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Research paper

Unravelling the polysorbate 20 composition: A fusion of UPLC-MS analysis and stochastic modelling☆

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ABSTRACT

Polysorbate 20 (PS20) is one of the most commonly used non-ionic surfactants in cosmetics, pharmaceuticals and food products. Considered as biocompatible and non-irritating, it is further valued for its solubilising and protein stabilising properties. PS20 is manufactured through a multi-stage reaction of sorbitol with various fatty acids and ethylene oxide, resulting in a complex mixture of components with different molecular weights and polarity. Since variations in the distribution of these components can influence its performance, such as the emulsifying or solubilising efficiency, a detailed understanding of the PS20 composition is of importance.

Herein we introduce a combined approach of reversed-phase chromatography with mass detection and automated stochastic modelling that enables the quantitative characterisation of PS20 at the component level. With two straightforward sample preparations and two methods for an ultra-high performance liquid chromatography (UPLC) system coupled to a single quadrupole mass (QDa) detector, this technique ensures efficient data acquisition. Seven PS20 products of different manufacturers, age and qualities were studied using the presented approach. Molar contents and weight percentages were calculated for each of the more than 27′700 components of the PS20, which were fully characterised by i) the substance class (i.e. sorbitan, isosorbide or polyoxyethylene (POE)), ii) the number of esters, iii) the fatty acid combination and iv) the number of OE units. The obtained results allowed not only an accurate prediction of bulk parameters, such as hydroxyl and saponification values, but also a detailed product comparison.

1. Introduction

Polysorbates belong to the most frequently used excipients in pharmaceutical and biopharmaceutical products. They are administered by various routes, including peroral, subcutaneous, intravenous, ophthalmic and topical [1–4]. In addition, polysorbates are utilised in cosmetics and food products, where polysorbate 20 is assigned the E number E 432 in accordance with European regulations [5–7]. As of April 2025, the FDA's Inactive Ingredient Database (IID) listed 129 entries for polysorbates, 29 of them specifically for PS20 [8]. In fact, the IID is not exhaustive, as Mieczkowski (2023) listed 40 antibody formulations containing PS20 [9]. Moreover, Wuchner et *al.* (2022) showed that the use of PS20 in antibody formulations is further increasing [10]. In a survey of 16 globally acting major biotechnology companies, all stated their intention to use PS20 for antibody formulations in the future [10].

This increasing trend is mainly attributable to the multifunctionality of PS20, which serves multiple purposes across various formulations. In biopharmaceutical formulations it acts as stabiliser to protect proteins and peptides from particle formation or denaturation during storage, processing or shipment [11]. Although the exact mechanisms are not fully understood, surfactants likely act stabilising against interfacial stresses at various interfaces encountered during drug product manufacturing (e.g., air–liquid, glass-liquid) [12–14]. Furthermore, PS20 is an important solubiliser for dissolving poorly water-soluble drugs in aqueous media. For this purpose, typical concentrations of 1 % to 5 % are used with a linear correlation of polysorbate concentration and solubility [15]. A widespread, traditional use of PS20 is as hydrophilic emulsifiers for the stabilisation of lotions and creams. An additional role in semi-solid dosage forms is its participation in the formation

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of liquid crystalline structures. These are formed from hydrophilic surfactants such as polysorbates and consistency agents such as fatty alcohols at a distinct ratio and are responsible for building up the viscosity of creams [3].

The wide-ranging functionality of PS20 stems from its complex composition, which is the result of a multi-step manufacturing process [16,17]. The synthesis begins e.g. with the acid-catalysed dehydration of sorbitol leading in the formation of sorbitan and isosorbide. In the subsequent step, the hydroxyl groups of both compounds are esterified by fatty acids in numerous combinations. In the final oxyethylation step, ethylene oxide (OE) chains of various lengths are incorporated between the fatty acid residues and the sugar backbone by rapid transesterification [18–20]. Thus, several factors such as the ethoxylated core structure (sorbitan, isosorbide or polyoxyethylene (POE)), the type of fatty acids, number of esters and number of OE units contribute to the heterogeneity of PS20 [21]. Further variations introduced by different manufacturers and batch-to-batch variability result in a complex mixture that cannot be linked to a well-defined composition [16]. For these reasons, it is not surprising that polyoxyethylene sorbitan monolaurates, which formally represent the predominant ester components of PS20, were previously found to constitute only 20 % by weight of the overall mixture [22].

Constituents of PS20 cover a broad spectrum of polarity, suggesting that different species may exhibit different physicochemical properties and functions in formulations. Nonetheless, research in this area is still scarce. In a study on protein stabilisation effects of PS20, it was demonstrated that the complete material as well as the isosorbide-POE-monolaurate fraction had superior stabilisation efficiency compared to the sorbitan-POE-dilaurate and sorbitan-POE-trilaurate fractions. However, the data does not yet allow generalised conclusions [23]. Another potential difference between PS20 components is their respective HLB (hydrophilic-lipophilic balance) value, which may result in different solubilisation and emulsifying properties. In the HLB system, first introduced by Griffin (1954) [24], each emulsifier is assigned a dimensionless numerical parameter, with a high number describing a more hydrophilic character and a low number suggesting a more lipophilic nature. Since there is no universally accepted method for determining this empirical parameter, reported HLB values for the same molecule can differ considerably, depending on the underlying approach [25,26]. The Handbook of Pharmaceutical Excipients states an HLB value of 16.7 for PS20 and further lists HLB values of 15.6, 14.9 and 15.0 for palmitate, stearate and oleate polyoxyethylene (20) sorbitan monoesters as well as 10.5 and 11.0 for the stearate and oleate polyoxyethylene (20) sorbitan triesters. While substitution of the fatty acid has little influence on the HLB value, it differs by approx. 4 to 5 units between mono- and triester species of the same fatty acid [27]. In view of these polarity differences, a detailed insight into the composition of the raw material appears to be advantageous with regards to its solubilisation and emulsifying properties, especially in the stage of formulation development.

Analytical methods able to characterise different types of PS20 subspecies are essential in the biopharmaceutical industry [16]. Two main objectives are pursued with the analytical methods available, namely the determination of the content of intact PS20 or its components and the analysis of degradation products [10]. A further subdivision can be made according to the type of the sample origin, i.e. either the raw material, or a PS20 containing formulation. The most widely adopted analytical characterisation approach involves (ultra-) high performance liquid chromatography. As PS20 has no significant absorption in the UV–Vis range, detection techniques such as charged aerosol detection (CAD), evaporative light scattering detection (ELSD) or mass detection (MS) have been used [28,29]. The first mentioned detectors typically show a non-linear response and are limited by insufficient specificity for individual PS20 constituents. MS-based methods, on the other hand, allow the detection of in-source fragments common to a particular component group or of selected PS20 components. Characteristic dioxolanylium ions, for example, are generated by collision-induced dissociation (CID) from fatty acid residues of esterified PS20 components and were repeatedly used as diagnostic ions. On this basis, Borisov et *al.* (2011) presented a semi-quantitative approach capable of profiling the distribution of predominant fatty acids in crude PS20 and PS80 materials, suitable for preliminary batch-to-batch comparisons [30]. Kopf et *al.* (2023), on the other hand, demonstrated the suitability of these ions for quantifying the content of intact or degraded PS20 or PS80 in protein formulations in relation to the raw material used [31]. A similar field of application for PS20 is addressed by our previous validated method, which in contrast monitors selected species, the so-called markers [21]. Even though these examples illustrate useful analytical tools, no holistic approach has yet been developed to capture the overall composition at the level of individual molecular masses. To date, the heterogeneity of PS20 has not been fully understood, and the differences between PS20 materials have not yet been studied at this detailed level.

In this work, we report on the development of UPLC-MS methods to experimentally access the i) molar ratio of sorbitan, isosorbide and POE components, and the distributions within these substance classes with respect to ii) the number of esters and iii) the number of OE units. By means of stochastic modelling, we demonstrate a high correlation of the latter two with binomial distributions. Based on these findings and the determination of the fatty acid distribution, as described in our previous work [21], the molar composition of PS20 is calculated using an automated tool. This approach is verified by comparison of the predicted hydroxyl and saponification values with those provided in the respective certificates of analysis for seven PS20 products studied.

2. Materials and methods

2.1. Materials

Two different batches each of polysorbate 20 high purity (PS20 HP) and polysorbate 20 Super Refined (PS20 SR) were obtained from Croda GmbH (Nettetal, Germany). An additional batch of PS20 was sourced from Kolb Distribution Ltd. (Hedingen, Switzerland) and two further batches were procured from Nanjing Well Pharmaceutical Co. Ltd. (Nanjing, China).

Analytical-grade reagents were used throughout the study. Glacial acetic acid and acetone were purchased from Merck KGaA (Darmstadt, Germany), while acetonitrile and methanol were obtained from Scharlab S.L. (Barcelona, Spain). Formic acid was supplied by Honeywell Fluka (Seelze, Germany), ammonium acetate by VWR International (Radnor, PA, USA), and sodium hydroxide by PanReac AppliChem GmbH (Darmstadt, Germany).

For chromatographic analyses, the following columns were employed: Acquity UPLC™ HSS Cyano (1.8 µm, 2.1 × 50 mm) and XSelect HSS Cyano (3.5 µm, 3.0 × 50 mm), both from Waters GmbH (Eschborn, Germany), as well as the Zorbax 300 SB-C8 column (1.8 µm, 2.1 × 50 mm) from Agilent Technologies, Inc. (Santa Clara, CA, USA).

2.2. Chromatographic conditions

All analyses were conducted using a UPLC system (ACQUITY UPLC H-Class PLUS, Waters, Milford, MA, USA) coupled to a single quadrupole mass spectrometer (QDa, Waters) operating in a mass range of 30–1250 *m/z* for peak detection. The chromatographic separation was performed using the following mobile phases: mobile phase A – 10 mM ammonium acetate; mobile phase B – acetonitrile; mobile phase C – methanol; and mobile phase D – ammonium acetate buffer (10 mM ammonium acetate and 5 mM acetic acid) prepared in water. Data acquisition, evaluation, and interpretation were carried out using Empower 3 software (Waters).

2.2.1. Polysorbate-20-ester method

PS20-esters were analysed using a cyano phase column (Acquity

UPLC™ HSS Cyano, 1.8 µm, 2.1 × 50 mm, Waters GmbH, Eschborn, Germany). The column was maintained at 30 °C, while the autosampler was held at 25 °C. A sample volume of 2 µL was injected for each run at a flow rate of 0.5 mL/min. The total method run time was 45 min. The chromatographic gradient started with a polar mobile phase composition (A: 95 vol%, B: 5 vol%) and linearly shifted to a non-polar composition (A: 15 vol%, B: 85 vol%) over 40 min. This non-polar condition was held isocratically from 40 to 42 min. Subsequently, the gradient was returned to the initial polar composition at 42.1 min, followed by a re-equilibration phase lasting until the end of the run at 45 min.

Mass spectrometric detection was performed using a single quadrupole detector operated in positive ion mode. The capillary voltage was set to 0.8 kV, and the probe temperature was maintained at 600 °C. Nitrogen was used as the desolvation gas at a flow rate of 1200 L/h, according to instrument default settings. Full-scan MS data were acquired in the *m/z* range of 150–1250 using a cone voltage of 15 V and a scan rate of 5 points per second. The positive scan data was used to generate the Total Ion Chromatograms (TICs).

For quantification purposes, Selected Ion Recording (SIR) mode was employed with a sampling rate of 2 points per second. Single Ion Chromatograms (SICs) were generated from the data recorded. The detected *m/z* values corresponded predominantly to ammonium adducts, comprising both single and double charged species.

Further details, including the exact masses of the detected ions, were described by Evers et *al.* (2020) [21].

2.2.2. Homologue distribution and composition method

The homologue distribution of PS20 was characterised using a Zorbax 300 SB-C8 column (1.8 µm, 2.1 × 50 mm, Agilent Technologies Inc., Santa Clara, CA, USA). The column temperature was 50 °C, while the autosampler was maintained at 25 °C. A sample volume of 1 µL was injected for each analysis at a flow rate of 0.5 mL/min. The total run time was 14 min. The chromatographic separation began with a polar mobile phase composition (C: 15 vol%, D: 85 vol%) and transitioned linearly to a less polar composition (C: 80 vol%, D: 20 vol%) over the first 10 min. At 10.01 min, the gradient was shifted to a strongly non-polar composition (C: 95 vol%, D: 5 vol%), which was maintained isocratically until 12 min. At 12.01 min, the mobile phase composition was returned to the initial gradient conditions, which were held constant until the end of the run at 14 min for column re-equilibration.

Mass spectrometric detection was performed in positive ion mode using a capillary voltage of 0.8 kV and a constant probe temperature of 600 °C. Nitrogen was employed as the desolvation gas with a flow rate of 1200 L/h, as per the instrument's default settings. Full-scan data were acquired in the *m/z* range of 50–1250 using a cone voltage of 15 V and a scan rate of 5 points per second.

For the quantification of the oxyethylene homologues, a higher cone voltage of 100 V was applied to induce source fragmentation. Measurements were carried out in Selected Ion Recording (SIR) mode, with a sampling rate of 2 points per second, targeting singly charged positive ions at 45 Da ($OH\text{-}CH_2\text{-}CH_2^+$, one ethylene glycol subunit).

2.2.3. Upscaled HPLC method for fractionation

Fractionation of PS20 esters was performed on a cyano column (XSelect HSS Cyano, 3.5 µm, 3.0 × 50 mm, Waters GmbH, Eschborn, Germany). The method from 2.2.1 was scaled considering the dimensions of the HPLC column and the dwell volumes of the instruments. The column was maintained at 30 °C, and the autosampler was set to 25 °C. For the analysis, 10 µL of a 20 mg/mL stock solution of PS20 HP were injected at a flow rate of 1.0 mL/min. The total run time was 45 min. The method started with a short isocratic phase using a polar mobile phase composition (A: 95 vol%, B: 5 vol%) until 0.32 min. A linear gradient was then applied from 0.32 to 40.33 min, shifting to a non-polar composition (A: 15 vol%, B: 85 vol%). This non-polar condition was held isocratically from 40.33 to 42.33 min. At 42.43 min, the gradient was returned to the initial polar conditions, which were maintained until the end of the run at 45 min for column re-equilibration. No detection was applied during this run; instead, individual fractions of 1 mL were collected each minute, starting from the beginning of the chromatographic run until minute 40. The first 32 fractions contained detectable amounts of PS20 markers and were therefore used for further analysis.

2.3. Preparation of calibration samples

For calibration purposes, two PS20 solutions of identical concentration were prepared: one quantitatively hydrolysed and one non-hydrolysed (i.e. intact). Defined mixtures of the fully hydrolysed and intact PS20 solutions were prepared in varying ratios to generate calibration samples containing 40, 32, 24, 16, 8, and 0 µg/mL of intact PS20, corresponding inversely to 0, 8, 16, 24, 32, and 40 µg/mL of the fully hydrolysed PS20, respectively. A high-purity PS20 sample from Croda GmbH (Nettetal, Germany) served as the source material for both solution types. Further methodological details were described by Evers et *al.* (2020) [21].

2.4. Sample preparation of collected fractions

The 32 fractions collected as described in section 2.2.3, were used to experimentally determine the distribution of the esterification degree within each substance class of PS20. Each fraction was divided into three aliquots (2 x 200 µL and 600 µL). One aliquot of 200 µL was used to visualise the non-hydrolysed sample (= sample A), another 200 µL for the fully hydrolysed sample (= sample B), and the third aliquot of 600 µL was designated for the Total Ion chromatograms (TICs). Initially, the solvent was evaporated from all aliquots.

The aliquots intended for hydrolysis were then mixed with 2 mol/L sodium hydroxide and left to react for three hours. After quantitative hydrolysis, these samples were neutralised with the appropriate amount of acetic acid and diluted with a water/acetone mixture to achieve a final concentration corresponding to 40 µg/mL PS20.

The non-hydrolysed aliquots were similarly diluted with a water/acetone mixture to a concentration corresponding to 40 µg/mL PS20.

The aliquots designated for the TICs were diluted to a higher concentration corresponding to 200 µg/mL PS20, in order to compensate for the lower sensitivity in the scanning process.

2.5. Final sample preparation

In the present study, various PS20 samples of differing quality grades, ages, and manufacturers were analysed to determine the composition of their individual components. For this purpose, an 8 mg/mL stock solution of each PS20 sample was prepared in water.

One aliquot of each stock solution was quantitatively hydrolysed using 1 mol/L sodium hydroxide solution and subsequently neutralised with 1 mol/L acetic acid, yielding the hydrolysed form of PS20. To maintain the solubility of the liberated fatty acids, the hydrolysed solutions were diluted with equal volumes of acetone and water, resulting in a final concentration equivalent to 40 µg/mL PS20.

A second aliquot of each stock solution was also diluted to the same final concentration (40 µg/mL) using a water/acetone mixture, representing the non-hydrolysed (intact) form of PS20.

3. Results and Discussion

3.1. Distribution of different esterification degrees within the substance classes

3.1.1. Experimental approach

Our initial study focused on the experimental determination of the molar distribution of the esterification degree within the sorbitan,

isosorbide and POE classes. The starting point was the validated UPLC-MS method of our previous work, which focused on the analysis of 41 individual, most representative PS20 components, the so-called markers [21]. This approach has been shown to reliably quantify PS20 markers in biopharmaceutical formulations, making it suitable for e. g. monitoring changes caused by degradation processes [21]. In the meantime, three additional markers have been validated and added to the method (sorbitan OE26 C12/C10-diester, sorbitan OE26 C12/C12/C10-triester and isosorbide OE13 C12/C10-diester). As a result, the selected components were 18 different sorbitan POE fatty acid esters, 13 different isosorbide POE fatty acid esters, 7 different POE fatty acid esters and the three unesterified markers sorbitan OE26 (S00), isosorbide OE13 (I00) and OE13 (OE00), each with the most frequently occurring OE number. The published method allows the calculation of the relative amount of each marker in a formulation compared to its amount in the intact PS20 batch used. However, due to different ionisation properties, particularly between components of a different number of esters, it is not suitable for an inter-marker comparison. To overcome this obstacle, we established three reference scales based on a common hydrolysis product of each substance class. These were the nonesterified markers S00, I00 and OE00, that result from the corresponding esterified components by quantitative hydrolysis. In order to access the molar proportions of the component groups studied by the reference scales, a chromatographic fractionation of PS20 was performed.

The published gradient method was upscaled for HPLC analysis to ensure sufficiently high marker concentration during collection. The separation efficiency and the status of the chromatographic run were monitored in parallel using the reported UPLC-MS method [21]. Total Ion Chromatograms (TICs) were recorded of collected fractions and compared to the TIC of a reference sample containing an equivalent total amount of PS20. Fig. 1 illustrates an overlay of all obtained TICs. A high agreement was observed between the sum of areas under the curve of the fractions and the reference, indicating a high precision of the experiment.

In the next step, the analysis of individual fractions regarding the selected markers of each substance class was performed. For this purpose, two aliquots were withdrawn from each fraction. One aliquot was diluted with a buffer/water mixture (sample A). The second aliquot was treated with a 2 mol/L sodium hydroxide solution prior to dilution, resulting in quantitative hydrolysis of the esterified PS20 components (sample B, see 2.4) [21]. All 64 samples were reanalysed with the published UPLC-MS method [21], and the relative amounts of the individual markers were determined using the validated calibration scale. The latter is comprised of mixtures of the quantitatively hydrolysed (= 100 % degraded) and intact (0 % degraded) PS20 solutions at different ratios. For each marker, a total of six calibration levels in the range between 0 and 100 % hydrolysis was obtained.

Table 1 exemplary shows the analytical results for sorbitan markers. The left side of the table illustrates the distribution of individual markers across the fractions in % with regard to the intact calibration standard. A sum of 100 % across all fractions of a marker corresponds to a complete recovery after chromatographic fractionation. For the sorbitan mono-, saturated diester and the sorbitan C12/C12/C12-triester markers, the recoveries found were within the analytical variation of the method of 80 – 120 %. The recoveries of late eluting sorbitan components, however, ranged between 62.9 – 81.6 %. We attribute this deviation to a reduced solubility of the late eluting components in the sample solvent used.

In contrast to the esterified markers, the relative amount of the marker S00 is expressed according to the reference scale. Here, 0 % S00 refers to PS20 containing fully esterified sorbitan OE26 components, whereas 100 % S00 corresponds to complete hydrolysis of the latter. For this reason, the sum of 25.9 % S00 represents the actual molar proportion of this marker in the entire sorbitan OE26 group. This value agrees well with the 26.8 % S00 in samples B of same fractions (right side of Table 1, column denoted as S00 Hynorm), as there is no analytical difference between samples A and B. In the following fractions, however, the results of samples B relate to the molar proportions of all sorbitan OE26 species present in the respective sample. To determine the relative ratios between the non–, mono-, di- and triesterified sorbitan OE26 groups, we subdivided the fractions based on contained markers with the same ester number. The molar proportions of the assigned groups were added and normalised to 100 % (see Table 1, column denoted as S00 Hynorm). Since only one overlap of markers from different groups was observed, the chromatographic fractionation experiment can be considered successful. The error in the molar percentages of the affected groups is negligible, as fraction F22 contained 2.4 % of sorbitan OE26 species. The recovery of S00 across all samples B was 102.3 %. This result not only highlights the high precision of the analysis, but also strongly indicates that all esterified sorbitan OE26 components were entirely collected. Deviations observed for the late eluting markers in samples A are most likely related to a solubility phenomenon. The

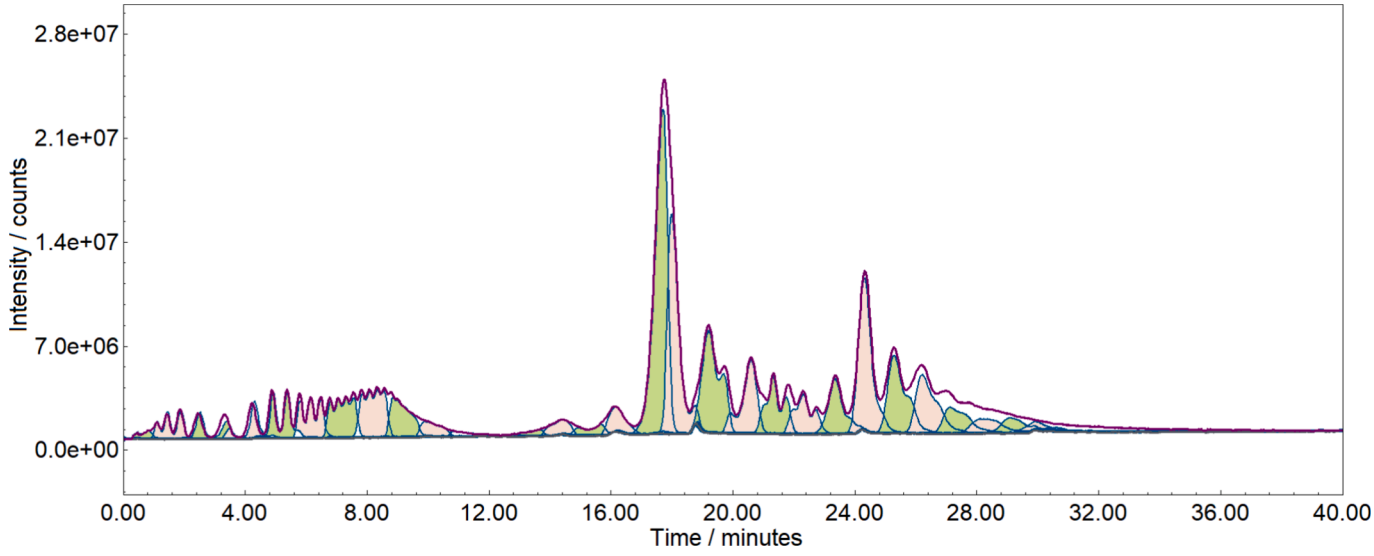

Fig. 1. Overlay of Total Ion Chromatograms (TICs) of blank sample (grey), of reference sample containing PS20 dissolved in acetone/water to approx. 200 µg/mL (purple) and of individual fractions with PS20, whose areas under the curve are alternately displayed in green and light pink. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Overview of the experimentally determined distribution of selected sorbitan OE26 markers across collected fractions F01-F32 in intact PS20 samples A (on the left side) and in the quantitatively hydrolysed samples B (on the right side) in %.

Fraction	S00	S08	S10	S12	S14	S16	S18:1	S18	S12/08	S12/10	S12/12	S12/14	S12/16	S12/18:1	S12/12/08	S12/12/10	S12/12/12	S12/12/14	S12/12/16	S00 Hy	S00 Hy norm
F01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F03	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F04	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F06	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F07	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	26.8
F08	24.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.3	
F09	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	
F10	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	
F11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F14	0.0	106.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	
F15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F16	0.0	0.0	110.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	
F17	0.0	0.0	0.0	85.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	21.4	42.7
F18	0.0	0.0	0.0	18.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	
F19	0.0	0.0	0.0	0.0	95.0	91.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.6	
F20	0.0	0.0	0.0	0.0	0.0	0.0	24.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.3	
F21	0.0	0.0	0.0	0.0	0.0	0.0	60.9	47.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	
F22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	40.4	90.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	
F23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	96.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	
F24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	90.4	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.4	21.6
F25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	89.7	13.2	0.0	0.0	0.0	0.0	0.0	0.0	5.1	
F26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	80.3	78.5	0.0	0.0	0.0	0.0	0.0	4.0	
F27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	71.2	0.0	5.7	0.0	0.0	2.3	
F28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	76.7	16.4	0.0	0.0	1.5	
F29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	59.5	24.8	0.0	2.4	8.9
F30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	44.9	30.5	1.7	
F31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	32.4	1.2	
F32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	25.9	106.5	110.7	103.6	95.0	91.0	85.4	87.8	90.5	96.2	90.4	95.1	93.5	78.5	71.2	76.7	81.6	69.7	62.9	102.3	100.0

lipophilic components of sample A were hydrolysed by the addition of sodium hydroxide to the highly soluble marker S00 and corresponding fatty acids which were not detected via the applied method.

The data analysis of individual fractions for the isosorbides was carried out analogously (see Table 2). The determined recoveries of the esterified markers were within the analytical tolerance of the method of 100 ± 20 %. A clear separation between the groups of non–, mono- and diesterified isosorbide markers was achieved, with no overlap in the fractions. The recovery of the fully hydrolysed I00 across all fractions was 89.9 %.

Within the POE class (see Table 3), the unesterified and the monoester markers were initially evaluated in accordance with the published method [21]. However, the detection of a total of 7.1 % of OE00 Hy norm in fractions F23 – F32 was an indication of further POE26 components that were not adequately represented by the selected markers. Based on the elution order and the chemical structure of POE, the diester components were the only plausible candidates. For this reason, we re-evaluated samples A including five diester markers (OE12/08, OE12/10, OE12/12, OE12/14 and OE12/16), which were chosen in analogy to the isosorbide markers and correspond to the most probable fatty acid combinations. As assumed, these diester markers were present in the later fractions (F23 – F28). High individual recoveries in the range of 84.7 – 112.5 % were determined for all POE markers studied, which were within the analytical tolerance limit of the method. Similarly to sorbitan and isosorbide components, sufficient separation between markers of the same ester number was achieved. The recovery of the OE00 marker among hydrolysed samples was 94.6 %, suggesting complete acquisition of POE components within the analytical limits of the method.

3.1.2. *Stochastic approach*

Through a sequence of analytical methods, we were able to determine the molar proportions of component groups with the same number of esters within the individual substance classes. However, the presented procedure requires high analytical and time-related effort and is therefore not suitable for routine analysis. For this reason, we searched for a universal stochastic model that is capable of accurately describing the data, and which is transferable to other PS20 mixtures.

Our approach involved a combinatorial representation of the transesterification reaction, which takes place upon addition of ethylene oxide in the final step of the manufacturing process [17–19]. The terminal hydroxyl groups of ethoxylated sorbitans, isosorbides or POEs were considered as equivalent reaction sites, since they are all primary [17]. It was assumed that esterification occurs at each site with the same probability and independently of each other, meaning that potential steric effects or intermolecular interactions were neglected. No distinction was made according to the type of fatty acids residues, as only the esterification degree of the resulting product is relevant for the present model. Therefore, the two considered outcomes for the individual reaction sites were i) an esterification or ii) a free, unreacted hydroxyl group.

The characteristics described meet all criteria of a binomial distribution B (n, p, k), that is given by equation (1). 'n' is the number of hydroxyl groups in a nonesterified component with $n = 4$ for sorbitans

Table 2
Overview of the experimentally determined distribution of selected isosorbide OE13 markers across collected fractions F01-F32 in intact PS20 samples A (on the left side) and in the quantitatively hydrolysed samples B (on the right side) in %.

Fraction	I00	I08	I10	I12	I14	I16	I18:1	I18	I12/08	I12/10	I12/12	I12/14	I12/16	I00 Hy	I00 Hy norm
F01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F03	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F04	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	
F05	44.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	46.9	
F06	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	55.5
F07	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F08	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F09	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F13	0.0	98.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	
F14	0.0	17.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	
F15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F16	0.0	0.0	106.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	
F17	0.0	0.0	0.0	9.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.4	40.7
F18	0.0	0.0	0.0	91.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	21.3	
F19	0.0	0.0	0.0	0.0	83.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.2	
F20	0.0	0.0	0.0	0.0	10.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F21	0.0	0.0	0.0	0.0	0.0	104.4	76.2	0.0	0.0	0.0	0.0	0.0	0.0	3.8	
F22	0.0	0.0	0.0	0.0	0.0	0.0	16.0	91.6	0.0	0.0	0.0	0.0	0.0	0.4	
F23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	95.7	0.0	0.0	0.0	0.0	0.0	
F24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	93.3	0.0	0.0	0.0	0.0	
F25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	75.7	0.0	0.0	1.1	
F26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	19.3	74.5	0.0	0.2	
F27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.0	60.0	1.1	3.8
F28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	28.7	1.0	
F29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	46.9	115.5	106.1	101.1	94.5	104.4	92.2	91.6	95.7	99.1	95.0	98.5	88.7	89.9	100.0

and $n = 2$ for isosorbides and POE; 'k' is the number of esters within the studied class, with $k = 0$ for the nonesterified compound, $k = 1$ for the monoester group, etc. and 'p' is the esterification probability.

$$B\ (n,\ p,\ k) = \binom{n}{k} \cdot p^{k} \cdot (1-p)^{n-k} \tag{1}$$

Eq. (1): General equation of a binomial distribution [32].

Since the probability 'p' was unknown, we calculated this variable for each substance class using the chromatographically determined molar percentages of the nonesterified S00, I00 and P00 markers (see method 1). In these cases, k equals 0 and the above Eq. (1) simplifies to Eq. (2).

$$B\ (n,\ p, 0) = \binom{n}{0} \cdot p^{0} \cdot (1-p)^{n-0} = (1-p)^{n} \tag{2}$$

Eq. (2): Binomial distribution with k = 0

The calculated probability values 'p' were 0.3111, 0.3227 and 0.3110 for the sorbitan, isosorbide and POE components. The match of these values, with an RSD = 2.1 %, appears plausible, since the terminal hydroxy groups of the OE chains are primary and have a comparable electronic environment.

Using the mean value of 'p', binomial distributions were calculated for each substance class and compared to the experimentally determined molar proportions of component groups with the same number of esters (see Fig. 2). In addition, the calculated relative amount of the tetraesterified sorbitan group was included. The stochastically predicted value

Table 3
Overview of the experimentally determined distribution of selected polyoxyethylene (13) markers across collected fractions F01-F32 in intact PS20 samples A (on the left side) and in the quantitatively hydrolysed samples B (on the right side) in %.

Fraction	OE00	OE08	OE10	OE12	OE14	OE16	OE18:1	OE18	OE12/08	OE12/10	OE12/12	OE12/14	OE12/16	OE00 Hy	OE00 Hy norm
F01	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F02	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F03	45.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	47.3	
F04	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	
F05	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F06	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F07	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	51.4
F08	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F09	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F13	0.0	110.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	
F14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F15	0.0	0.0	61.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3	
F16	0.0	0.0	46.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	
F17	0.0	0.0	0.0	25.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.9	
F18	0.0	0.0	0.0	75.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	17.5	41.5
F19	0.0	0.0	0.0	0.0	90.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	
F20	0.0	0.0	0.0	0.0	9.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F21	0.0	0.0	0.0	0.0	0.0	103.7	53.9	0.0	0.0	0.0	0.0	0.0	0.0	4.6	
F22	0.0	0.0	0.0	0.0	0.0	0.0	32.8	87.4	0.0	0.0	0.0	0.0	0.0	1.5	
F23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	97.7	0.0	0.0	0.0	0.0	0.6	
F24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	39.1	0.0	0.0	0.0	0.0	
F25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	73.4	10.8	0.0	0.0	0.4	
F26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	88.4	0.0	0.0	2.3	
F27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	89.4	0.0	1.4	
F28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	84.7	1.4	7.1
F29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	
F30	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
F32	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	47.1	110.6	107.8	101.5	99.5	103.7	86.7	87.4	97.7	112.5	99.2	89.4	84.7	94.6	100.0

of this group is only 0.9 %, thus explaining why the tetraester sorbitan markers were inaccessible by analysis. Remarkably, the purely combinatorial approach showed a high degree of agreement with the experimentally determined distributions for all three substance classes. This result implies that potential intermolecular interactions and steric effects play a minor role in the transesterification reaction of oxyethylated components. The suitability of the model was demonstrated using experimental results of markers of a fixed OE number, i.e. the most frequently occurring number within each substance class. Since the correlation found is of a purely stochastic nature, it can be postulated that the model is transferable to homologues with other OE numbers.

The modelled results for the distribution of the esterification degree within the class of sorbitans and isosorbides are in high accordance with the data published by Brandner (1998) [17]. In a theoretical approach, the author used the median hydroxyl and saponification values given in the National Formulary to approximate the esterification probability. Although not explicitly termed as a binomial distribution, the subsequent mathematical calculations refer to the summed probabilities of individual branches of a binomial tree. However, the correctly identified mathematical relationship has not been applied to the POE class or verified by experimental measurements.

In contrast, our stochastic model is based on a chromatographic analysis of the molar proportions of the fully hydrolysed markers S00, I00 and P00. Since the presented chromatographic method is specific to selected PS20 components, it offers a distinct advantage over the titration procedure used for the determination of hydroxyl and saponification values. Thus, higher accuracy is assumed with the presented model compared to calculations based on bulk parameters.

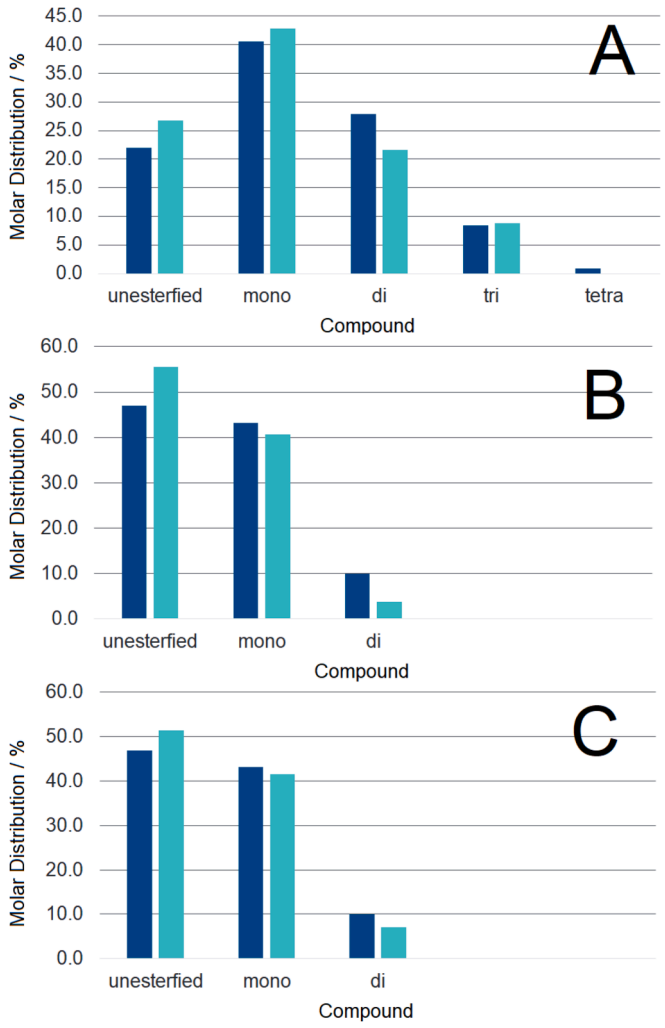

Fig. 2. Modelled (blue) and experimentally determined (turquoise) molar distributions of the esterification degree within the class of A) sorbitans, B) isosorbides and C) POEs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Determination of the oxyethylene distribution for each substance class

In the next step, we addressed the distribution of homologues with respect to the number of OE units. Birkmeier and Brandner (1958) applied the Poisson distribution to describe the variation of OE chain lengths in the ethoxylation reaction of stearic acid [18]. However, the Poisson fit was insufficiently accurate, particularly at the maximum of the distribution. In view of this limitation, we revisited the study on the distribution of OE homologues to find a more suitable stochastic model.

To minimise the number of analytes, the quantitatively hydrolysed sample B of the previous analysis was chosen for evaluation. The latter contained besides free fatty acids a mix of nonesterified components from the three substance classes differing in the number of OE units.

For analysis, we developed a chromatographic method with mass detection (UPLC QDa) based on parameters described in the literature. In the context of poloxamer quantification, Kopf et *al.* (2023) applied an increased cone voltage of 80 V to generate diagnostic ions by in-source fragmentation [31]. The authors evaluated signals at 45 *m/z* and 89 *m/z* corresponding to ethylene glycol ions of one or two subunits, as well as 59 *m/z* referring to the propylene glycol ion. With the aim to detect a single mass channel of an ethylene glycol fragment of PS20, our studies focused on the 45 Da signal. We performed experiments with different cone voltages to increase the ion yield of the 45 *m/z* signal and found 100 V to be the optimal setting. For peak assignment, a Total Ion Chromatogram in full scan mode was additionally recorded.

Fig. 3 shows the obtained Single Ion Chromatogram (SIC), in which the most prominent peaks were identified and assigned based on their mass spectra in full scan mode. Two superimposed effects add complexity to the interpretation of the chromatogram: i) the proportionality to the number of OE units and ii) the relative frequency of components with the same OE unit number in the mixture. Thus, to extract information solely on the distribution of the OE homologues, normalisation was performed by dividing the peak area by the respective number of OE units. For isosorbides and POE chains, six neighbouring and separated peaks were evaluated, and for sorbitans eight such peaks were considered. In Fig. 4, the normalised peak areas were plotted against the sum of OE units for the nonesterified sorbitan, isosorbide and POE representatives. The maximum value for ethoxylated POE is in good agreement with the previous publication by Borisov *et al.* (2011), who reported an average of 12–13 OE units for POE and isosorbide laurate esters [30]. In the case of isosorbides, we found a slight shift towards a maximum of 11 OE units, while for sorbitans, a slight shift towards a maximum of 25 OE units was observed, compared to the previously reported 26 OE units [21,30]. Considering the discrete nature of the distribution and in analogy to the previous chapter (3.1.2), we applied a scaled binomial distribution to model the data (see Eq. (1)). '*n*' corresponds to the total number of OE units, '*k*' is the sum of OE units in a molecule and '*p*' is the chain elongation probability by one OE unit. Since the parameters '*n*' and '*p*' were experimentally inaccessible, the closest fit for each substance class was determined by an approximative optimisation approach. The corresponding distributions, normalised to the theoretical total peak area, were included in Fig. 4. A high level of agreement was observed between the theoretical and experimental data, with deviations in individual values of less than 10 %. This result supports the suitability of this model.

It is worth noting that a binomial distribution with high '*n*' and small '*p*'-values approximates the Poisson distribution. However, based on the calculated results, this does not apply here, which could explain the deviations observed by Birkmeier and Brandner (1958) [18].

3.3. Determination of the molar proportions of substance classes

A further variable within the profiling of the mixture are the molar proportions of individual substance classes. Attempts have been previously made to calculate the percentages of sorbitans and isosorbides, however, a quantitative approach, including the POE class, is not yet available [17].

We addressed this issue based on the scaled binomial distributions of the previous chapter, that were demonstrated to closely fit the molar distribution of ethoxylated homologues within each substance class. Since the experimental data originated from a single chromatogram and the peak areas were normalised to the OE-count of individual components, the comparison of peak areas between different distributions was justified. Therefore, the molar ratios between the substance classes were determined from the ratio of the total areas in the individual calculated histograms. Under the assumption of a negligible content of free fatty acids, the molar proportions of sorbitans, isosorbides and POE were obtained by normalisation of these ratios to 100 %. For the product studied, the molar proportion was 51.9 % for sorbitans, 30.3 % for isosorbides and 17.8 % for POE.

3.4. Final method and its application to different batches

In the previous chapters, combined approaches of analytical methods and stochastic models were introduced, which enable quantitative characterisation of a PS20 batch by component groups. The components were classified based on common characteristics of the i) backbone, ii) number of ester groups and iii) sum of OE units. So far, no differentiation

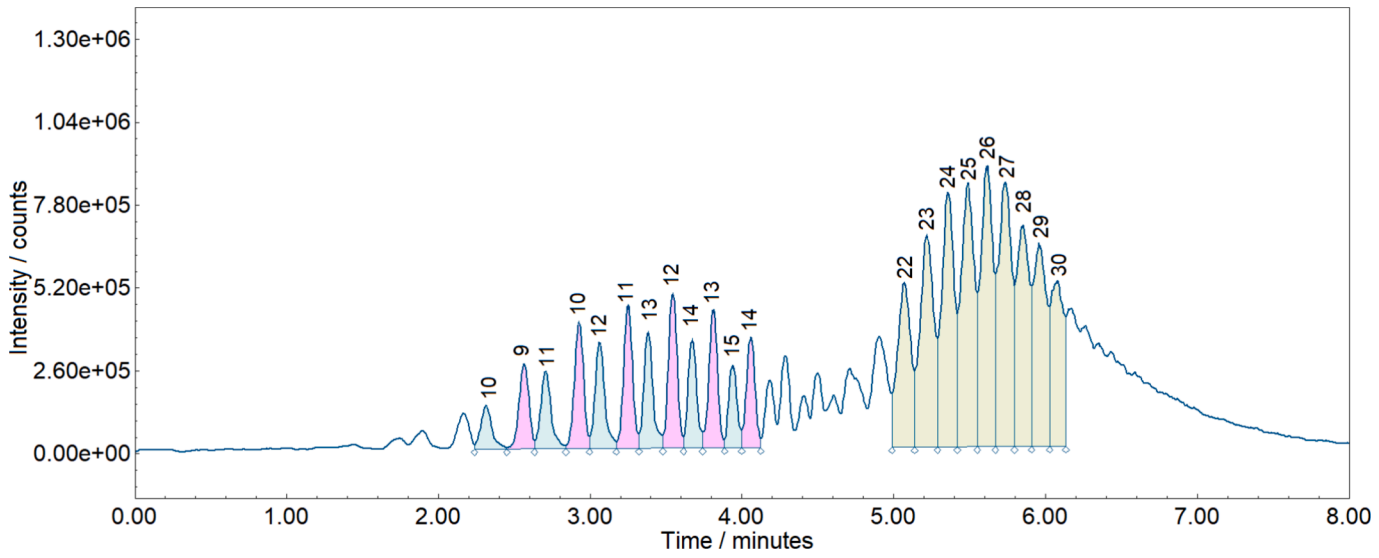

Fig. 3. Single Ion Chromatogram (SIC) at *m/z* 45 of a quantitatively hydrolysed PS20 sample, recorded with a cone voltage setting of 100 V. Peak assignment was performed based on the Total Ion Chromatogram (TIC) recorded in parallel. Assigned peaks are shown in green for sorbitans, pink for isosorbides and light blue for POE. The determined numbers of OE units are displayed at the top of the peaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was made between the individual fatty acid residues, although their distribution is necessary for the final disassembly of the mixture. To close this gap, we used a combinatorial approach described in the literature that utilises the test results of the batch-specific fatty acids content of the certificate of analysis [21]. The certificate values were converted to mol% before use. The general formula for the individual probabilities of mono-, di-, tri- and tetraesters in the PS20 mixture is given in Eq. (3).

$$\text{Probability of individual esters} = \frac{k!}{l_1!\cdot l_2!\cdot l_3!\cdot l_4!}\cdot\left(\frac{\%FA\ I}{100}\cdot\frac{\%FA\ II}{100}\cdot\frac{\%FA\ III}{100}\cdot\frac{\%FA\ IV}{100}\right)\cdot 100\,\% \tag{3}$$

Eq. (3): Probability of individual esters of PS20, based on the amount of fatty acids in mol% (%FA I – %FA IV)

'k' corresponds to the esterification degree and '$l_1 - l_4'$' is the number of same fatty acid residues of a component. *%FA I – %FA IV* are the molar percentages of the corresponding fatty acids calculated from the weight percentages of the certificate of analysis. For the isosorbide and POE species and in the case of multiple esterification with the same fatty acid, the non-applicable factors 'l_x' are set to 1 and the variables *%FA* to 100 %. Accordingly, the first factor of the trilauryl myristyl sorbitan tetraester, for example, is equal to 4 (= 4! / 3! ·1!). Since the equation represents the individual probabilities of esters with a specific fatty acid composition, each possible combination of fatty acids contained in the PS20 mixture must be considered individually. For a component group with the same degree of esterification, the number of these combinations is defined by Eq. (4). The variable 'm' represents the number of different fatty acids specified in the certificate of analysis.

$$\text{number of possibilities} = \frac{(m+k-1)!}{(m-1)!\cdot k!} \tag{4}$$

Eq. (4): Number of possibilities for mono-, and higher-order esters

The calculations were automated using a program written with Visual Basic for Applications (VBA) within Microsoft Excel. Hereby, the results of previous chapters on the molar proportions and the number of variants for corresponding component groups were included. A total of 27′775 individual components were computed, which were characterised by the molar mass, the sum of the OE units and, if applicable, the number and type of esterified fatty acids. For each component, the molar proportion and the weight percentage of the total mixture were calculated, providing a detailed overview of the quantitative composition of the analysed PS20. Since our results were presented in a tabular format, the tool's sorting and filtering features allowed for the grouping of components by any common property. We calculated e.g. the proportion of POE sorbitan monolaurate esters in the product tested at 15.6 % by weight of the total mixture. This value is comparable to the previously reported range of 18–23 % (w/w) for commercial PS20 lots of Croda [22]. Remarkably, our calculations further revealed that POE (20) sorbitan monolaurate, which is listed as the formal structure of PS20 according to the European Pharmacopoeia [33], and the U.S. Pharmacopeia – National Formulary [34], made up less than 1 % by weight.

By filtering esterified components, we determined the molar amount of KOH theoretically required to saponify the fatty acid residues. Conversion of the result to mg KOH per gram PS20 gave the saponification value. In an analogue manner, we calculated the mole equivalents of free hydroxyl groups and thus derived the hydroxyl number. Remarkably, both calculated values agree well with the titration results given in the certificate of analysis (see Table 4).

We repeated the chromatographic analysis required for stochastic modelling, as well as the computer assisted data evaluation for six further PS20 samples. Different qualities, ages and manufacturers were included in the study (see Table 4). The saponification and hydroxyl values of the corresponding certificates of analysis were used as verification parameters for the method. A high prediction accuracy was achieved for the individual saponification and hydroxyl values, which was within the range of 100 ± 10 %. In view of the number and the different origins of the PS20s studied, the result clearly demonstrates the capability of the method.

Based on the same results, but with a different group classification, we were able to acquire information on the general composition of these seven products. Table 5 illustrates the calculated proportions of sorbitans, isosorbides and POE for each PS20. The comparison of batch pairs of same quality with respect to the molar percentages of individual substance classes shows only minor batch-to-batch variability. In the two PS20 HP products investigated, for example, the molar proportions of sorbitans, isosorbides and POEs range between 51.8 – 51.9 %, 30.3 % and 17.8 – 17.9 %, respectively. Although the results indicate a high

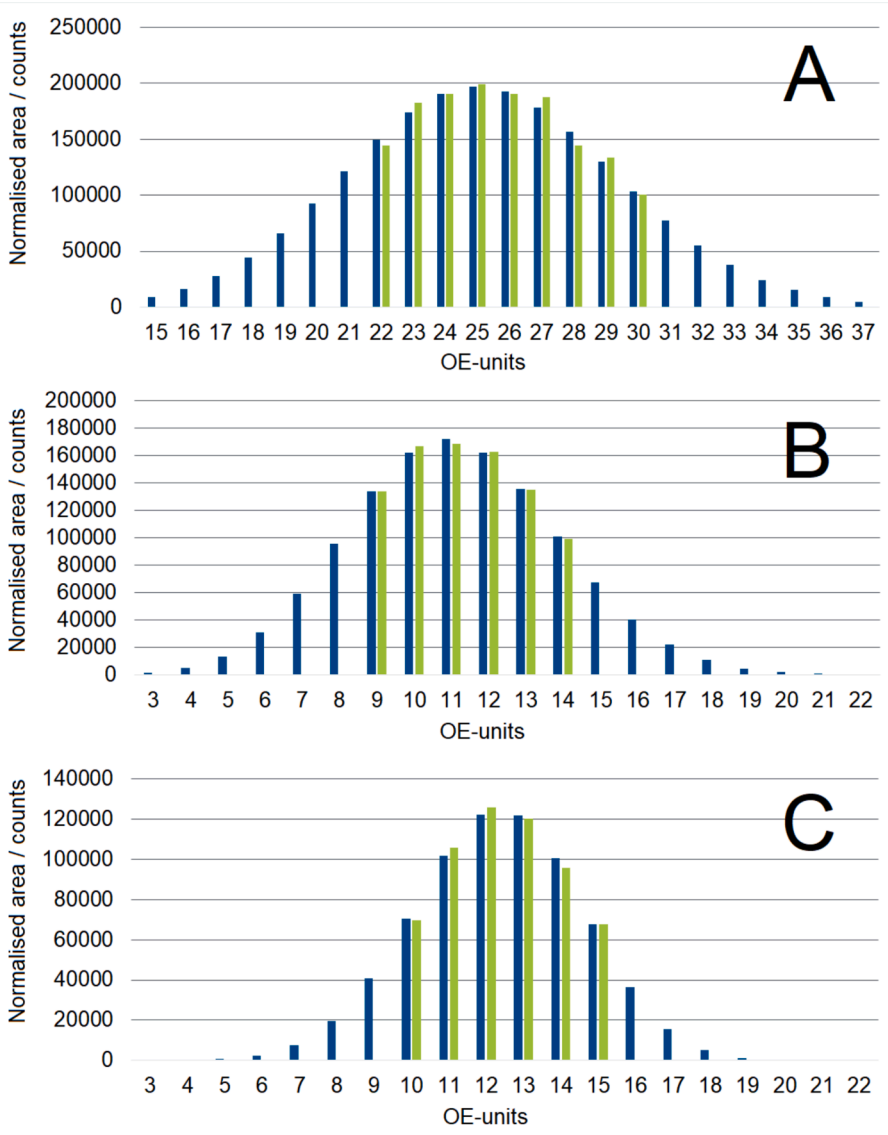

Fig. 4. Modelled (blue) and experimentally determined (green) molar distributions of the number of OE units within the class of A) sorbitans, B) isosorbides and C) POE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Comparison of the calculated saponification and hydroxyl values with test results in the certificate of analysis for studied PS20 samples.

Manufacturer	Type / Quality	Batch	Saponification value			Hydroxyl value		
			Calculated	Certificate	Recovery	Calculated	Certificate	Recovery
Croda	HP	1938157	46.6	45	103.6	102.2	105	97.3
Croda	HP	1244268	45.5	46	98.8	106.8	103	103.7
KLK Kolb	Kotilen-L/1	1–2508621	46.9	45	104.3	104.4	101	103.4
Nanjing Well Pharma Co LTD.	PS 20 for injection	20171030–1 k	47.3	47	100.6	100.8	101	99.8
Nanjing Well Pharma Co LTD.	PS 20 for injection	20190501–1 k	47.6	45	105.8	101.4	101	100.4
Croda	Super Refined	0001204756	52.4	49	107.0	97.3	100	97.3
Croda	Super Refined	0001308151	54.5	50	109.0	93.4	98	95.3
				Mean:	104.2		Mean:	99.6
				%RSD:	3.4		%RSD:	3.2

degree of similarity between materials of the same product type, this assumption cannot be generalised due to the small sample size. Evaluation across all studied materials revealed a pronounced variation in the molar composition. The highest variability of 63.8 % RSD was observed in the molar POE content, that ranged from 1.8 % for batch 20190501–1 k of Nanjing Well Pharma Co LTD to 25.9 % for batch 0001308151 of Croda. Interestingly, the molar content of isosorbides showed an inverse pattern in these products, with 33.0 % for the first mentioned and 16.5 % for the latter. In contrast, the molar amounts of sorbitan components were nearly constant, with a mean value of 56.8 % and a relative standard deviation (RSD) of 8.6 %. This result could suggest a similar distribution of sorbitan species in the PS20 products studied. However, the deviating % RSD of the corresponding weight proportions points to differences in the composition at a more detailed level. We therefore

Table 5
Comparison of the calculated molar and weight proportions of sorbitans, isosorbides and POE for seven different PS20s studied in this work.

Manufacturer	Type / Quality	Batch	Mol% sorbitan	isosorbide	POE	Weight% sorbitan	isosorbide	POE
Croda	HP	1938157	51.9	30.3	17.8	69.0	20.3	10.6
Croda	HP	1244268	51.8	30.3	17.9	68.4	20.8	10.8
KLK Kolb	Kotilen-L/1	1–2508621	53.3	35.9	10.8	69.8	23.6	6.5
Nanjing Well Pharma Co LTD.	PS 20 for injection	20171030–1 k	58.2	37.2	4.6	71.9	25.3	2.8
Nanjing Well Pharma Co LTD.	PS 20 for injection	20190501–1 k	65.2	33.0	1.8	77.2	21.8	1.0
Croda	Super Refined	0001204756	59.4	15.4	25.1	75.1	10.4	14.5
Croda	Super Refined	0001308151	57.7	16.5	25.9	73.6	11.0	15.4
		Mean:	56.8	28.4	14.8	72.1	19.0	8.8
		%RSD:	8.6	31.3	63.8	4.6	31.2	63.1

compared the ester number distribution of sorbitans, with the focus on the mono- and triester groups. These groups make up a relevant proportion of the total mixture and, based on their opposite polarities, are expected to have the greatest influence on the emulsifying ability of PS20. The calculated ratios between the mono- and triester sorbitan species ranged from approx. 2.47: 1 for the Super Refined product #0001308151 of Croda to approx. 5.11: 1 for the HP product #1244268 of Croda (see Fig. 5). To interpret this result in terms of its potential impact on the solubilising and emulsifying properties of the overall product, we used the HLB concept. The literature data on HLB parameters of polysorbates is limited to commercial products, such as POE (20) sorbitan monolaurate, POE (20) sorbitan mono- and tristearate with experimentally determined values of 16.7, 14.9 and 10.7, respectively [35]. Since these HLB values refer to complex mixtures and not to individual fractions, they rather correspond to the mean value over the entirety of components instead of representing the formal structure indicated. Therefore, these data do not allow for a direct comparison between specific component groups. Having characterised PS20 by its individual constituents, however, we were able to calculate the HLB distributions of all PS20s studied using a procedure described by ICI (see Fig. 6) [35]. Although the plausibility of our calculations is supported by the fact that weighted mean values were within ± 10 % of the reported HLB parameter for PS20 of 16.7 [35], the results of this purely theoretical approach should be considered as an approximation. The distributions of all tested materials covered a wide HLB range from approx. 10

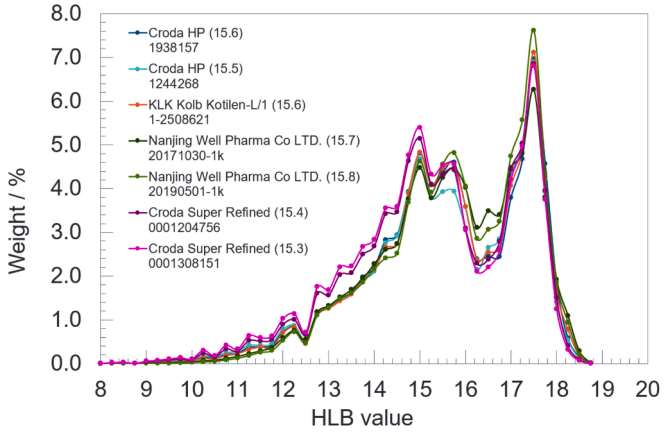

Fig. 6. Calculated HLB value distribution of seven analysed PS20 materials based on the HLB value calculation of the individual esterified components according to the described ICI method [35]. The weighted, calculated HLB average for each PS20 product is listed in parentheses following the product name.

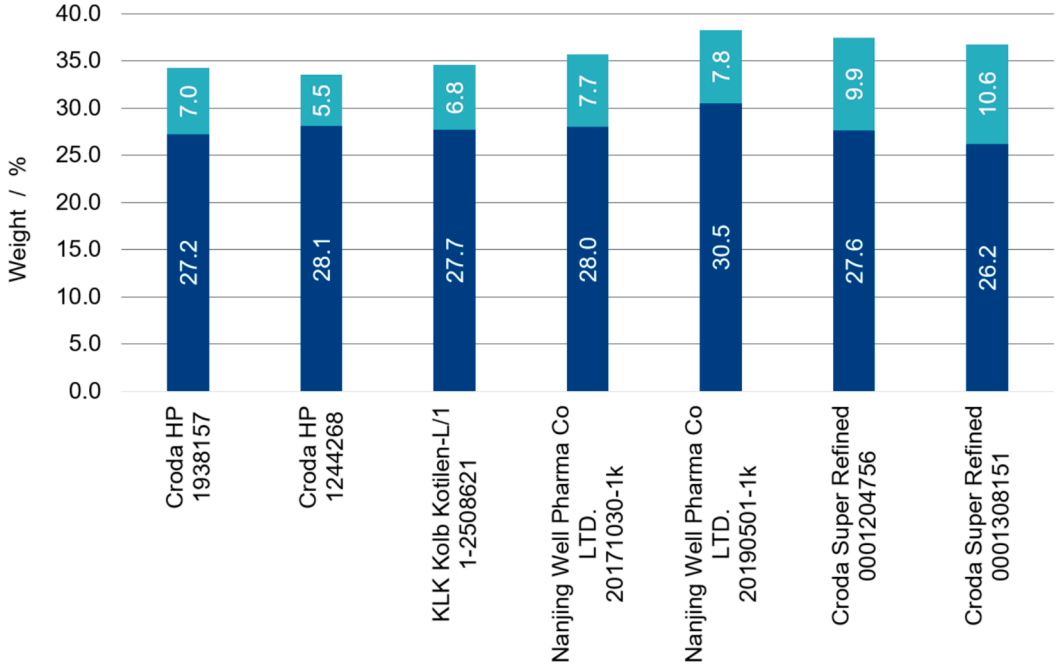

Fig. 5. Weight percentages of monoester (dark blue) and triester (light blue) sorbitan components in the PS20 products studied. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to 19, with a sharp maximum at approx. 18. According to Griffin's original classification [24], the entire range of components is suitable for use as O/W emulsifiers. However, only surfactants with an HLB value greater than 15 are considered solubilisers.

Interestingly, the products of Nanjing Well Pharm. were characterised by a significantly higher weight proportion of components with an HLB value of approx. 16 to 17, while for the Super Refined products, PS20 constituents with an HLB value of approx. 13–15 were more represented. A detailed analysis of components in this last-mentioned region reveals the prevailing presence of POE sorbitan triester species, which is consistent with the higher tri- to monoester ratios for the Super Refined products illustrated in Fig. 5. Considering the variations observed in the HLB profiles, it can be assumed that, despite the very similar weighted HLB mean values, the materials may exhibit different emulsifying properties. Detailed characterisation of PS20 may therefore help explain deviations in the behaviour of different product qualities and contribute to the robustness of the formulations.

The ability to resolve the full compositional complexity of PS20 down to individual ester species may further open new possibilities for a more informed selection and understanding of PS qualities in biopharmaceutical formulations. Recent studies have demonstrated that the stabilising effect of PS20 is not solely dependent on its overall concentration but is also influenced by the distribution of esterified species. For instance, Tomlinson et *al.* (2020) and Diederichs et *al.* (2023) highlighted the superior interfacial protection offered by isosorbide monolaurate and monoesters [23,36], respectively, while Gregoritza et *al.* (2024) and Glücklich et *al.* (2023) reported protein-specific effects of selective ester degradation [37,38]. These findings suggest that the ester composition of PS20 can modulate critical formulation attributes such as surface activity, micelle formation, and resistance to agitation-induced aggregation. The method presented in this study enables detailed characterisation of these ester species and could therefore provide an understanding of the stabilising properties. It may help determine whether different PS20 compositions provide similar stabilising effects or whether specific protein interactions require a more targeted approach.

4. Conclusions

Polysorbate 20 (PS20) is a widely used nonionic surfactant known for its emulsifying and stabilising properties in pharmaceutical and biopharmaceutical applications. It is a complex mixture of numerous constituents that results from a multistep reaction of sorbitol with fatty acids and ethylene oxide. In addition to the analytical focus on determining the PS20 content and monitoring the degradation products in biopharmaceutical formulations, there is a growing interest in structure–activity relationships of its fractions or individual components. To approach this aspect, a thorough understanding of the composition, as well as the ability to identify and analyse variations in the quality of the raw material, is of importance. Previously, Brandner (1998) presented a theoretical calculation of weight percentages of sorbitan and isosorbide fractions, grouped according to the number of esters [17]. This approach, based on the hydroxy and saponification numbers, did not account for polyoxyethylenes (POEs) and is unable to capture differences between different PS20 material at a more detailed level. As demonstrated by several groups, high analytical accuracy in the identification and quantification of selected PS20 components is achieved using liquid chromatography with mass spectrometric detection [21,30,31]. However, varying ionisation potentials across different substance groups do not permit a quantitative comparison. Further, due to the vast number of constituents, capturing all structures solely based on their m/z values would be highly time-consuming.

In this work, we introduced a unique approach using two UPLC-MS methods and computational data evaluation, which deciphers the composition of PS20 at the molecular mass level. Our calculations were based on the realisation and experimental evidence that the esterification degree and the sum of OE units are binomially distributed within individual substance classes. The presented approach only requires the following PS20 sample preparations of the intact stock solution in water: i) dilution to a concentration of 40 µg/mL, and ii) quantitative hydrolysis with subsequent dilution to 40 µg/mL.

In the first step, both samples were analysed by the previously reported and validated UPLC-MS method to determine the relative percentage of the fully hydrolysed markers S00, I00 and P00. These results were used to calculate the binomial distribution of the esterification degree within each substance class (see Fig. 7). In the second step,

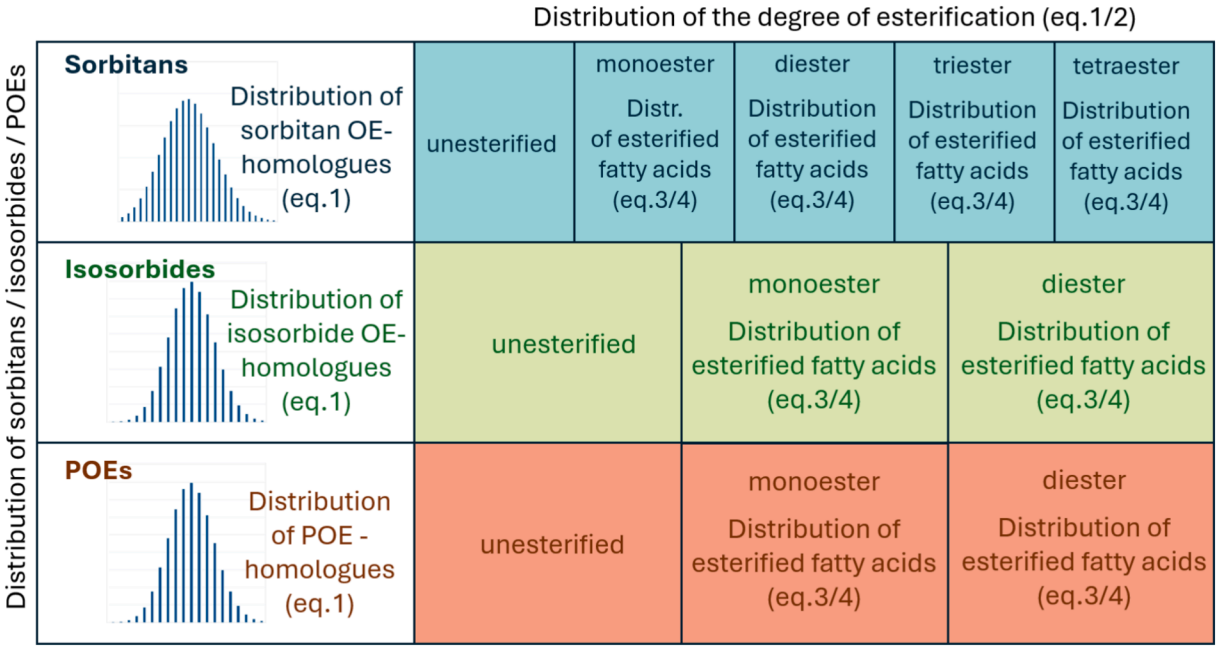

Fig. 7. Overview of the PS20 architecture subdivided into component groups, as investigated in the present study.

analysis of the hydrolysed sample was performed with the developed UPLC-MS method. The ionisation conditions of the single ion mass channel included a cone voltage of 100 V to ensure specific fragmentation of oxyethylene (OE) chains to single OE units. In combination with the full scan MS detection, 6 – 9 representative OE homologues of the respective substance classes were identified, and their molar ratios were determined. The data was modelled using a scaled binomial distribution, with the parameters p and n determined through an approximative optimisation process. This mathematically well-defined correlation provided information on the molar distribution of the OE homologues. It was further used to calculate the total areas, from which the molar distributions of sorbitans, isosorbides and POE were derived. The fatty acid composition listed in the certificate of analysis finally served as the basis for determining the fatty acid distribution by a combinatorial equation.

A total of 27′775 components were calculated with a program written in VBA in Microsoft Excel and individually displayed in a tabular format. The molar contents and weight percentages were determined for each component that was fully characterised by i) the substance class (sorbitan, isosorbide or POE), ii) the number of esters, iii) the fatty acid combination and iv) the number of OE units. With the filter and sort functions, the results table enabled a targeted extraction of information on the molar or weight content of component groups, summarised according to variable, user-defined characteristics. On this basis, we e.g. identified esterified components or those with free hydroxyl groups to access the theoretical saponification or hydroxyl value. For a total of seven different PS20 products studied, we found a very high agreement between the predicted values and those provided in the certificate of analysis. By grouping the components of individual products according to the substance class, a general comparison of the PS20 composition was made. High consistency was found between pairs of batches of the same quality, indicating compositional reproducibility of a specific material type originating from the same manufacturer. Across different products, however, high variability was observed for the molar and weight percentages of isosorbides and POEs. A further difference was identified in the sorbitan mono- to triester ratio, which was lowest in the Super Refined materials. By computing HLB profiles that were based on structures of the individual PS20 constituents, we were able to link this observation to an increased appearance of HLB values in the range of 13–15. It is likely, that the found HLB differences may affect the emulsifying or solubilising properties of the overall material. Thus, the analytical method presented herein may offer a systematic approach during pharmaceutical formulation development and for root cause analysis.

Breaking down the heterogeneity, a detailed understanding of this complex excipient is provided. This may further be instrumental for manufacturers to design and optimise polysorbate products and monitor batch-to batch variability.

In summary, a comprehensive and routine-compatible protocol was presented capable of quantifying PS20 on molecular mass level by two short UPLC-MS methods. Currently, work is ongoing on developing methods to transfer the methodology presented in this study to PS80.

CRediT authorship contribution statement

Dirk-Heinrich Evers: Conceptualization, Investigation, Methodology, Writing – original draft, Formal analysis. **Janek Giebel:** Investigation, Writing – original draft. **Finnya Nienau:** Formal analysis, Writing – original draft. **Stefan Carle:** Resources, Writing – review & editing. **Sascha Gorissen:** Formal analysis, Writing – review & editing. **Julia Buske:** Resources, Writing – review & editing. **Michael E. Herbig:** Formal analysis, Writing – review & editing, Writing – original draft. **Patrick Garidel:** Resources, Writing – review & editing. **Elina Hagelskamp:** Writing – original draft, Conceptualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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