

Investigating the Molecular Heterogeneity of Polysorbate Emulsifiers by MALDI-TOF MS

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a new technique that can be used to determine the molecular composition of polysorbate emulsifiers, which are commonly used as food additives. This is the first study to offer such a detailed examination of these heterogeneous compounds. MALDI-TOF MS is a powerful tool that can provide a polysorbate mass profile in less than two minutes. 2',4',6'-Trihydroxyacetophenone monohydrate was chosen to be an ideal matrix, as it easily facilitated desorption and ionization, provided good resolution, and allowed for fast and simple preparation of the sample. By addition of aqueous 0.01 M potassium chloride, species were resolved exclusively as potassium adducts in the positive ion mode. MALDI-TOF MS analysis before and after saponification indicated the presence of unbound ethylene oxide polymers, as well as free and esterified sorbitan- and sorbide-based species. Some evidence for the presence of disorbitan-based species was provided. Also illustrated were the polydispersity of the oxyethylene chains, the degree of esterification, and the identity of esterified fatty acids.

Keywords: Polysorbates; Tween; emulsifiers; MALDI-TOF MS; compositional analysis

INTRODUCTION

Polysorbates are nonionic emulsifiers composed of the partial fatty acid esters of sorbitol-derived cyclic ethers (sorbitans and sorbides) condensed with approximately 20 mol of ethylene oxide per mol. Although sorbitan esters themselves exhibit emulsifying properties, polymerization with ethylene oxide improves their water solubility, thereby expanding their applications. Currently, three types of polysorbates are approved for food use in Canada: polysorbate 60, polysorbate 65, and polysorbate 80 (1). In the United States, polysorbate 20 is also permitted (2). These types of additives are found in a wide variety of food products, including ice cream, salad dressings, cake mixes, coffee whiteners, alcoholic cocktails, and breath freshener products. Levels of addition are generally restricted to less than 0.7%, a limit which varies depending on the product.

The capacity of polysorbate emulsifiers to perform particular functions in foods is dependent on their chemical nature, as dictated by the structure of the sorbitol derivative core, the alkyl chain length of the fatty acids, the degree of esterification, and the number of polymerized oxyethylene residues. For example, both the size of the hydrophilic headgroup and the size of the lipophilic tailgroup have been shown to influence the degree of lipid oxidation that occurs in oil-in-water emulsions (3, 4). Understanding the chemical nature is the key to predicting properties, and by extension, to selecting emulsifiers to suit many different applications.

The molecular heterogeneity of polysorbates presents an analytical challenge. Since the introduction of the hydrophile-lipophile balance (HLB) system several decades ago, few attempts have been made to further elucidate the nature of polysorbate molecular complex-

ity, although many analytical methods have been developed. Techniques such as colorimetry (5, 6), infrared spectroscopy (6), and gravimetric determination by precipitation (7, 8) focus on qualitative detection of polysorbates in food products. Quantification and rudimentary characterization have been attempted using techniques such as thin-layer chromatography (5, 6, 9, 10), gas chromatography (6, 11, 12), high-performance liquid chromatography (10, 13), OH⁻ negative ion chemical ionization mass spectrometry (14), and recently, an optical chemical sensor method (15). Unfortunately, none of these methods allows satisfactory separation or resolution of the individual polymer species, nor identification of the components. Several of these methods require saponification prior to analysis, which destroys and simplifies the original composition of the heterogeneous emulsifiers, and all of these methods can be laborious and time-consuming. In a recent report where matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to analyze polyoxyethylene-type emulsifiers (10), the spectra were of poor quality and peak identity was not addressed.

The technique known as MALDI-TOF MS was first demonstrated in 1987 (16), and though still in its infancy with regard to food analysis, shows great potential toward a variety of analytical problems (17). Its advantages over other methodologies include ease of sample preparation, speed of analysis, high sensitivity, and minimal fragmentation allowing direct access to molecular weight. A limitation, however, is the inability to differentiate between compounds of the same molecular weight. Very recently, MALDI-TOF MS has been successfully applied to analyze other types of carbohydrate-based polymers found in foods such as fructooligosaccharides (18) and maltooligosaccharides (19). This study describes a simple method to determine,

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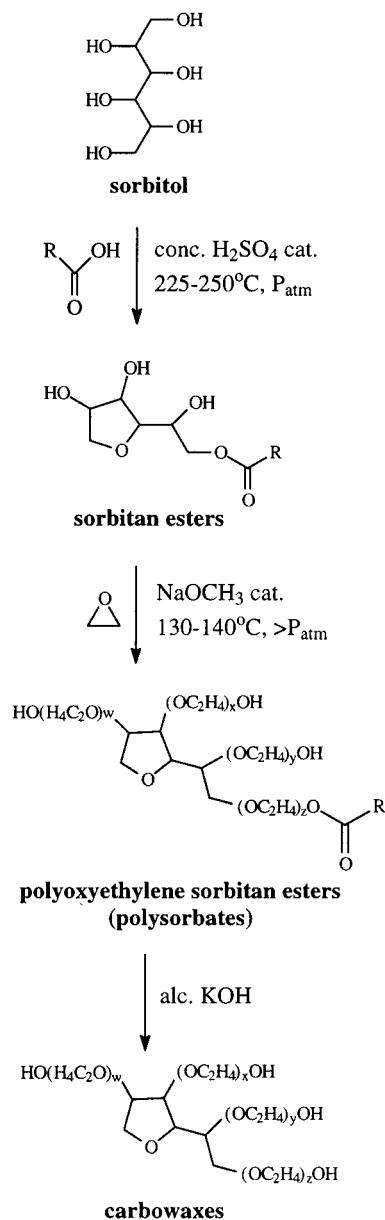


Figure 1. Synthesis and saponification of polysorbates.

for the first time, the molecular complexity of polysorbate emulsifiers using MALDI-TOF MS. The objectives of this research were to select a proper matrix for polysorbates and to identify the polysorbate molecular components before and after saponification by means of their mass profiles.

MATERIALS AND METHODS

Materials and Reagents. Samples of Tween 60K (polyoxyethylene (20) sorbitan monostearate; polysorbate 60) and Tween 80K (polyoxyethylene (20) sorbitan monooleate; polysorbate 80) were donated by Quest International (Lachine, Quebec, Canada). 2',4',6'-Trihydroxyacetophenone monohydrate and 3-aminoquinoline were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxy- α -cyanocinnamic acid was purchased from Sigma Chemical Co. (St. Louis, MO). All water was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA), and all reagents were of analytical grade.

Saponification. Saponification was carried out as described in the literature (8). Each polysorbate sample (200 mg) was refluxed with 12 mL of 1 M ethanolic KOH. After 45 min of reflux, 2.5 mL of water was added to the reaction flask.

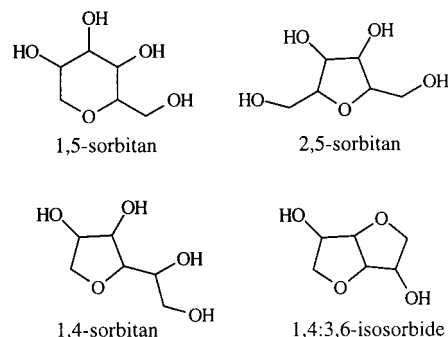


Figure 2. Structures of cyclic sorbitol-derived ethers.

Reflux continued for an additional 15 min. The mixture was then transferred to a separatory funnel containing 12 mL of water. The solution was acidified with concentrated HCl, mixed, allowed to cool, and extracted twice with 12 mL of hexane. The aqueous layer was desalted by swirling with a mixed-bed ion-exchange resin (TMD-8, Sigma Chemical Co.) until some of the blue indicator color remained in the resin. The resin was washed with 40 mL of water. The wash was combined with the aqueous layer and the solution was evaporated to dryness using a rotary evaporator at 40 °C. The polyoxyethylene sorbitan (carbowax) residue was reconstituted in 40 mL of aqueous 0.01 M potassium chloride to give ca. 5 mg/mL concentration for MALDI-TOF MS analysis.

Methyl Esterification and Gas Chromatography (GC) of Fatty Acid Fraction. The method of methyl esterification and gas chromatography of the fatty acid fraction was a variation of a standard procedure (20). Polysorbates (10–20 mg) were dissolved in 5 mL of boron trifluoride reagent (14% BF₃ in methanol/methanol/hexane (7:9:4, v/v/v)) and heated for 30 min. Fatty acid methyl esters were extracted by shaking with 4 mL of water and 4 mL of hexane. The hexane layer was diluted 1:10 for GC analysis.

MALDI-TOF MS. MALDI-TOF MS analysis was performed using a Proflex III instrument with a linear flight tube (Bruker Analytical Systems Inc., Billerica, MA). External mass calibration was performed using maltotetraose and angiotensin II standards. To prepare the probe, 1 μ L of 2',4',6'-trihydroxyacetophenone saturated in acetone was applied and allowed to air-dry. Then 1.5 μ L of the polysorbate solution (5 mg/mL in aqueous 0.01 M potassium chloride) was spotted onto the matrix crystals and further air-dried. The polyoxyethylene sorbitan esters were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. Laser attenuation was adjusted to optimize the signal-to-noise ratio, and samples were analyzed in the positive ion mode. Spectra were acquired as the sum of 200 laser shots.

RESULTS AND DISCUSSION

The degree and type of esterification and the number of oxyethylene residues described in commercial names of polysorbate products reflect an estimation of the dominant species. For example, polysorbate 60 is commercially known as polyoxyethylene (20) sorbitan monostearate even though the molecular species to which this name refers is only one member of a family of related compounds. Complete polysorbate analysis presents a challenge, as there are several factors contributing to molecular heterogeneity. In the preliminary steps of polysorbate manufacture, mixtures of fatty acids are heated (generally at 225–250 °C) with sorbitol in the presence of an acid catalyst (21) (Figure 1). These conditions drive the dehydration reaction of sorbitol, whereby one water molecule may be lost to produce cyclic sorbitol ethers known as sorbitans, or two water molecules may be lost to produce cyclic sorbitol diethers

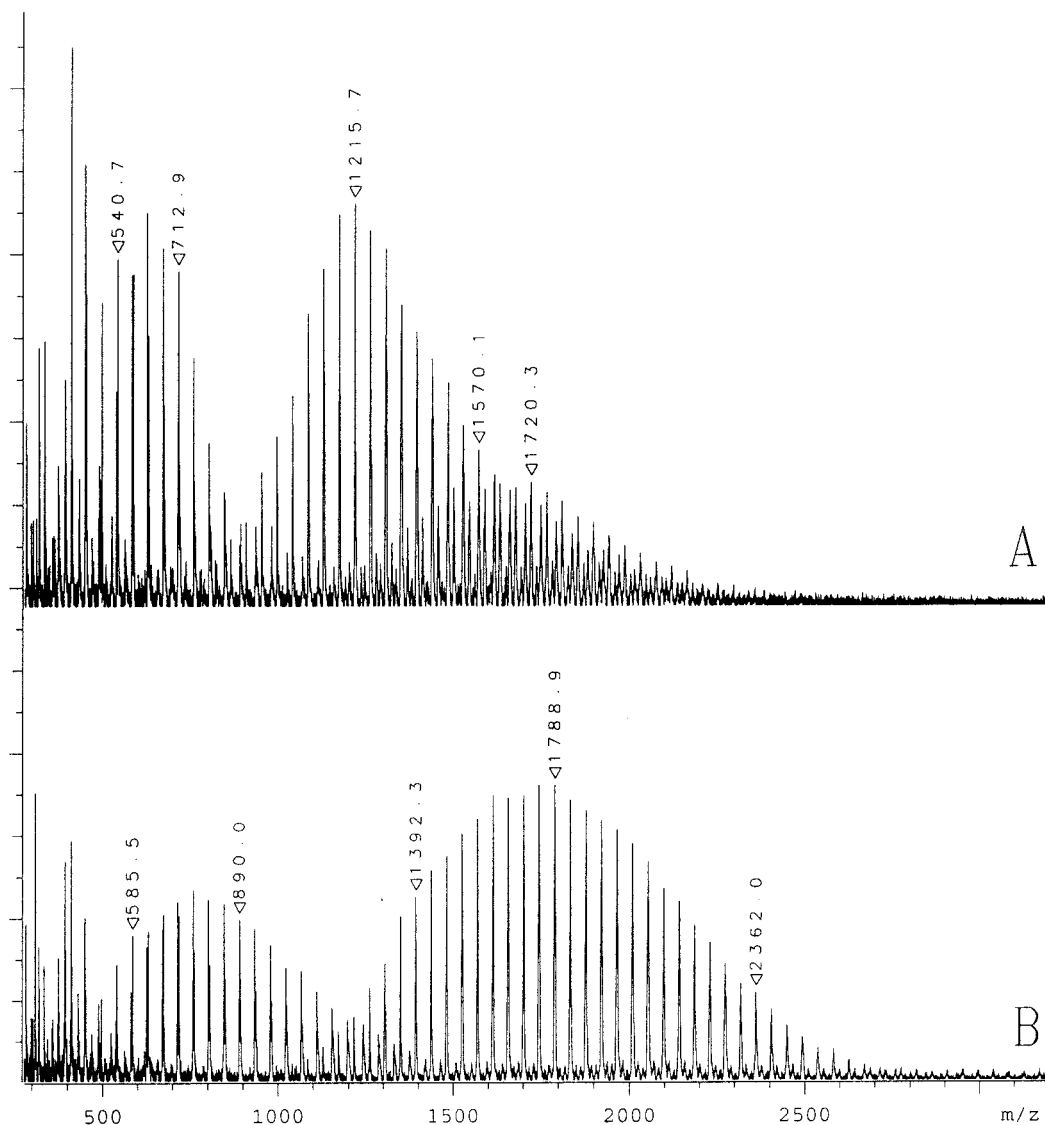


Figure 3. MALDI-TOF MS positive ion spectra of PS 60 (A) and PS 80 (B) at 5 mg/mL in aqueous 0.01 M potassium chloride. All major peaks correspond to potassium adducts. For identification of labeled peaks, see Table 1. Extraneous peaks below m/z 500 result from fragmentation of the matrix.

known as sorbides (22) (Figure 2). Under these conditions, virtually no acyclic sorbitol would remain in the final product (23).

Following cyclization, esterification occurs (24), probably on the primary alcohol, because of steric effects (23). Subsequent condensation of sorbitan esters with ethylene oxide at high pressure in the presence of an alkaline catalyst favors rapid interesterification, essentially scrambling the fatty acid moieties among many possible sites. The result is a complex mixture of esters and partial esters of sorbitans and sorbides, with varying degrees of esterification, and varying alkyl chain lengths of the fatty acids, exhibiting the polydispersity characteristic of any polymeric species. Furthermore, due to the asymmetric nature of sorbitol, many stereoisomers would also be produced. MALDI-TOF MS can elucidate the presence of sorbitan-, sorbide-, and disorbitan-based species, the polydispersity of ethylene oxide chains, the degree of esterification, and the types of esterified fatty acids.

Three common matrixes were examined for desorption and ionization of polysorbates. Of these matrixes, 2',4',6'-trihydroxyacetophenone monohydrate, produced the highest quality spectra. It easily facilitated desorp-

tion and ionization, and demonstrated good spot-to-spot repeatability and high resolution. 4-Hydroxy- α -cyano-cinnamic acid saturated in acetonitrile/0.01% trifluoroacetic acid (1:2) also produced high quality spectra, but sample preparation was more laborious. 3-Aminoquinoline was not suitable as response was low and resolution was poor.

Dissolving the samples in aqueous 0.01 M potassium chloride assisted in sample ionization and simplified the spectra by enhancing the potassium adduct peaks to the exclusion of other adduct peaks (particularly $[M+Na]^+$). Species were resolved exclusively as potassium adducts with m/z values ranging approximately from 400 to 3000. Peaks in any given series are separated by a mass of 44.1 which corresponds to the difference of one ethylene oxide residue. The polydispersity of the ethylene oxide polymer is responsible for the observed Gaussian distributions (Figures 3 and 4).

The composition of fatty acids in each sample was confirmed by GC analysis of the corresponding fatty acid methyl esters. Stearate and palmitate were identified in PS 60, in approximately a 1:1 ratio. Present in PS 80 were oleate, stearate, and palmitate, in roughly an 8:1:1 proportion.

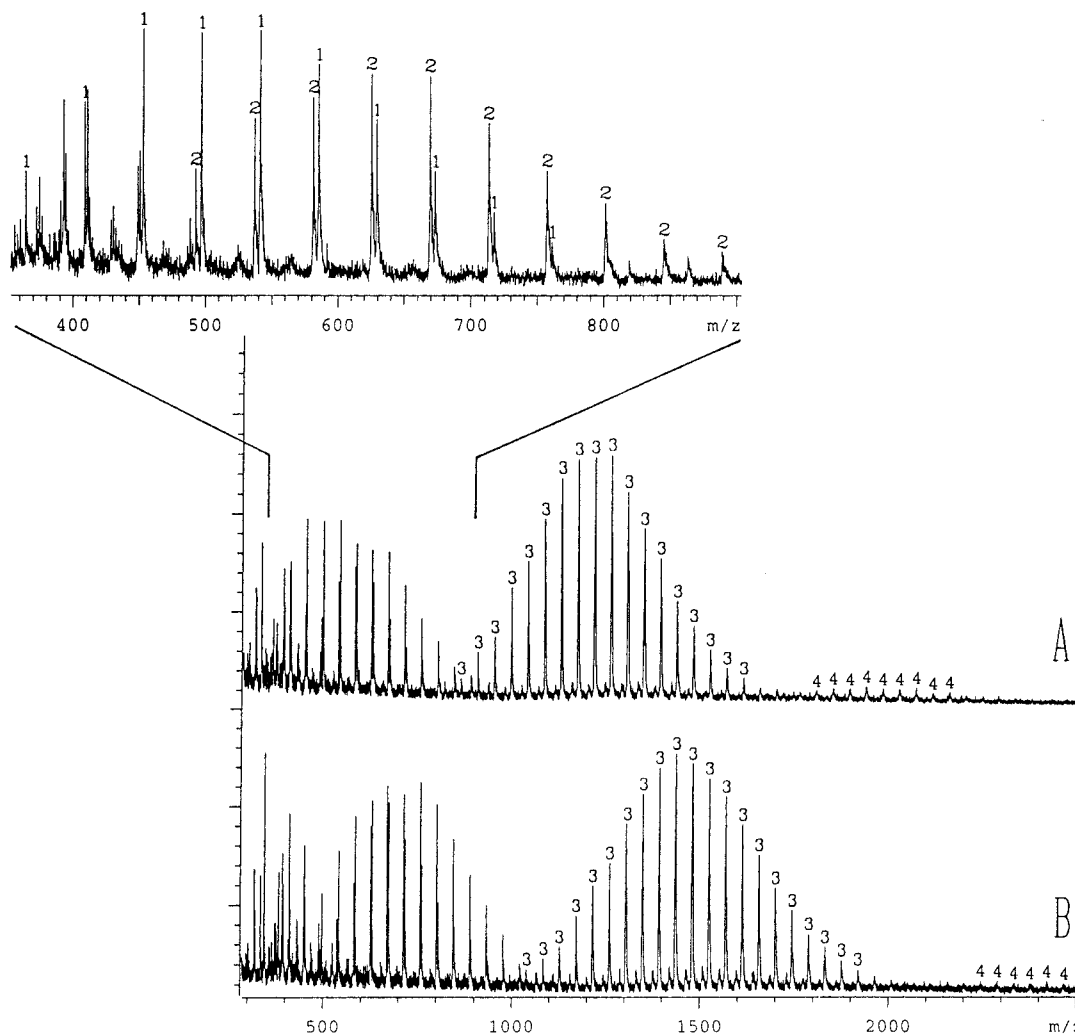


Figure 4. MALDI-TOF MS positive ion spectra of PS 60 (A) and PS 80 (B) after removal of the fatty acids by saponification. Concentrations were 5 mg/mL in aqueous 0.01 M potassium chloride. The peaks were identified as belonging to the following four series: 1, polyoxyethylenes; 2, isosorbide carbowaxes; 3, sorbitan carbowaxes; 4, disorbitan carbowaxes. The small peaks among series 3 are sodium adducts of the sorbitan carbowaxes. Unlabeled peaks below m/z 500 result from fragmentation of the matrix.

Table 1. Identification of Polysorbate Peaks Labeled in Figure 3

sample	observed MW	possible identities	theoretical MW
PS 60 (Figure 3A)	540.7	polyoxyethylene (11)	541.6
	712.9	polyoxyethylene (12) isosorbide	713.7
	1215.7	polyoxyethylene (17) isosorbide monopalmitate	1216.3
		polyoxyethylene (23) sorbitan	1216.4
	1570.1	polyoxyethylene (25) sorbitan monostearate	1570.8
PS 80 (Figure 3B)	1720.3	polyoxyethylene (29) sorbitan monopalmitate	1719.0
	585.5	polyoxyethylene (12)	585.7
	890.0	polyoxyethylene (16) isosorbide	890.0
	1392.3	polyoxyethylene (22) isosorbide monopalmitate	1392.6
		polyoxyethylene (27) sorbitan	1392.7
		polyoxyethylene (21) sorbitan monooleate	1392.5
		polyoxyethylene (15) sorbitan dioleate	1392.7
	1788.9	polyoxyethylene (36) sorbitan	1789.2
		polyoxyethylene (30) sorbitan monooleate	1789.1
		polyoxyethylene (24) sorbitan dioleate	1789.2
2362.0	polyoxyethylene (43) sorbitan monooleate	2361.9	
	polyoxyethylene (37) sorbitan dioleate	2362.0	

Although the degree of esterification can be controlled somewhat by the molar ratios of reactants during production, ultimately a probability distribution occurs. The spectra shown in Figure 3 reflect the complexity introduced by the varying degrees of esterification and different types of fatty acids. Complete compositional analysis of PS 60 and PS 80 was unachievable because of limitations inherent in a mass spectrometric analysis,

which is unable to differentiate among compounds with the same or similar molecular weights. Peak identities by mass included unesterified polyoxyethylenes, sorbitides, and sorbitans; monoesterified sorbitides and sorbitans; diesterified sorbitans; and trace amounts of disorbitan-based species. Many of the peaks had several possible identities because several plausible molecules matched the observed molecular weights (Table 1).

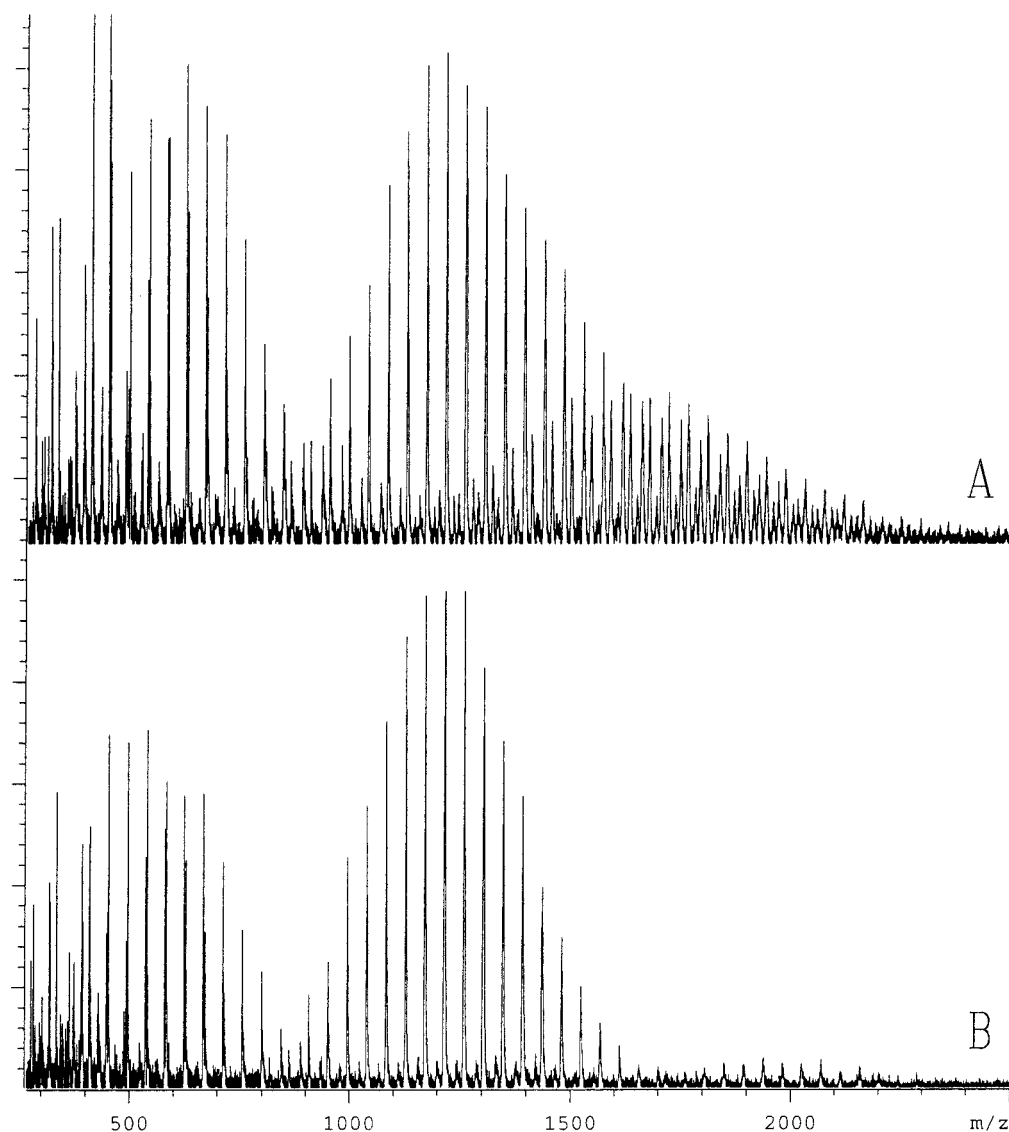


Figure 5. Comparison of the MALDI-TOF MS positive ion spectrum of PS 60 before (A) and after (B) saponification.

Thus, the observed pattern is the sum of all of the possible components, with the relative contributions from each individual species unknown.

To simplify the problem, fatty acids were removed from the polysorbates by saponification. Complete hydrolysis was confirmed by unchanged MALDI-TOF MS spectra following two subsequent rounds of re-saponification. Examination of spectra acquired after saponification (Figure 4) revealed four distinct molecular series which form the core in the absence of fatty acids: polyoxyethylenes (simple linear chains not bound to sorbitol-derived ethers), isosorbide carbowaxes, sorbitan carbowaxes, and trace amounts of species speculated to be disorbitan carbowaxes. Often, species present in trace amounts exhibit poor resolution, resulting in molecular weight "drift". Thus, identification of trace species is tentative when relying solely on molecular weight. These compounds appear in both carbowax spectra in an approximate ratio of 1:1:2:0.2. The fact that both the PS 60 and PS 80 spectral distributions are essentially the same shape after saponification confirms that the esterified fatty acids are indeed a defining attribute of various polysorbate formulations.

Although similar in shape, the mass distributions for the saponified PS 80 occur in a higher-molecular-weight

range than do the saponified PS 60 mass distributions (Figure 4). This phenomenon suggests that PS 80 exhibits a higher degree of ethylene oxide polymerization than does PS 60, which is notable given that these compounds are both defined to contain a molar average of 20 ethylene oxide residues. It is clear from these spectra that the average number of ethylene oxide residues is substantially higher in PS 80 than in PS 60, a situation which may contribute to differences in emulsification properties.

Interestingly, it appears that a significant proportion of the species present in both PS 60 and PS 80 do not contain a fatty acid at all, as revealed by the comparison of mass distributions before and after saponification, shown in Figure 5. Many of the molecular weights observed before saponification are identical to those appearing after saponification, implying the absence of esterified fatty acids in the original product. Lacking a hydrophobic region, these components would not be considered good emulsifiers.

MALDI-TOF MS analysis provides the most complete and detailed account to date of the molecular composition of polysorbate emulsifiers. It confirms the polydispersity of the ethylene oxide chains, and offers insight into the degree of esterification and the relative distri-

bution of sorbitan- and sorbide-based species. For a more complete description of attributes such as stereochemistry of the constituents or the position of esterification, complementary analytical methods would be required, but MALDI-TOF MS is an excellent tool for providing a snapshot of polysorbate composition. The technique also demonstrates potential for future quantitation of the various polysorbate components, as well as polysorbate analysis in complex food matrixes.

As a result of this analysis, it can be seen that the commercial names of polysorbate products are simplifications of the true nature of the product. The efficacy of polysorbate emulsifiers is probably due largely to their heterogeneity, which until now could only be estimated by statistical probabilities. MALDI-TOF MS offers a fast and simple method for characterizing their molecular complexity, with potential applications for quality control during manufacture. Furthermore, by gaining an understanding of polysorbate composition, more informed choices can be made when selecting polysorbates for both new and existing food formulations.

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