TOF system with a dual Agilent Jet Stream source. Data were collected with Agilent MassHunter acquisition (11.00) workstation software, using an Auto MS/MS method. Targeted analysis was processed using MassHunter Qualitative Analysis 12.0 software with the Find by Formula (FBF) algorithm and Personal Compound Database and Library (PCDL) Manager B.8.00.00. Suspect screening analysis was processed with PolyMatch Flow and Visualizer.[9] Note that polyethylene glycol (PEG) and polyoxyethylene (POE) are chemical synonyms. The entries in the PCDL use the term POE, which is more commonly used when describing the components of polysorbate. PolyMatch uses the term PEG, as this software was designed to analyze all polymers. Therefore, both terms are used interchangeably throughout this application note.

Method conditions

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Surfactant Profiling 300 Å, 3.5 µm, 2.1 × 100 mm (part number 861775-907) with guard (part number 821126-927)
Mobile Phase A	10 mM Ammonium acetate
Mobile Phase B	Methanol
Flow Rate	0.3 mL/min
Injection Volume	2 µL
Column Temperature	50 °C
LC Gradient	Time (min) %B 0 to 1 5 1 to 10 5 to 91 10 to 14 91 14 to 14.1 91 to 5 14.1 to 15 5
Post Time	3 min
Needle Wash	20:80 methanol:water, 10 s

Table 2. Agilent 6546 LC/Q-TOF parameters.

Parameter	Value
Source	Dual Agilent Jet Stream
Gas Temperature	200 °C
Gas Flow	12 L/min
Nebulizer	30 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Voltage Capillary (VCap)	3,500 V
Nozzle	0 V
Fragmentor	145 V
Reference Mass	121.0509, 922.0098
Acquisition Rates (MS; MS/MS)	5 and 3 spec/sec
MS and MS/MS Ranges	*m/z* 50 to 3,000
Max Precursors Per Cycle	5
Collision Energies	10, 20, 40 V

Results and discussion

Polysorbate oxidation is a complex process, partially due to the heterogeneous structure of polysorbates. Polysorbates consist of four different chains of POE groups, with 20 POE moieties divided among the four chains. In addition, each chain can terminate in a fatty acid. Oxidation can occur on the unsaturated sites of the free fatty acids and at any of the 20 POE moieties. To add to the complexity, oxidation is radically induced and can continue to propagate, resulting in numerous potential degradation products. High-resolution accurate mass liquid chromatography/tandem mass spectrometry (LC/MS/MS) is often the method of choice for unambiguous identification of this complicated problem. Two approaches are described in this application note to maximize the number of oxidation by-product identifications.

Data acquisition

Due to the complexity of polysorbate oxidation, LC/MS/MS runs tend to be lengthy. The AdvanceBio Surfactant Profiling column was designed to provide high-resolution separations of polysorbate components with shorter run times. A 100 mm length column was selected to provide higher resolution, using an 18-minute method. To ensure that the data were suitable for both a targeted and suspect screening approach, data were acquired with Auto MS/MS, a data-dependent method where the top five peaks per spectrum are targeted for MS/MS with active exclusion applied. Because the control sample was not freshly purchased and had some exposure to light, there is a possibility that some oxidation occurred. The oxidized sample, which was exposed to artificially oxidized conditions, is expected to have more oxidation. In Figure 1, the total ion chromatograms show high reproducibility between replicates. In the oxidized chromatograms specifically, a reduction in signal where the mono-, di-, and triesters elute as well as a slight signal increase earlier in the chromatogram indicates some degradation.

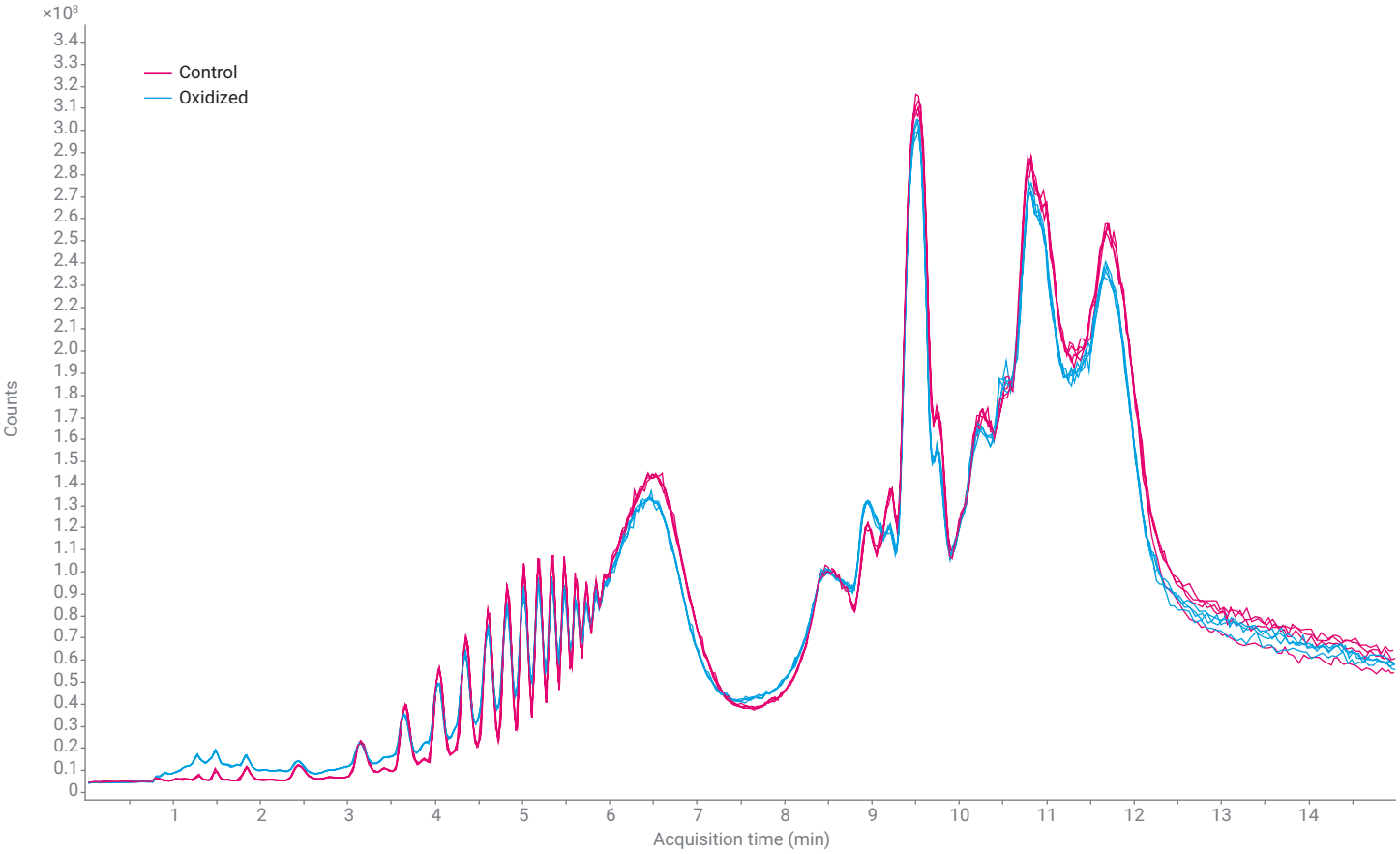

Figure 1. A comparison of total ion chromatograms between the control and oxidized samples indicates that some degradation has occurred.

Targeted analysis

As previously mentioned, both polysorbate heterogeneity and the radical-initiated oxidation process result in a complicated scenario, with hundreds and possibly thousands of potential degradation products. A targeted approach can be used if known degradants have previously been observed. An increasing amount of literature has been published on known polysorbate degradation products, and the data from these articles can be incorporated into a targeted approach. A recent publication includes over 4,800 potential degradation products and provides a diagnostic MS/MS ion for each product.[2] Based on this list, a PCDL was compiled and incorporated into the FBF algorithm in the Qualitative Analysis 12.0 software. The FBF algorithm scores compounds based on mass accuracy, isotopic abundance, and spacing. Additionally, this algorithm has a feature capable of assigning chemical formulas to MS/MS ions.

Figures 2 and 3 show an example of one identified compound, POE1 monosuberate. Four replicates were collected for each of the control and oxidized samples. The control shows a small amount of this compound, verified by the FBF algorithm. However, the abundance in the control is not high enough to trigger MS/MS. A low abundance of an oxidized peak in the control sample is expected. Because the retention time of the control matches that of the oxidized sample, and the FBF score is 82.5 out of 100, the compound can be identified with high confidence. As this is a low-abundance compound, the isotopic fidelity is slightly decreased, explaining why the FBF score is not in the 90s. Compared to the control sample, the oxidized sample shows an order of magnitude increase in the abundance of POE1 monosuberate, with a much higher FBF score of 99.5. The diagnostic MS/MS ion from the referenced publication[2] is present, along with additional annotated MS/MS peaks.

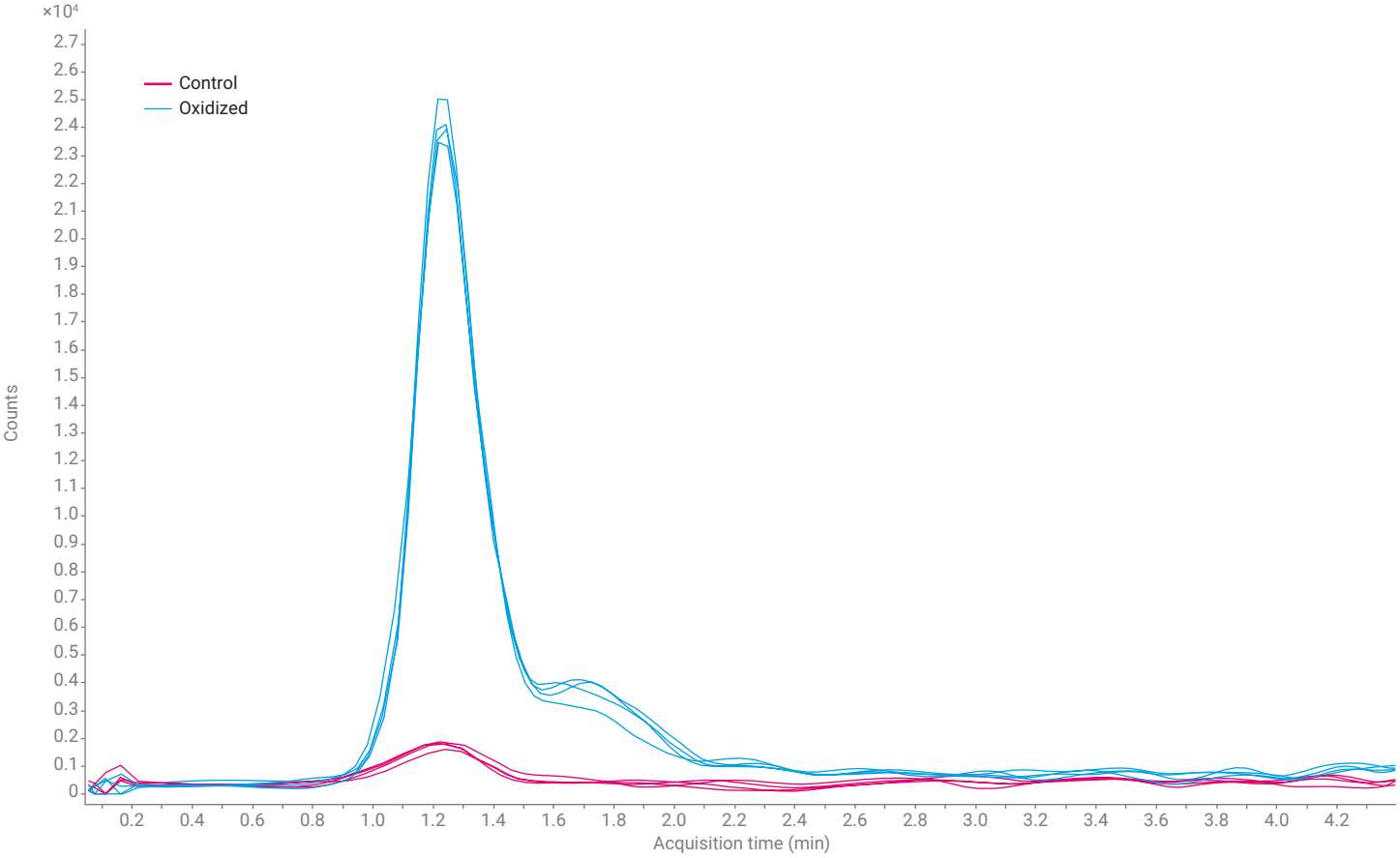

Figure 2. Extracted ion chromatograms of POE1 monosuberate for the control and oxidized samples. Replicates show high peak area precision, 13.4 and 1.9%, respectively.

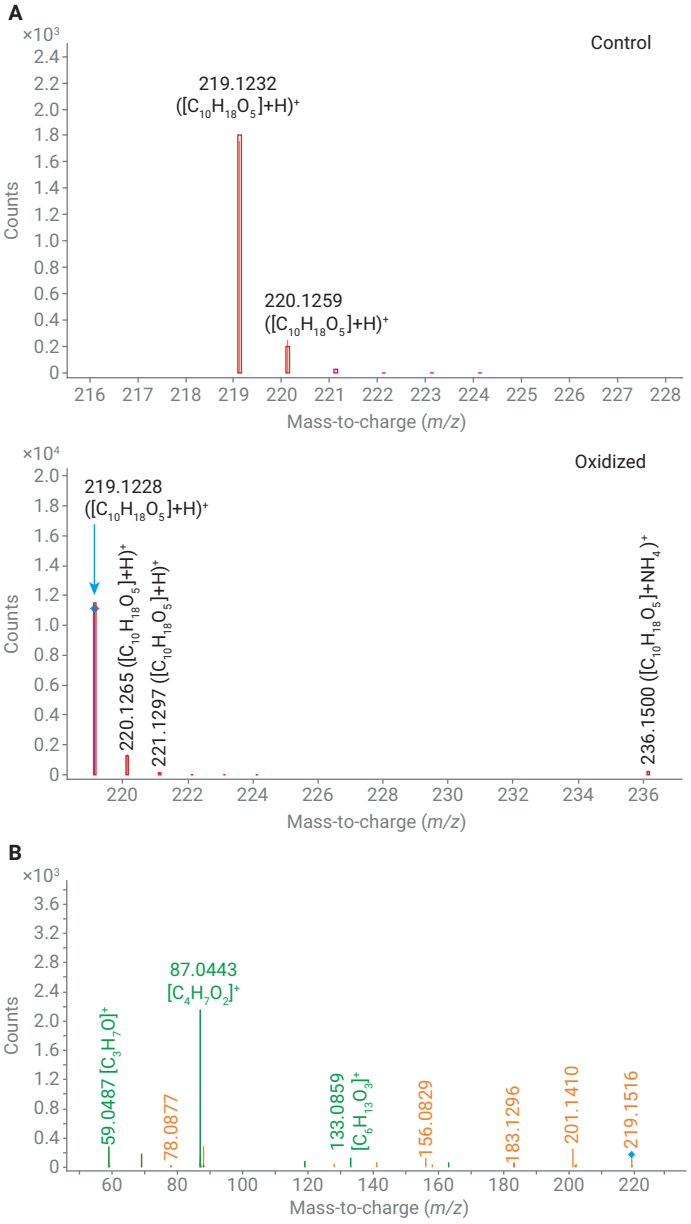

Figure 3. (A) Spectra results for MS1 only, annotated by the FBF algorithm for POE1 monosuberate. The oxidized sample also shows evidence of the compound with an ammonium adduct. Only the oxidized sample had enough abundance of this compound to trigger MS/MS (B).

A second example of identified compounds is a series of homologous species, POE11 to POE15 monohydroperoxy oleates, each differing by a single POE group. FBF scores for this series are all very high, ranging from 96.3 to 99.7, with an average of 98.8. Mass errors for all eight replicates of all five compounds are under 1 ppm, as shown in Figure 4. While some hydroperoxy oleate species are found in the control sample, there is a consistent increase in hydroperoxy oleate species in the oxidized sample. Peak area reproducibility for all replicates is maintained, ranging between 1.9 and 5.9% RSD. Figure 5 shows an example of the POE13 monohydroperoxy oleate in one of the oxidized samples. There are several advantages to using a targeted approach. Setting up a PCDL is straightforward, provided a name and molecular formula are available. The FBF algorithm processes quickly, and visualizing differences between samples is clear. However, targets excluded from the library will not be identified. Alternative data analysis methods offer complementary benefits.

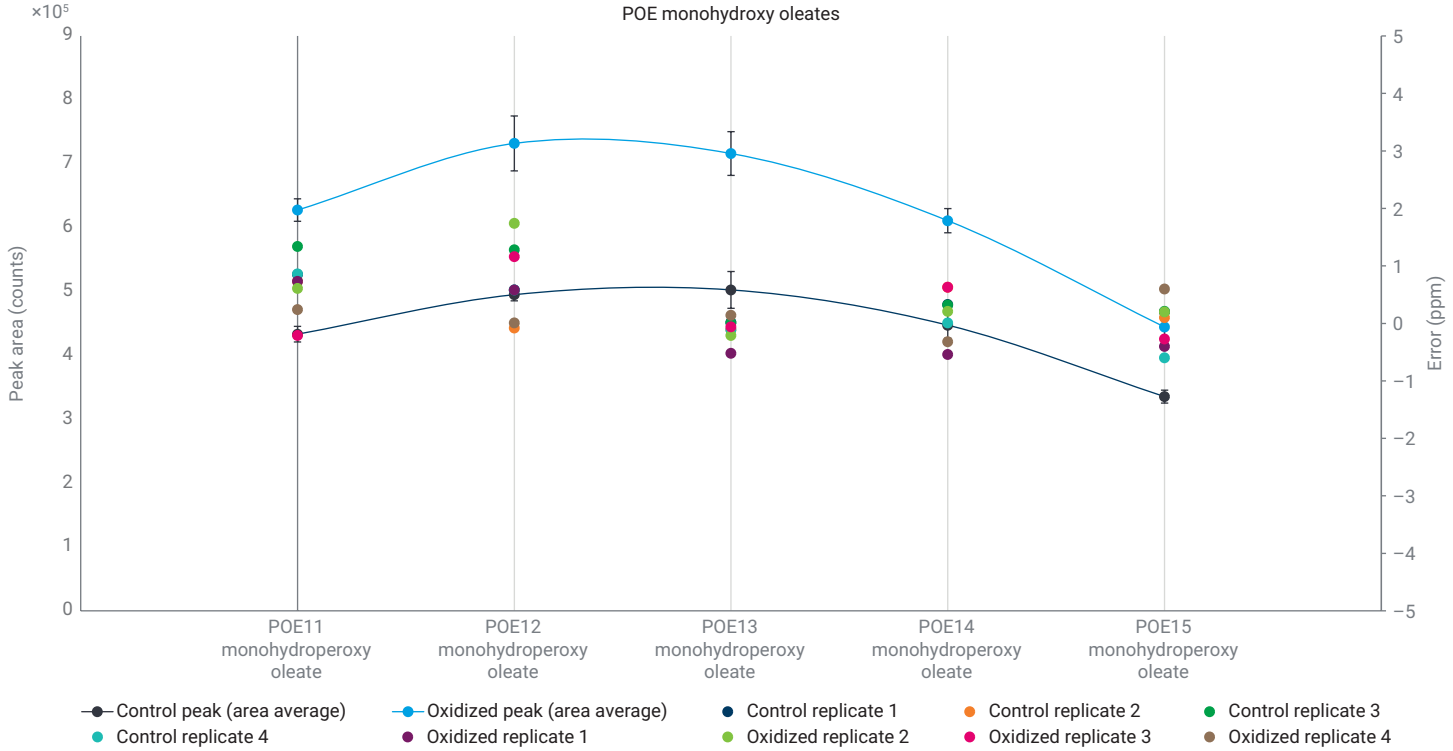

Figure 4. A summary of the POE(n) monohydroperoxy oleate series. The degradant products are increased in the oxidized sample and show excellent reproducibility. All replicates have a high FBF score, ranging from 96.3 to 99.7, influenced by mass errors of less than 1 ppm.

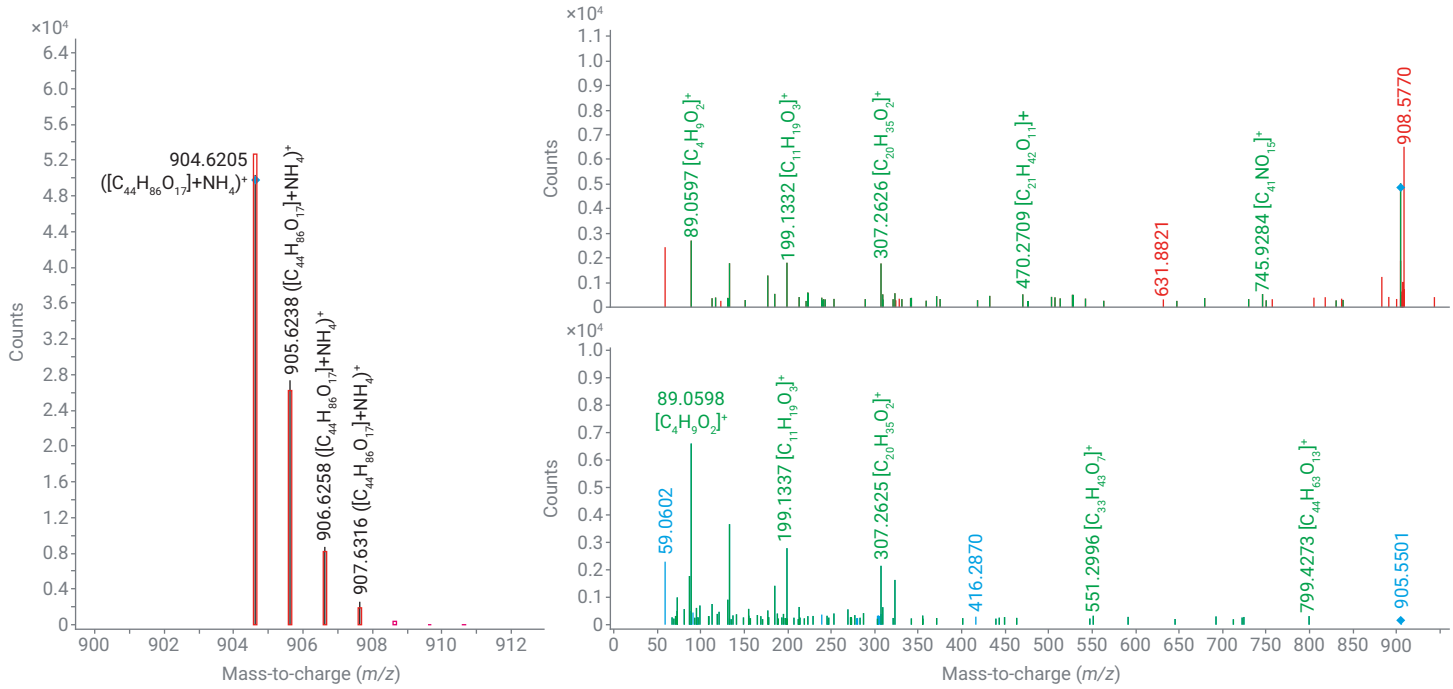

Figure 5. An example of the POE13 monohydroperoxy oleate in one of the oxidized samples, which has an FBF score of 98.4. The diagnostic MS/MS ion, 307.2632, is present in two of the three MS/MS spectra, with mass accuracies of −2.3 ppm or less. Another MS/MS ion, 323.2572, is present in the bottom spectrum and is indicative of oxidation.

Suspect screening analysis

While a targeted approach is an excellent starting point for analyzing polysorbate oxidation, an untargeted approach that considers several features, including retention time, tandem mass spectral data, and mass defect, provides greater confidence and can identify new degradation products. PolyMatch integrates a wide range of evidence to classify polymers, including mass defect, retention time, and exact mass, which can be used alongside homologous series to compile groups of chemicals that likely belong to the same class. MS/MS evidence can provide structural information pertaining to class- or species-level assignments.[9]

PolyMatch can currently be used for the detection of unknown polymers, including oxidized species, through fragment screening, homologous series detection, and a "generalized" library. Homologous series detection with fragment screening for PEG-related fragments will pull out all respective polymers with pegylated units, regardless of whether they are contained in the PolyMatch rule-based libraries containing known polymer structures. Furthermore, the "generalized" library will also capture oxidized polymers (Figure 6). This library contains polymers characterized by double bond equivalents, the addition of various functional groups and adducts, the size of pegylated units, and the presence of CH_2 groups. Figure 7 shows examples of PEGs and their features in the PolyMatch library, including illustrations of some of the structures identified in Figure 10. Although the PolyMatch library does not yet include specific oxidized polysorbate species and their fragmentation patterns, this feature will be added in the future with an expanded library. The software program includes automatic peak picking, blank filtering, annotation, and visualization.[9]

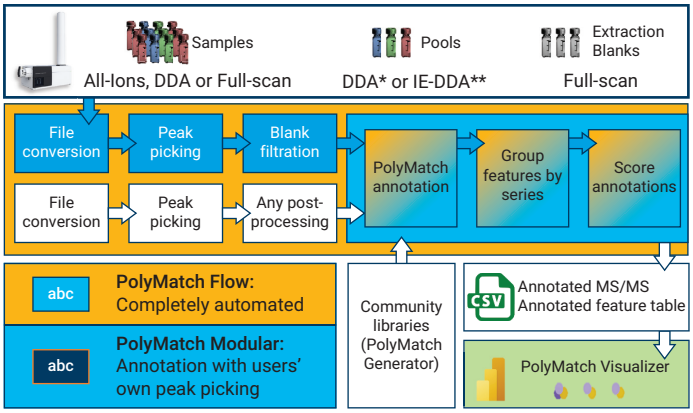

Figure 6. An overview of the PolyMatch workflow. The fully automated PolyMatch Flow interface was used in this experiment.

Simulated PEGs: 64,450 species

Example	Definition	Built-In Range
n = 3	Number of PEG units	3 to 44
NH_4^+	Adduct	NH_4^+, Na^+, H^+
CH_2 = 6	Number of carbons (non-PEG)	0 to 19
DBE = 4	Double bond equivalents	0 to 6
(4-OH)	Functional groups	OH (0 to 4), NH_3 (0 to 2), =O (0 to 2)

Structures and naming conventions:

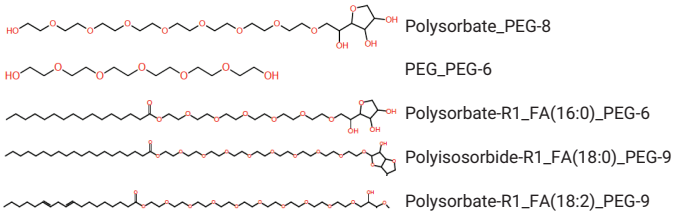

Figure 7. The PolyMatch library contains structures and functional groups such as PEGs/POEs. The structures shown were generated from Simplified Molecular Input Line Entry System (SMILES) files produced by PolyMatch, then drawn from https://www.cheminfo.org.

The use of mass defect is particularly powerful because it leverages the repeating POE groups in polysorbates. PolyMatch can confirm the degradation products' series and offers a clear visual representation, making it easy to exclude false positives. Figure 8A shows a series of polysorbate components and their repeating POE/PEG units, identified as Polysorbate-R1_FA(16:0)-PEG(n). The naming convention from PolyMatch differs from the PCDL. Therefore, the Polysorbate-R1_FA(16:0)-PEG(n) is the same structure and series as the POE(n) monohydroperoxy oleate referenced in Figures 4 and 5.

The advantage of the AdvanceBio Surfactant Profiling column coupled with the PolyMatch software is highlighted by Figure 8B. While Figure 8A shows a single series of related polysorbate components, Figure 8B reveals a set of isomers that have been chromatographically separated within the mass defect series. The selectivity of the LC column separates the isomers without requiring a lengthy method. Figure 9 shows an example MS/MS spectrum from each set of isomers. The top spectrum is from the set that eluted later, and the fragments are mostly annotated. The bottom spectrum is from the set that eluted earlier and has some of the same annotations. However, this bottom spectrum differs greatly from the top as it includes a highly abundant not-yet annotated peak of *m/z* 323.2576. The literature indicates that this MS/MS ion is a keto oleic acid, which provides evidence of polysorbate oxidation. This is an example of an ion that will be added to the PolyMatch library.[4-6]

This study focused largely on one set of polysorbate components, but many other components were detected. PolyMatch uses a scoring system with letters A to E. Compounds with an A score are considered very confident identifications, based on exact mass and a class-specific fragmentation pattern. There were 261 compounds with an A score. Figure 10 shows the mass defect and corresponding retention time versus *m/z* plots for these high-scoring compounds. Because polysorbate oxidation is a complex and radical process, powerful software tools are essential for determining the extent of oxidation. Additionally, using an AdvanceBio Surfactant Profiling column, which expedites the data acquisition process, is highly beneficial.

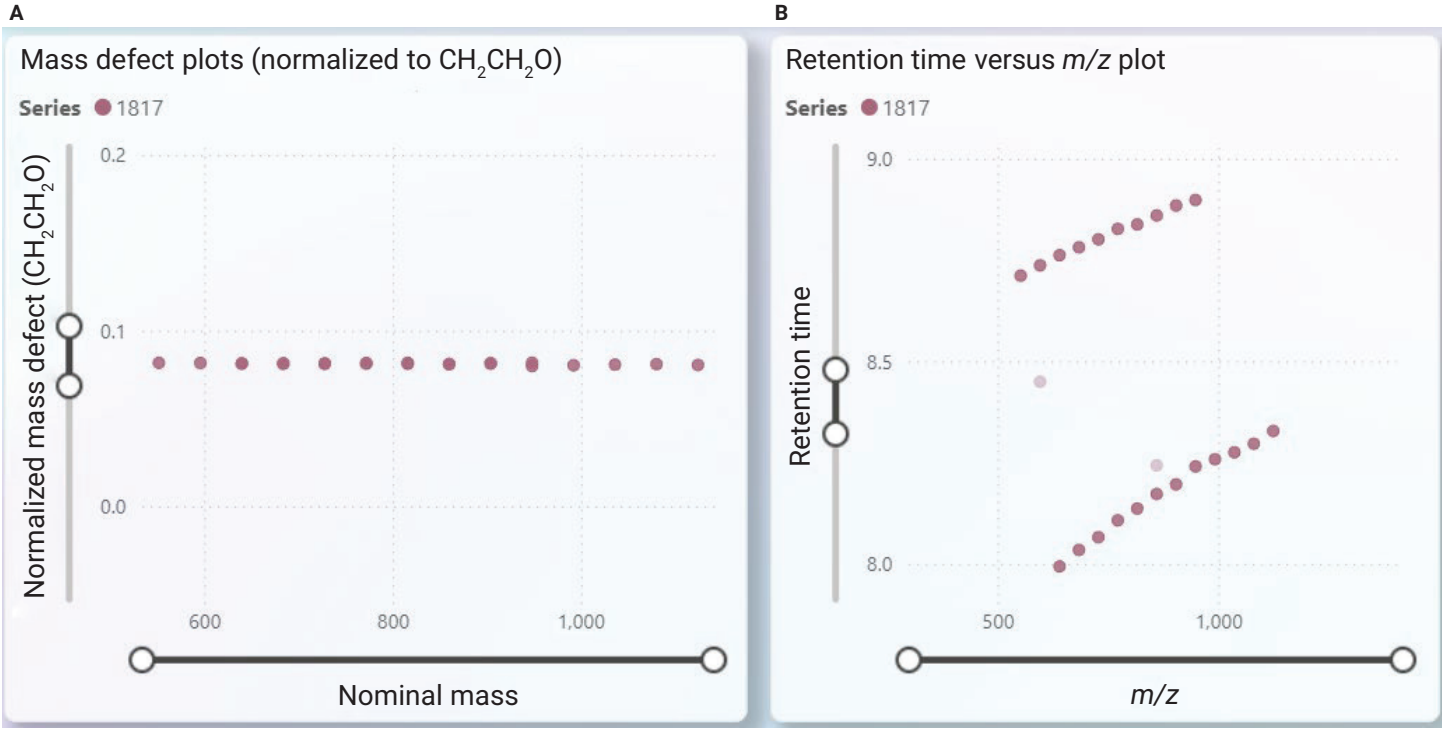

Figure 8. The Kendrick mass plot (A), normalized to CH_2CH_2O, shows a series of polysorbate components identified as Polysorbate-R1_FA(16:0)-PEG(5–18). The retention time versus *m/z* plot (B) shows a set of isomers within the mass defect series that have been chromatographically separated.

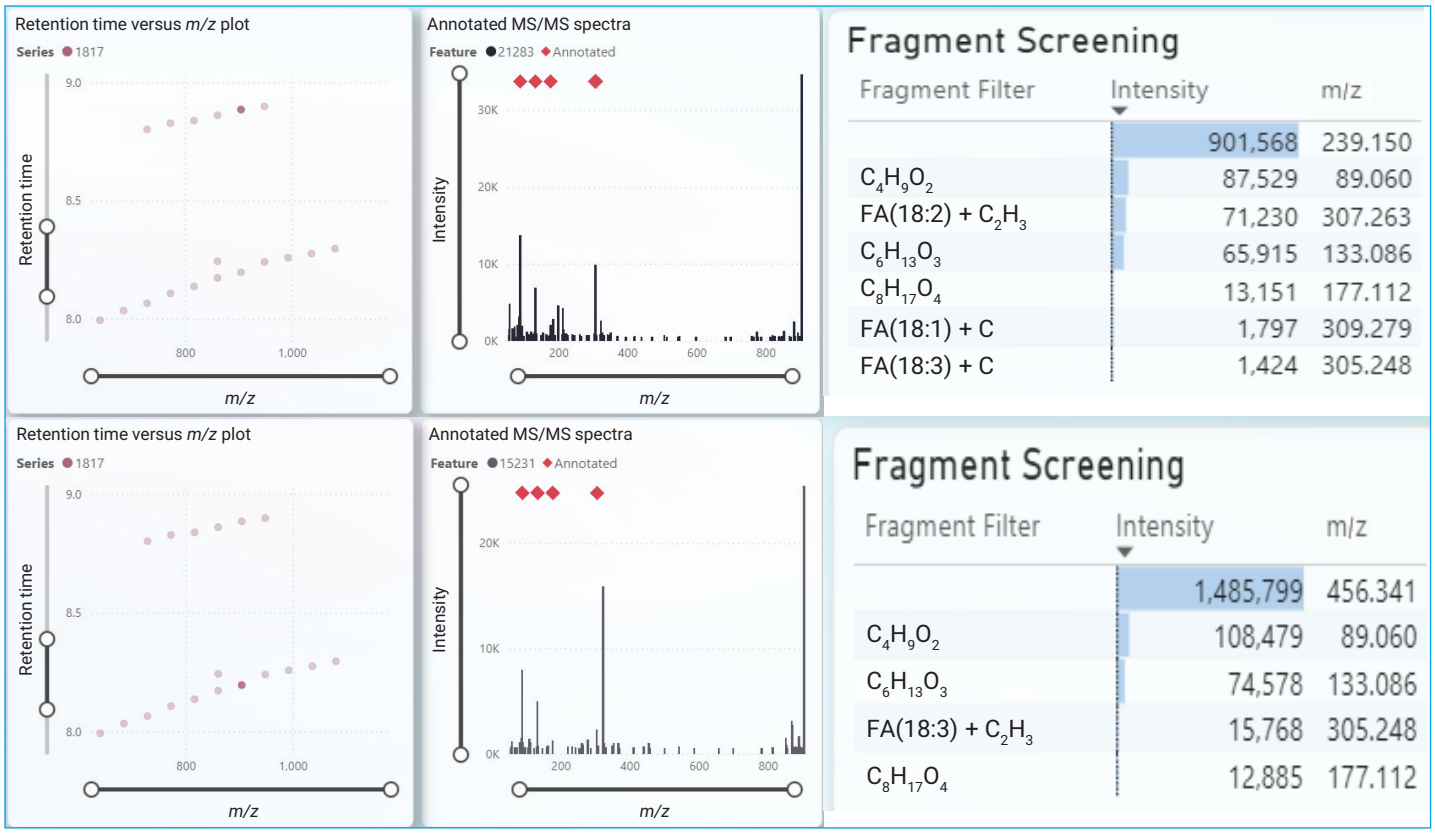

Figure 9. Examples of MS/MS spectra and annotated fragments from each set of isomers. MS/MS confirms that the bottom compound is oxidized with the diagnostic ion, *m/z* 323.2576.

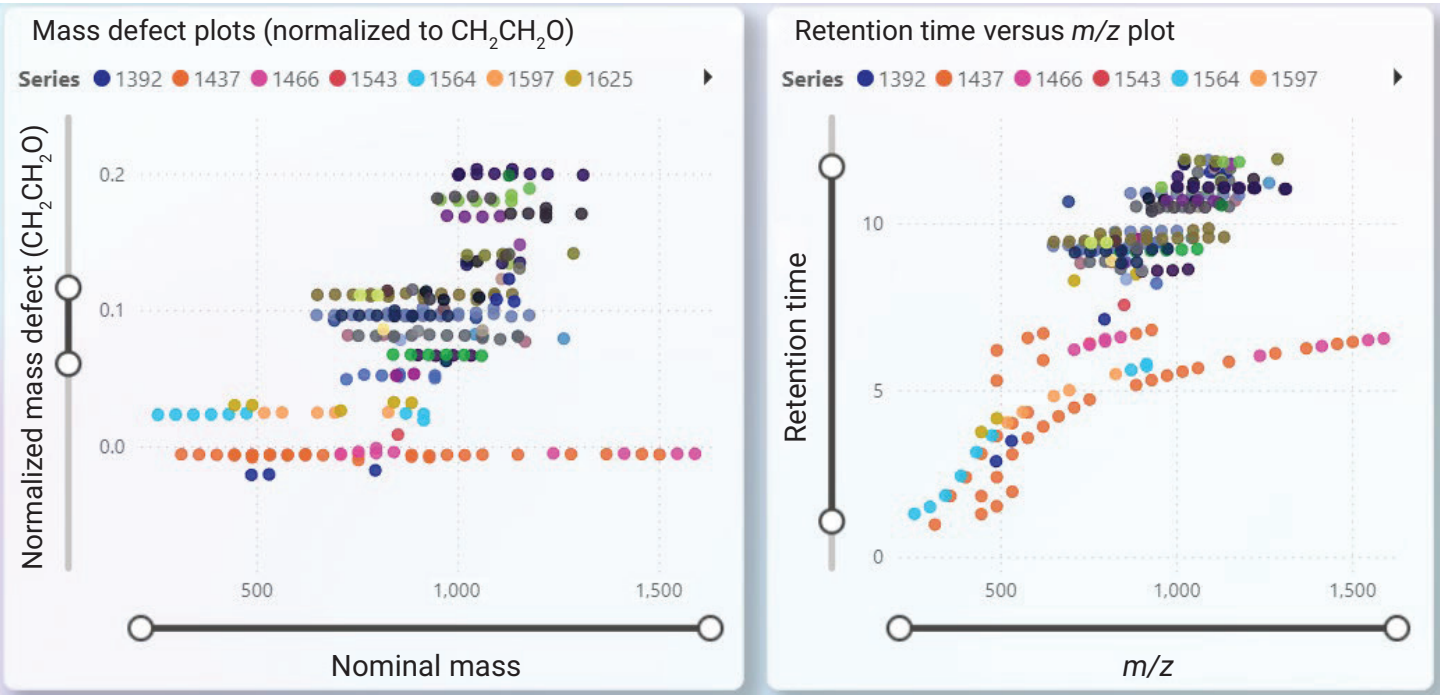

Figure 10. The mass defect plot and retention time versus *m/z* plot for all high-scoring compounds.

Conclusion

An LC/MS/MS workflow for identifying polysorbate oxidation by-products has been developed as a screening tool for labs monitoring biotherapeutic product and formulation stability. The Agilent AdvanceBio Surfactant Profiling column provides high-resolution separation of polysorbate components with a short run time. The Agilent 6546 LC/Q-TOF system produces high-resolution and accurate mass data, with exceptional isotopic fidelity for identifying degradation products. The Agilent MassHunter Qualitative 12.0 Find by Formula (FBF) algorithm can be used for a fully targeted approach, while PolyMatch software is best for a more untargeted, suspect screening approach.

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