Analysis of Polysorbate 80 and its Related Compounds by RP-HPLC with ELSD and MS Detection

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The chemical composition of polysorbate 80 strongly influences the physicochemical properties and performance of many products. Consequently, a reliable characterization of polysorbate 80 is crucial for many applications. However, the exact composition of these chemical mixtures cannot be determined by colorimetry, hydrolysis, size-exclusion chromatography, nuclear magnetic resonance or mass spectrometry (MS). Meanwhile, due to the strong retention of higher esters on the reversed-phase (RP) column, the published high-performance liquid chromatography (HPLC) methods suffered from inadequate elution. In the present paper, an HPLC–evaporative light scattering detection (ELSD) and an HPLC–electrospray ionization (ESI)-MS method were developed and validated for the separation and identification of the chemical composition of polysorbate 80. A full separation of the entire composition was achieved in 45 min. In the HPLC–ESI-MS spectra, each class of the compound in polysorbate 80 was directly confirmed and identified by $[M + NH_4]^+$ and $[M + 2NH_4]^{2+}$ ions. The number of polyoxyethylene groups and their distribution within the molecule were determined, in addition to the dehydration and esterification degree of sorbitol. Analysis showed that polysorbate 80 contained different proportions of components (polyoxyethylene sorbitan, polyoxyethylene isosorbide, polyoxyethylene sorbitan monooleate-dioesters-trioleates-tetraoleates and polyoxyethylene isosorbide monoester-dioesters). It was concluded that HPLC–ESI-MS is a useful tool for establishing the compositional profile of polysorbate 80.

Introduction

Polysorbate 80 (Tween 80) has been widely used in the preparations of drugs, cosmetics, skin care products and food as a solubilizer, emulsifier and stabilizer. Polysorbate 80 has been shown to have a typical structure (Figure 1) that contains approximately 20 groups of polyoxyethylene (POE) per molecule (1). However, the actual synthetic process not only yields the desired monoesters, but also some by-products.

Figure 2 shows the process of synthesizing polysorbate 80. In this process, sorbitol was first dehydrated to yield isomers of sorbitol monoanhydrides (sorbitans) and/or sorbitol dianhydrides (isosorbides) in the presence of a catalyst. Then the mixture of sorbitans and isosorbides was esterificated with oleic acid, followed by polymerization with ethylene oxide to yield polysorbate 80. The synthetic process implies that polysorbate 80, primarily composed of polyoxyethylene sorbitan monooleate (PSM), may contain other intermediates such as the polyoxyethylene isosorbide monooleate (PIM) series, polyoxyethylene sorbitan trioleates (PSTri) series, polyoxyethylene sorbitan tetraoleates (PSTetra) series, polyoxyethylene sorbitan

dioleates (PSD) series, polyoxyethylene isosorbide dioleates (PID) series, polyoxyethylene sorbitan (PS) series and polyoxyethylene isosorbide (PI) series. All possible polyethoxylated intermediates listed previously are shown in Figure 2.

The physicochemical behaviors of polysorbate vary markedly from batch to batch due to the inconsistency of the synthetic processes (2). However, it is very important to know the precise composition of polysorbate formulations. The surfactant's physicochemical behavior of polysorbate 80, such as the hydrophilic lipophilic balance (HLB) or the critical micell concentration (CMC) value, is dependent upon its chemical composition, governed by the structure of the sorbitol derivative core (i.e., sorbitan and isosorbide), the degree of esterification, the number of POE groups and their distribution within the molecule. Therefore, the development of an analytical method for the determination of the total chemical composition in polysorbate 80 would be a great help for quality control of the synthetic process and the finished product.

As macromolecular compounds, the polysorbates have a heterogeneous molecular structure; hence, analysis of their chemical composition poses many challenges to the analyst. Several methods for the quantification of the nonionic surfactants have been described in the literature. Greff *et al.* presented the colorimetric method based on the formation of a blue complex between ammonium cobaltothiocyanate and a polyethoxylated compound (3). However, the disadvantage of this method was that no distinction could be made between intact polysorbate and degraded polysorbate (4). Another technique was based on the quantification of the fatty acid content formed in the hydrolysis of polysorbates (5–7). This method, however, suffered from lack of selectivity, and could only demonstrate the average degree of esterification and not differentiate the internal chemical composition of polysorbates. Several direct methods for the analysis of polysorbate 80 have been developed by using high-performance liquid chromatography (HPLC) with different detection, such as evaporative light scattering detection (ELSD) (8–11), mass spectrometry (MS) (12), charged aerosol detection (CAD) (13) and condensation nucleation light scattering detection (CNLSD) (14). Other researchers, unaware of the possible existence of chemical compositions other than the classical structure, have used chromatographic methods that do not allow elution of the entire composition. The polysorbate is actually a mixture of polyoxyethylene sorbitan monoester and other intermediates that can be chromatographically separated. Wuelfing *et al.* presented an HPLC–ultraviolet (UV) method to detect polysorbate 80 (15), but UV absorption of the polysorbate was too weak to

detect polysorbate 80. The contribution to the response of the chromatographic peaks can primarily be attributed to its oxidative degradation or its polyunsaturated fatty acid impurities. Proton nuclear magnetic resonance (1H NMR) enables a rapid analysis of the overall polysorbate composition. However, both degraded and non-degraded polysorbate 20 samples displayed similar proton peak responses in the 1H NMR spectrum (4),

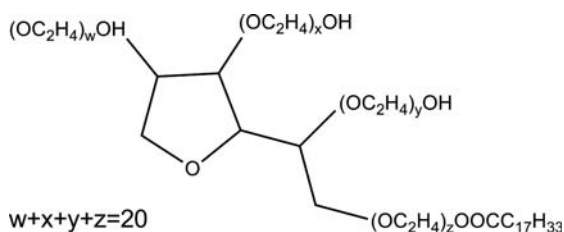which led to a conclusion that a simple 1H NMR experiment does not allow for the determination of the degree of degradation. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF-MS) could provide a means of elucidating the complex molecular distribution in polysorbate formulations, but polysorbate 80 is not suitable to be analyzed by MS because the PS, PSM, PSD, PSTri and PSTetra oligomers have the same molecular weights (16–18).

The analysis of such complex samples is a difficult task. The combination of the high separation capabilities of chromatography and the power of MS as an identification and confirmation method appear to offer a practical solution. ELSD can be utilized to optimize the separation condition and then LC–MS can be used to separate and identify each peak under the optimal chromatographic conditions. In the present paper, we attempt to develop an HPLC-ELSD-MS method for simultaneous identification of the chemical composition of polysorbate 80.
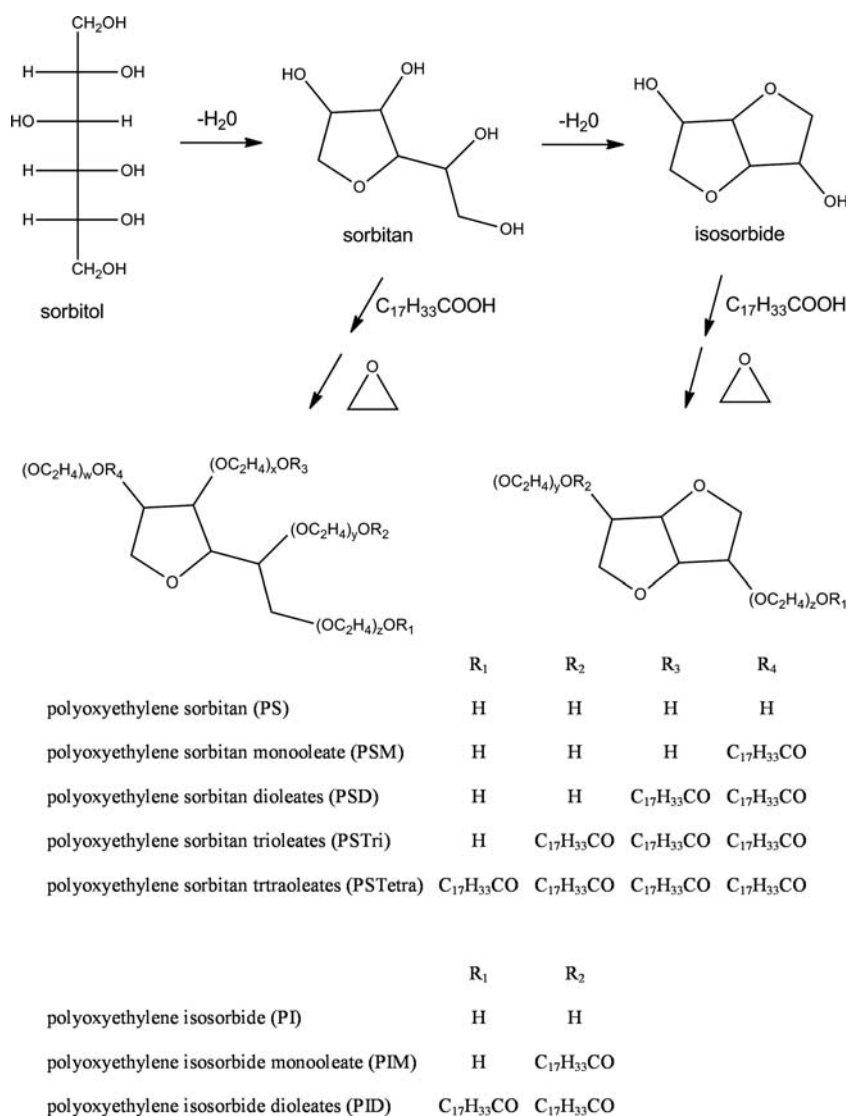

Figure 1. Typical structure of polysorbate 80.

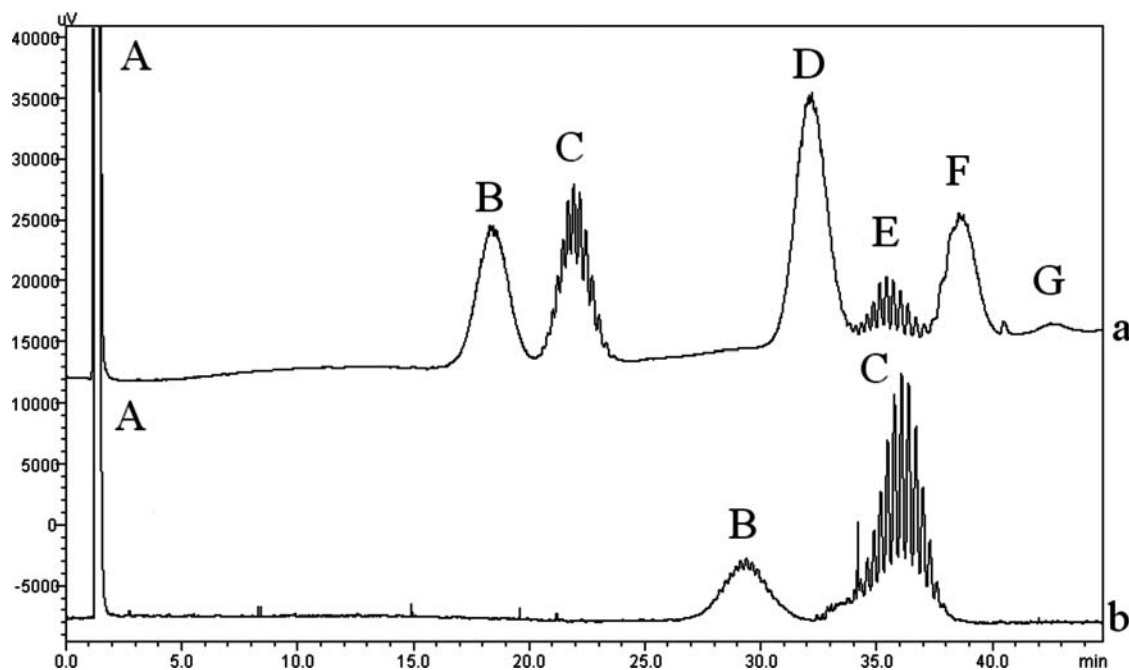**Figure 2.** Polysorbate 80 synthetic routes and possible products.

Figure 3. Chromatogram obtained from polysorbate 80 sample (Well, batch number 20080412): mobile phase: 50% CH$_3$CN–water (mobile phase A)–THF (mobile phase B); gradient elution (0–80% B in 40 min) (A); mobile phase: 50% CH$_3$CN–water (mobile phase A)–CH$_3$CN (mobile phase B) gradient elution (0–100% B, in 40 min) (B).

Experimental

Chemicals and reagents

Different batches of polysorbate 80 were purchased from Well (Nanjing, China), standard grade (oleic acid purity >60%, by fatty acids composition analysis), batch number 20070408, 20091018, 20100516; high purity grade (oleic acid purity >98%, by fatty acids composition analysis), batch number 20080412, 20080418, 20101019. NOF (Tokyo, Japan), high purity grade, batch number 807367D, CRODA (Rancho Cucamonga, CA); standard grade, batch number 0000285529. HPLC-grade acetonitrile (CH$_3$CN) and tetrahydrofuran (THF) were purchased from Sigma (St. Louis, MO), and Merck (Darmstadt, Germany), respectively. Water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA). All other chemicals were of analytical grade and purchased from Nanjing Reagent Company (Jiangsu, China).

LC–ELSD

Polysorbate 80 samples were analyzed using the LC–ELSD technique as follows. A Prominence LC-20A series HPLC system (Shimadzu, Tokyo, Japan) was coupled to an ELSD 2000 ES (Alltech, Lincolnshire, UK). The column used was an Eclipse XDB C18 column (150 × 4.6 mm, 3.5 μm, 100 Å, Agilent, Wokingham, UK). Ten microliters of sample (approximately 1 mg/mL in acetonitrile) was injected, using the following mobile phase program: flow rate 1 mL/min at 20°C; mobile phase A consisted of CH$_3$CN–water (50:50, v/v ratio), and mobile phase B with THF. The gradient program started with 0% B eluent and a 40 min gradient duration was applied up to 80% B. The column was then equilibrated with starting conditions for 10 min before the next injection. The ELSD was operated in

the impactor "off" mode, the drift tube temperature was set at 95°C and nitrogen flow was maintained at 2.5 L/min.

LC–MS

Polysorbate 80 samples were analyzed using the LC–MS technique as follows. An Agilent 1100 HPLC was fitted with an Eclipse XDB C18 column (150 × 4.6 mm, 3.5 μm, 100 Å, Agilent). Two microliters of sample (approximately 100 μg/mL in water) was injected, using the following mobile phase program: flow rate 1 mL/min at 20°C; mobile phase A consisted of CH$_3$CN–water–ammonium formate (50:50:0.1, v/v ratio), and mobile phase B with THF. The gradient program started with 0% B eluent, and a 40 min gradient duration was applied up to 80% B. The column was then equilibrated with starting conditions for 10 min before the next injection. Applying a splitter built in after the column, the mobile phase flow rate was split (in 1:4 ratio) toward an Agilent Ion Trap MSD instrument (20% flow), which was operated in electrospray ionization (ESI) positive mode with the ion source temperature set at 300°C, the drying and nebulizer gas at a flow rate of 8 L/min and the needle voltage at 4.5 kV, and scanning was performed between 100 and 2,200 amu.

Results and Discussion

LC–ELSD analysis

Fig 3A shows the chromatogram of polysorbate 80 supplied by Well. The results indicate that polysorbate 80 is composed of approximately seven different species (labeled A–G).

In previously published papers, a methanol–water or aceto-nitrile–water system was often used for the separation of polysorbate 80 (8, 12, 13). However, our preliminary studies

showed that the solvent strength of such systems was not sufficient for complete elution of all components of polysorbate 80. The chromatographic conditions for Figure 3B were the same as in Figure 3A, described previously, except that the mobile phase B was acetonitrile, indicating that the acetonitrile–water system can only elute the components with lower hydrophobicity than peak C. The chemical components with a higher degree of esterification show strong retention on the reversed column, and are often unnoticed due to the inadequate eluant strength. In the present study, we optimized the mobile phase condition to elute all components of polysorbate 80 in the sequence of hydrophobicity. The advantage of the proposed method lies in its simplicity, because it uses only common equipment found in most laboratories and therefore has a wide applicability.

Polysorbate 80 sample (Well, batch number 20080412) was analyzed with the established separation method to validate the method. The injection precision in both the RPLC separations was evaluated by six successive analyses of the same sample solution, and its repeatability was evaluated with six independently prepared sample solutions. The analysis of the same sample solution at different times (0, 1, 2, 4, 5, 8, 12, 15, 18 and 24 h) was used to evaluate the stability of sample solutions within 24 h. The relative standard deviations (RSD) of the retention times and peak areas of typical peaks were used to reflect the precision, repeatability and sample stability of the methods.

In the chromatograms, seven peaks were used to validate the method (Figure 3A). The injection precision, represented by the RSD, was 0.15–1.49% ($n = 6$) for the retention times and 1.23–6.28% ($n = 6$) for the peak areas. The repeatability (RSD) was 0.15–0.76% ($n = 6$) for the retention times and 2.03–6.87% ($n = 6$) for the peak areas. Stability was evaluated from 0 to 24 h. The stability (RSD) was 0.21–1.12% ($n = 10$) for the retention times and 0.98–8.30% ($n = 10$) for the peak areas, which confirmed that sample solution was stable for 24 h (Table I).

The absence of standards to determine response factors hindered absolute quantitative analysis of polysorbate 80 batches. However, the method can be used to qualitatively compare different polysorbate 80 batches by comparing the relative peak area proportions of the primary species and the purity analysis of polysorbate 80. The HPLC–ELSD chromatograms of eight batches of standard or high purity grade polysorbate 80 supplied by three different vendors and the matrix (ACN) are shown in Figure 4. shown in the chromatogram, the batches contained quite different proportions of components.

Table I

Precision, Repeatability and Stability of the LC–ELSD Method

Peak number	RSD of retention time (%)			RSD of peak area (%)		
	Precision ($n = 6$)	Repeatability ($n = 6$)	Stability ($n = 10$)	Precision ($n = 6$)	Repeatability ($n = 6$)	Stability ($n = 10$)
A	0.33	0.40	0.51	1.46	2.03	1.17
B	0.75	0.51	0.69	1.32	3.83	1.09
C	0.29	0.46	0.31	1.23	3.75	0.98
D	0.15	0.18	0.21	1.52	3.32	1.92
E	0.42	0.62	0.39	6.28	5.48	8.30
F	0.19	0.15	0.23	2.85	5.00	2.63
G	1.49	0.76	1.12	5.63	6.87	5.03
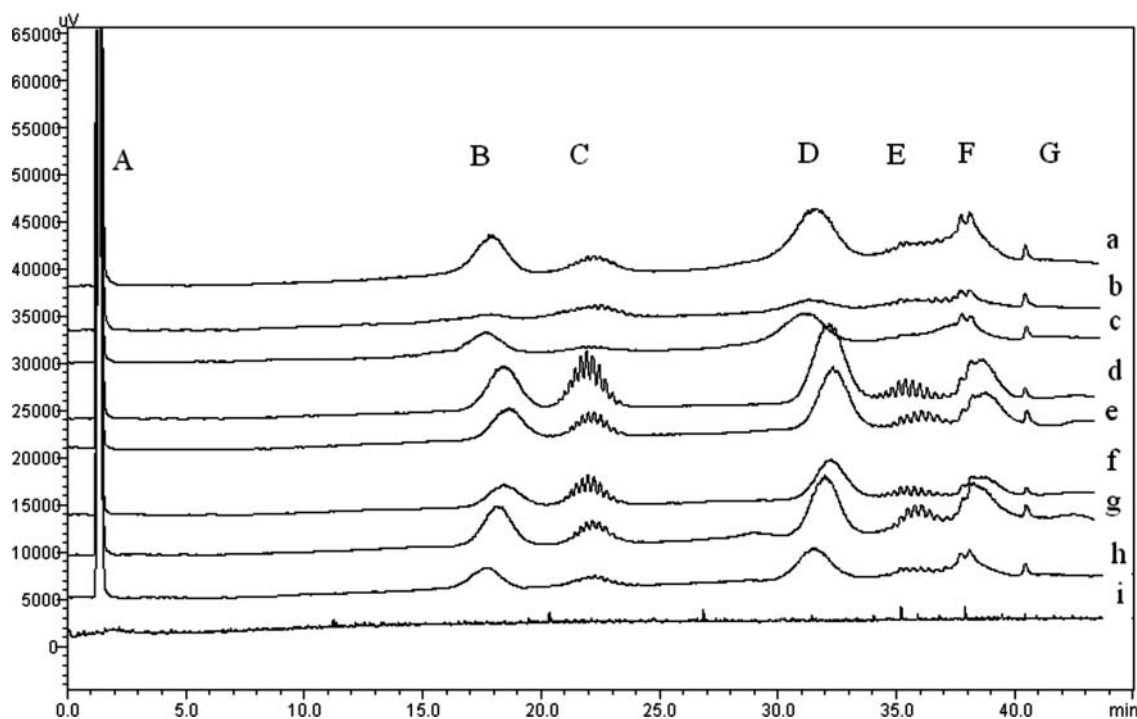
Figure 4. LC–ELSD chromatograms of polysorbate samples of standard grade, batch number 20070408 from Well (A); standard grade, batch number 20091018 from Well (B); standard grade, batch number 20100516 from Well (C); high purity grade, batch number 20080412 from Well (D); high purity grade, batch number 20080418 from Well (E); high purity grade, batch number 20101019 (F); high purity grade, batch number 807367D from NOF (G); standard grade, batch number 0000285529 from CRODA (H); ACN (I).
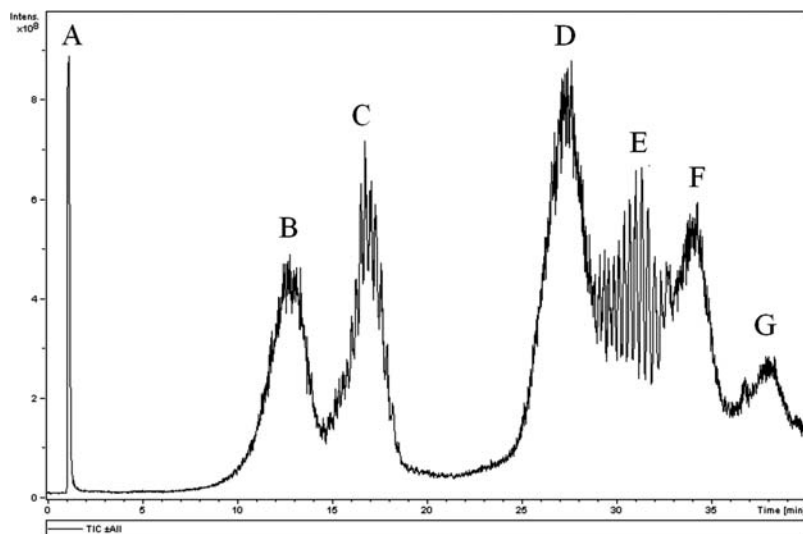
Figure 5. TIC traces obtained from polysorbate 80 sample (Well, batch number 20080412).
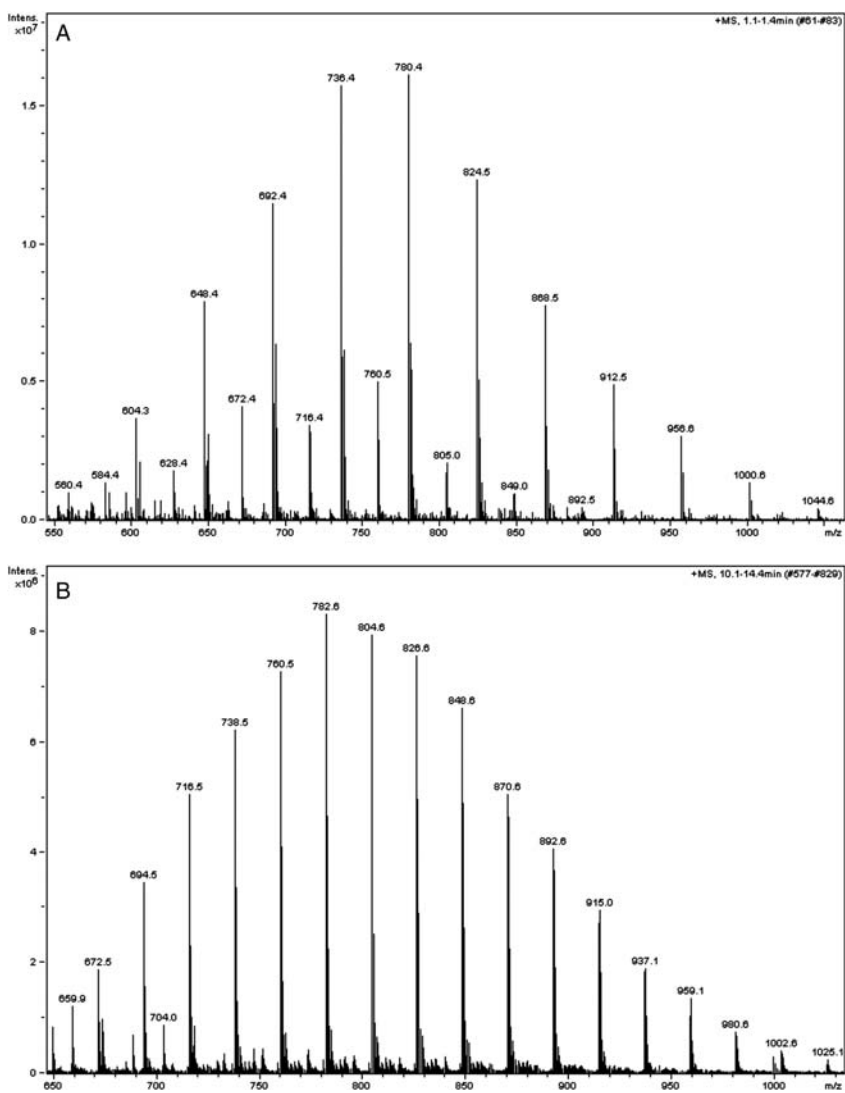
Figure 6. ESI-MS spectra of representative peaks in polysorbate 80: Peak A (A); peak B (B); Peak C (C); Peak D (D); Peak E (E); Peak F (F); Peak G (G).
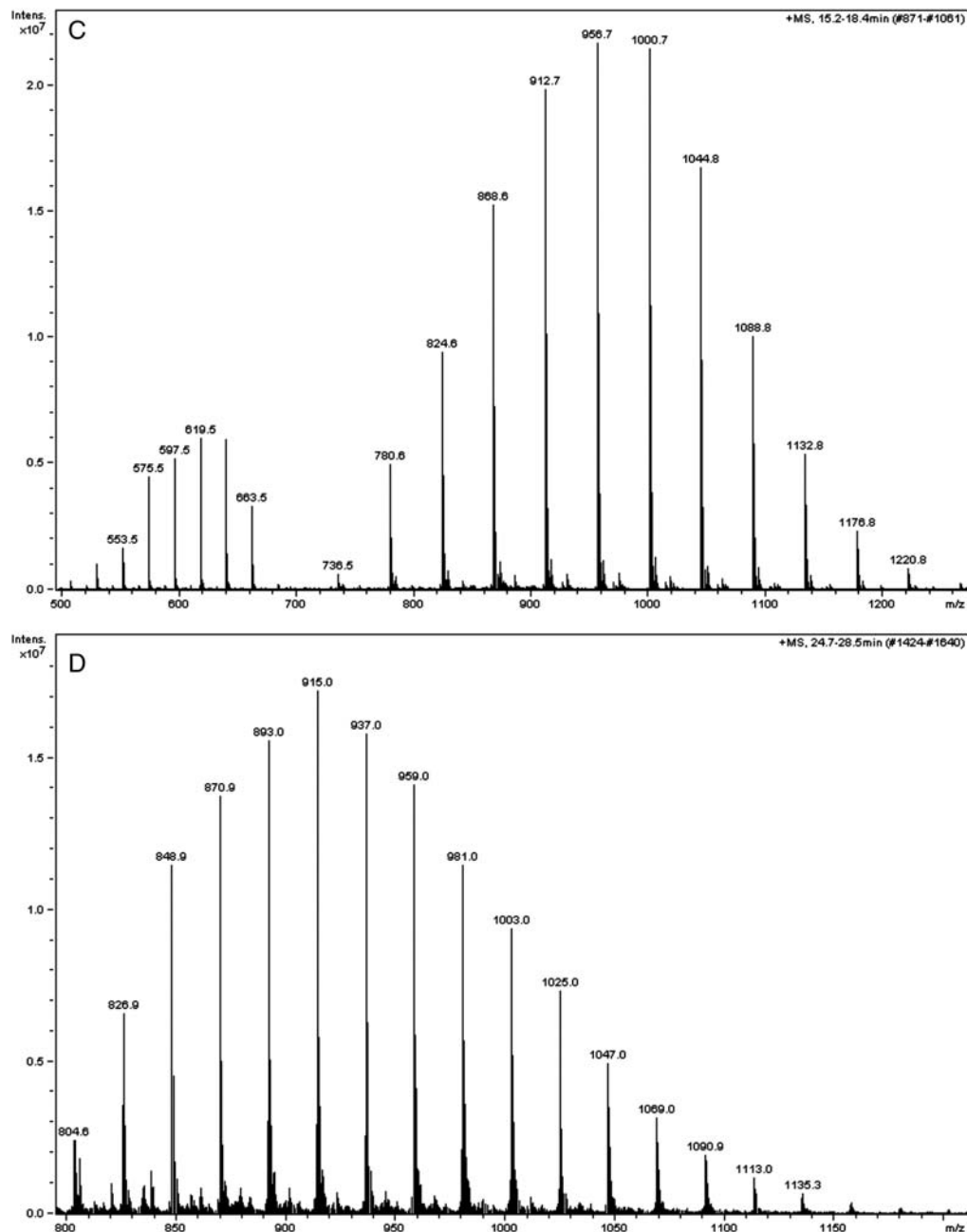
Figure 6. (Continued)

LC–MS analysis

Figure 5 shows total ion current (TIC) traces obtained from polysorbate 80 (Well, batch number 20080412). The results indicate that polar components (peak A) had a lower response in ESI mode than the esters (peaks B–F), which eluded later in the chromatogram. Compounds with high surface activity (i.e., esters) tend to give strong responses in ESI mode.

A probable explanation for the series of ions present in peak A at approximately 1 min is that they contain non-esterified PS and PI species. Figure 6A shows that the ions were attributable to PI NH_4^+ adducts containing approximately 9–20 POE groups and PS double NH_4^+ adducts containing approximately 22–36 POE groups. For instance, the ion at 780.4 is attributable to the NH_4^+ adduct of isosorbide + 14 POE units and the ion at 760.4 is attributable to the $2NH_4^+$ adduct of sorbitan + 30 POE units.

The major peak in the chromatogram around 12 min (peak B) was composed of doubly charged ions bearing two NH_4^+ adducts of PSM. The series of ions separated by 22 amu also showed a range of POE chains between 19 and 36 (e.g., the strongest ion at m/z 782.6 was attributable to sorbitan + 25 POE units–monooleate). (Figure 6B). Because sorbitan has four sites for POE groups, if the POE groups are evenly distributed, there are six or seven per site.
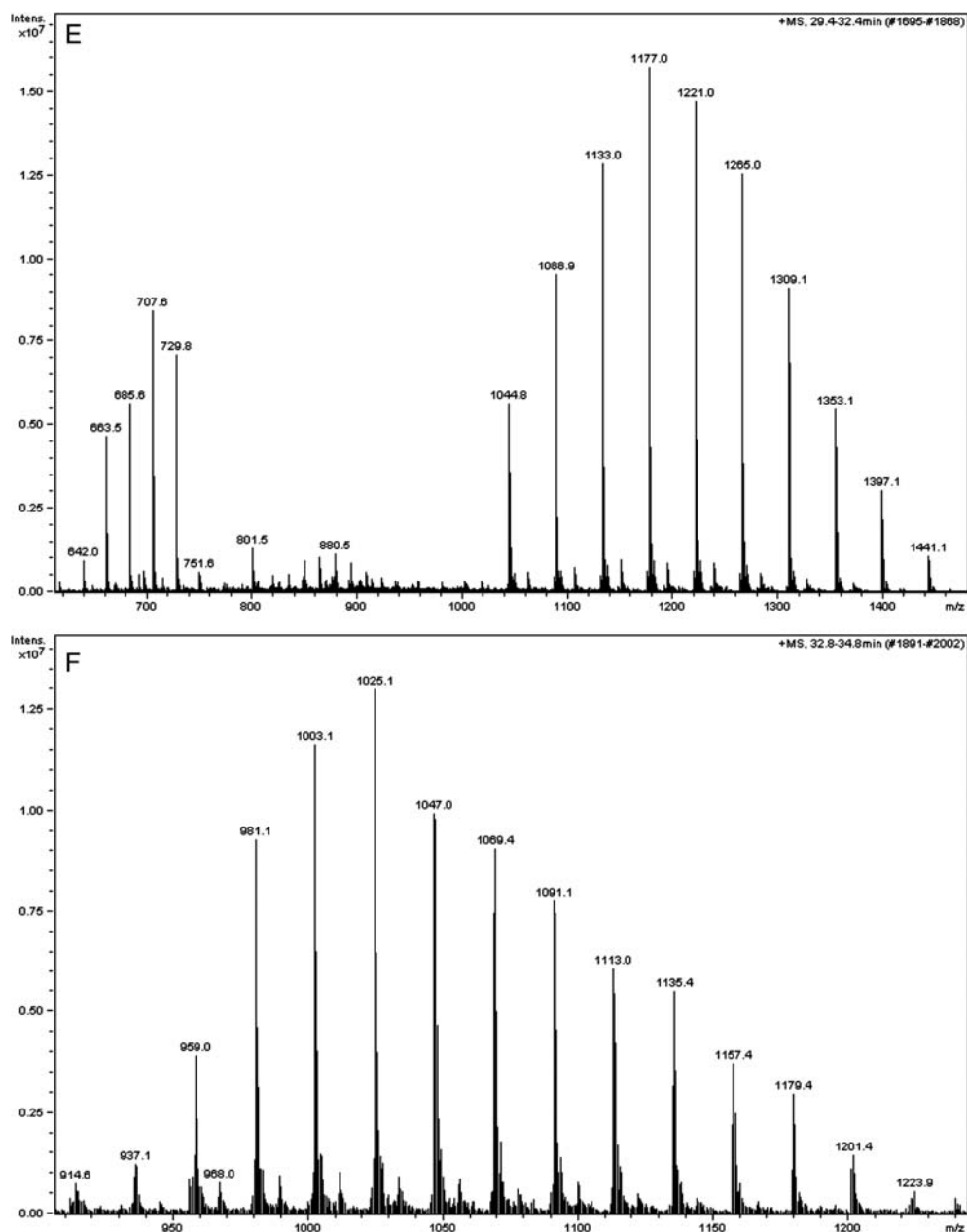
Figure 6. (Continued)

The PIMs are visible in peak C at 17 min. Figure 6C shows that this peak contained two envelopes. The ions in the higher mass group were attributable to PIM NH_4^+ adducts containing 7–18 POE groups (for instance, the ion at 956.7 is attributable to the NH_4^+ adduct of isosorbide + 11 POE units−monooleate). Because isosorbides have only two sites for POE groups, if the POE groups are evenly distributed, there are five or six per site. The second envelope of ions in the spectrum of this peak around m/z 509.6∼686.6 was attributable to doubly charged ions bearing double NH_4^+. For instance, the ion at 619.5 is attributable to two NH_4^+ adducts of isosorbide + 17 POE units−monooleate.

Peak D centred at 27 min, which had only one envelope, was attributable to double NH_4^+ adducts of PSD species (Figure 6D).

The strongest ion at m/z 915.0 was attributable to sorbitan diesters with 25 POE units. Peaks D and B have similar POE distribution.

Similar data obtained from peak C was repeated for peak E observed at 31 min, which had two envelopes (Figure 6E). One was composed of NH_4^+ adducts of PID species. In this case, the biggest ion at m/z 1,177.0 was due to an isosorbide dioleate with 11 POE units; i.e., five to six POE groups per chain if evenly distributed. The other was composed of double NH_4^+ adducts of PID species. In this case, the biggest ion at m/z 707.6 was attributable to an isosorbide dioleate with 16 POE units.

Peak F centerd at 32 min fits the NH_4^+ adducts of sorbitan POE trioleate. For instance, the ion at m/z 1,025.1 was
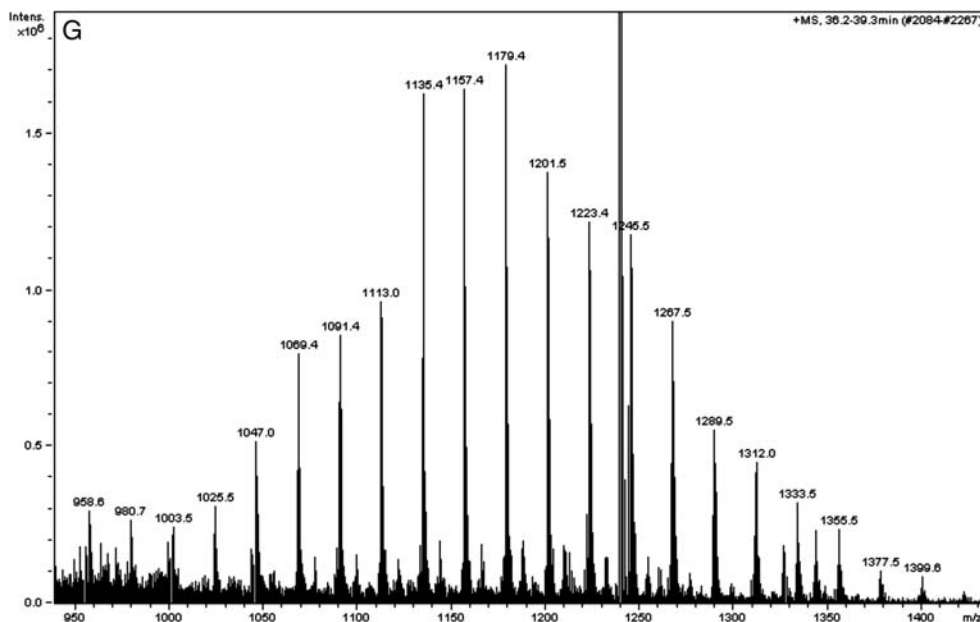
Figure 6. Continued

attributable to sorbitan trioleates with 24 POE units; i.e., approximately six POE groups per chain if evenly distributed (Figure 6F).

The broad indistinct peak G eluting towards the end of the run time was attributable to tetraoleate esters of sorbitan POE (Figure 6G). For example, the ion at m/z 1,179.4 was due to a sorbitan POE tetraoleate with 25 POE units; i.e., approximately six to seven POE groups per chain if evenly distributed.

The polysorbate 80 (Well, batch number 20080412) is composed of approximately eight different species with their identities shown in Table II.

The standard mass range of the mass spectrometer used in this study was 100~2,200 Da. However, the molecular weight of PSTetra was up to 2,800 Da, which exceeds the detectable mass range of our MS. To observe the complete mass distribution of PSTetra, we added ammonium formate to the mobile phase to allow sorbitan derivatives to form stable double charged ions with m/z shifted to smaller masses. This was crucial for the accomplishment of this study.

The chemical composition of polysorbate 80 is more complex than initially anticipated. In addition to monooleate, there are dioleates, trioleates and tetraoleates, especially the non-esterified PS, PI and fully-esterified PSTetra, PID, which only have hydrophilic or lipophilic groups and thus have no surface activity. These species are first shown in this study, and can play an important role in physicochemical behaviour of polysorbate 80.

Meanwhile, the strongest ions in peaks A–G imply the possibility of equal distribution of POE, with the chain length approximately 6. According to the Pharmacopoeial definition, polysorbate 80 contains 20 POE group units. However, the data in Table II indicate that the number of POE group units has exceeded 20. That is because the Pharmacopoeial definition only takes into account PSM but ignores the existence of

isosorbide derivatives. However, sorbitan has four reactive hydroxyl groups, while isosorbide has only two. One mole of sorbitol would yield a one mole mixture of sorbitan and isosorbide. Thus, after polymerization with 20 of EO, the one-mole sorbitan and isosorbide mixture would produce oxyethylates with an average of more than five EO moieties on each reactive hydroxyl site. However, the Pharmacopoeial definition of polysorbate 80 suggests that its average chain length is five. In fact, this single formula in the Pharmacopoeial definition does not adequately represent the real esterificated degree and even contradicts the formal definition of polysorbate 80, because esters of sorbitan copolymerized with approximately 20 moles of ethylene oxide for each mole of sorbitan. This study indicates that the classical definition needs to be updated.

Conclusions

In the present study, the optimized HPLC–ELSD and HPLC–ESI-MS method provides valuable information for the separation and identification of the sorbitan and isosorbide derivatives in polysorbate 80. This simple and rapid method confirmed that polysorbate 80 is a complex mixture of polymeric species containing POE groups. The surfactant contained not only PSM but also a number of POE intermediates (PS, PSD, PSTri, PSTetra, PI, PIM and PID). These have not been reported from polysorbate 80 until now. Therefore, the method can be used in rapid monitoring of the polysorbate 80 synthetic process and controlling of its quality. The analysis of the new composition in polysorbate 80 may contribute to the prediction of its physicochemical properties and rational use. However, the absolute content of each chemical component in polysorbate 80 could not be accurately determined due to lack of reference standards. The preparation of the reference standards is under further investigation.

Table II
Characteristic MS Adduct Ions of Seven Peaks in the Polysorbate 80 Sample (Well, batch number 20080412) and Peak Identities

Peaks	Observed mass distribution $[M + NH_4]^+$ (m/z)	Observed mass distribution $[M + 2NH_4]^{2+}$ (m/z)	Structure assignment	Most abundant mass signal (m/z) / assignment
A	560.4, 604.3, 648.4, 692.4, 736.4, 780.4, 824.5, 868.5, 912.5, 956.6, 1000.6, 1044.6	584.4, 606.3, 628.4, 650.3, 627.4, 694.4, 716.4, 738.4, 760.4, 760.5, 782.4, 805.0, 826.5, 848.4, 870.5, 892.5	POE (22–36): Sorbitan	760.4 / POE 30: Sorbitan
B	736.5, 780.6, 824.6, 868.6, 912.7, 956.7, 1000.7, 1044.8, 1088.8, 1132.8, 1176.8, 1220.8, 1264.8	650.4, 672.5, 694.5, 716.5, 738.5, 760.5, 782.6, 804.6, 826.6, 870.6, 892.6, 915.0, 937.1, 959.1, 981.6, 1002.6, 1025.1	POE (9–20): Isosorbide	780.4 / POE 14: Isosorbide
C		508.6, 531.5, 553.5, 575.5, 597.5, 619.5, 641.5, 663.5	POE (19–36): Sorbitan-monooleate / POE (7–18): Isosorbide-monooleate	782.6 / POE 25: Sorbitan-monooleate / 956.6 POE 12: Isosorbide-monooleate
D		804.6, 826.9, 848.9, 870.9, 893.0, 915.0, 937.0, 959.0, 981.0, 1003.0, 1025.0, 1047.0, 1069.0, 1090.9, 1113.0, 1135.3, 1157.4, 1179.9, 1201.4	POE (20–38): Sorbitan-dioleates	915.0 / POE 25: Sorbitan-dioleates
E	1044.8, 1088.9, 1133.0, 1177.0, 1221.0, 1265.0, 1309.1, 1353.1, 1397.1, 1441.1	619.4, 641.4, 663.5, 685.6, 707.6, 729.8, 751.6	POE (8–17): Isosorbide-dioleates	1177.0 / POE 11: Isosorbide-dioleates
F		914.6, 937.1, 959.0, 981.1, 1003.1, 1025.1, 1047.0, 1069.4, 1113.0, 1135.4, 1157.4, 1179.4, 1201.4, 1223.9	POE (19–32): Sorbitan-trioleates	1025.1 / POE 24: Sorbitan-trioleates
G		958.6, 980.7, 1003.5, 1025.5, 1047.0, 1069.4, 1091.4, 1113.0, 1135.4, 1157.4, 1179.4, 1201.5, 1223.4, 1245.5, 1267.5, 1289.5, 1312.0, 1333.5, 1355.5, 1377.5, 1399.6, 1421.6	POE (15–36): Sorbitan-trtraoleates	1179.4 / POE 25: Sorbitan-trtraoleates

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