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Separation of nonionic surfactants according to functionality by hydrophilic interaction chromatography and comprehensive two-dimensional liquid chromatography

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ABSTRACT

It is shown, that amphiphilic polymers—such as polysorbates and fatty esters of polyethylene glycol can be separated by comprehensive two-dimensional liquid chromatography using a reversed phase column (under critical conditions for the polyoxyethylene chain) and a HILIC column, which may arranged in different order. The mobile phases in both dimensions can be 93–97 wt% acetone water. As the retention of higher esters on the reversed phase column is very strong, this column should be used as the first dimension. On the HILIC column all fractions elute within a reasonably short time (at a flow rate of 2.5 ml/min within 2 min). With a flow rate of 0.1 ml/min in the first dimension, a full separation can be achieved in 90 min.

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1. Introduction

Amphiphilic polymers, which contain hydrophilic and hydrophobic blocks, are in widespread use as surfactants or emulsifiers for various applications. In most of these polymers, the hydrophilic blocks consist of polyoxyethylene; while there is a wide variety in the hydrophobic part of these molecules [1,2].

Typical examples are fatty alcohol ethoxylates (FAE), which are synthesized by ethoxylation of fatty alcohols. Similar molecules can also be obtained by ethoxylation of alkyl phenols of fatty esters [3], especially the methyl esters (FAME). If these samples are synthesized from pure starting materials, they can be characterized quite easily [4–8]: the required information is the molar mass distribution of the ethoxylates and the content of polyethylene glycol (PEG), which results from chain transfer to water. With technical starting materials, characterization is more complicated: as (for example) technical dodecanol typically contains also tetradecanol and hexadecanol, the resulting ethoxylate will contain several series with different end groups [9].

The situation is even more complicated with fatty esters of polyethylene glycol (PEG), which can be synthesized by ethoxylation of fatty acids or esterification of PEG. Obviously, these products will not only contain the monoesters, but also PEG and the diesters, which makes characterization more difficult [8,10–12].

An even higher degree of complexity is found in fatty esters of ethoxylated sorbitan, which are often referred to as polysorbates (trade name TWEEN) [13–22]. Polysorbates are synthesized from sorbitol by subsequent ethoxylation and esterification with fatty acids. In the synthesis of polysorbates, various side reactions may occur, which lead to different byproducts (Scheme 1).

At the temperature required for the ethoxylation, sorbitol undergoes a condensation to sorbitan (a tetrafunctional molecule), and to some extent a further condensation to isosorbide (a difunctional molecule). These molecules react with ethylene oxide (EO): sorbitan yields a four-armed star, isosorbide a linear chain. The water formed in the condensation reacts with ethylene oxide to yield PEG (also a linear difunctional chain). These three hydrophilic species are then esterified with a fatty acid: obviously, this will not only yield the desired monoesters, but also some diesters of PEG and ethoxylated isosorbide as well as di-, tri- and even tetraesters of ethoxylated sorbitan. Moreover, the products will contain the unreacted ethoxylates. For the use as emulsifiers, the ethoxylates without the fatty ester are useless, while the higher esters are very hydrophobic; hence they rather require an emulsifier. Obviously, the formation of side products in the synthesis cannot be completely avoided.

The key parameter for the application of amphiphilic molecules is the hydrophilic–lipophilic balance (HLB), which depends on

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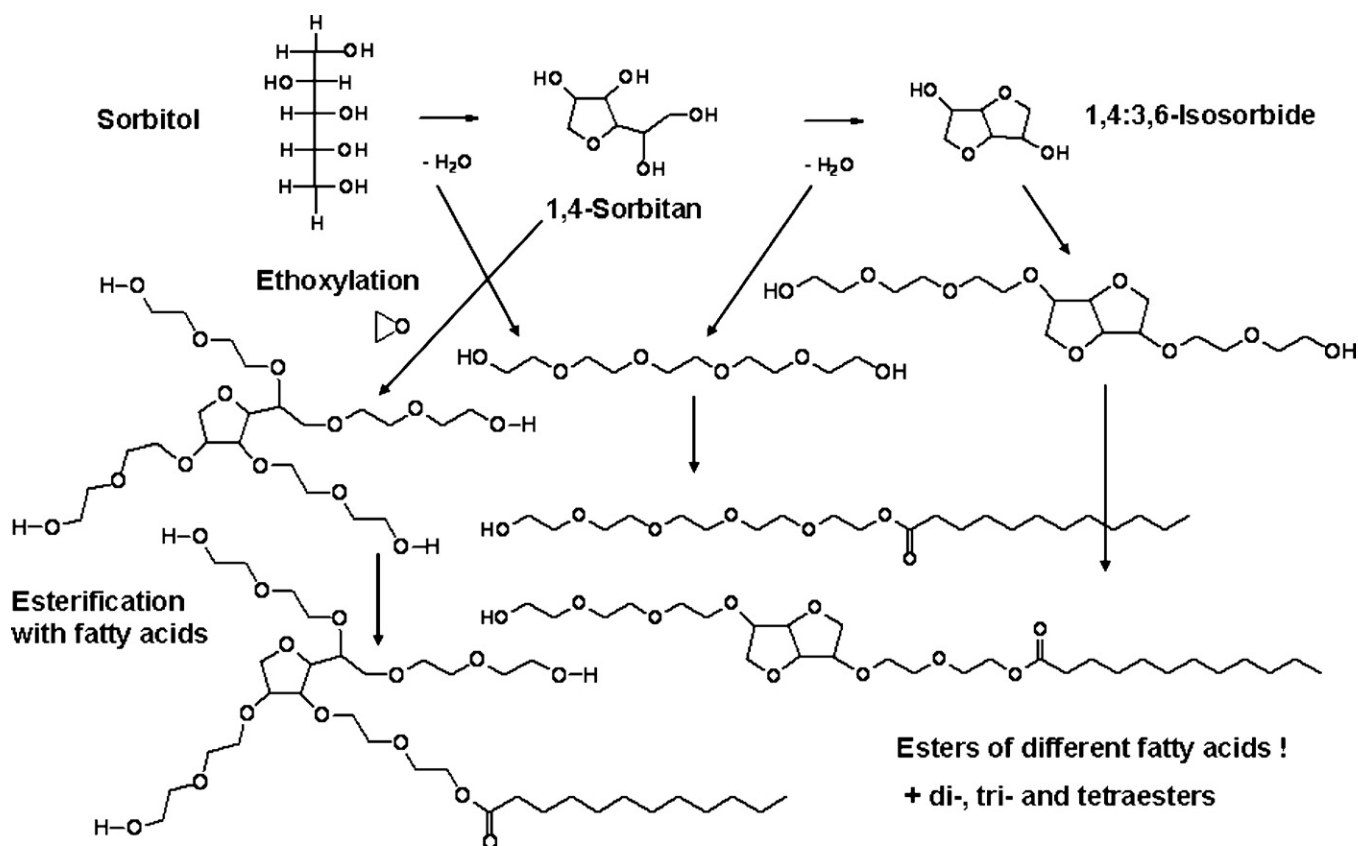

Scheme 1. Possible structures in the synthesis of polysorbates.

the relative length of the hydrophilic and lipophilic blocks, and – of course – on their number. This means, that possible side products with a different structure will strongly influence the physical properties and thus the performance of these products. Consequently, a reliable characterization of such products is crucial for many applications.

The analysis of such complex samples is, however, a difficult task, which can only be solved by sophisticated techniques, such as two-dimensional chromatography [9,10,23,24] or matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) [25]. While FAE can be reasonably characterized by MALDI-TOF-MS alone, this is not the case with highly complex polymers such as polysorbates, which may contain species with the same molar mass, but different structure. As will be shown in another paper [26], unambiguous results for such highly complex samples can only be obtained with fractions from liquid chromatography, in which all structures except one can be excluded. For example, there may be molar masses, which could correspond to PEG, the ethoxylates of sorbitan and isosorbide or their mono- and diesters with different fatty acids. In a spectrum of the raw material no discrimination is possible, but in a fraction containing just one of these series the other species cannot be present, and thus the assignment will unambiguous.

In principle, different chromatographic modes can be employed for this purpose.

1.1. Separation modes in liquid chromatography of polymers

Size exclusion chromatography (SEC), which is an entropic process, separates according to molecular size. In two-dimensional chromatography (2D-LC) of polymers, SEC may be applied, but because of its low selectivity is not the method of choice in the analysis of highly complex samples.

Liquid adsorption chromatography (LAC) is based on the interaction of the sample molecules with the stationary phase, which is typically governed by the enthalpy change. LAC can be performed on normal or reversed phases: NP-LAC separates according to the number of polar groups (EO, OH, etc.), and RP-LAC separates according to the number of non-polar groups (alkyl, fatty ester, etc.).

In both cases, the retention is also influenced by the other structural units, consequently, a big mess of peaks will be observed, which originate from the different homologous series.

This can be avoided at the critical point of adsorption (CAP) for a given structural unit: in a special mobile phase composition (and temperature) entropic and enthalpic contributions compensate each other. In this case the corresponding polymer chain becomes “chromatographically invisible”, which means, that all (non-functional) chains elute at the same volume, regardless their molar mass.

This is utilized in liquid chromatography at critical conditions (LCCC) [12,27–30]. If a polymer contains an end group, which is adsorbed at the CAP of the repeat unit, these chains will elute as a sharp peak (independent on molar mass) at an elution volume corresponding to that of the end group [31]. Under such conditions, monofunctionals and A–B diblocks (with an adsorbing block A) elute at the CAP for B as narrow peaks, and so do B–A–B triblocks.

A completely different behaviour is, however, observed with A–B–A triblocks (or difunctional chains) [11,28]. The probability of the second end group (or block) to be adsorbed depends on the size of the block between them: even at the CAP for B, the retention of A–B–A structures decreases with increasing length of the block B. Consequently, the individual oligomers will be separated in SEC order, but at elution volumes much larger than the void volume. This can be utilized for the separation of diesters [6,8]. On the other hand, small amounts of diesters will be difficult to detect, if

they are resolved into many very small peaks. In two-dimensional separations, this is an even more serious problem: in the second dimension such small peaks will often be below the detection limit.

Especially difficult is the situation with amphiphilic stars, such as the higher esters in polysorbates. A separation according to the number of hydrophobic groups at the CAP for PEG is rather difficult in such complex samples. As is shown in another paper [22], the hydrophilic part and the monoesters can be resolved quite well, but the higher esters cannot easily be quantified, as they do not elute as single peaks.

In this paper we have now tried to find a system, which allows a separation of amphiphilic molecules according to the number of terminal hydroxy groups. This would mean, that the separation according to the size of the center block in A–B–A structures must be suppressed, which should not be possible, if the separation is based on the adsorption on the surface of the stationary phase. The desired retention behaviour might, however, be observed, if the separation was based on partitioning, as is the case in hydrophilic interaction chromatography (HILIC) [32–35].

As will be shown in other papers [26,36], a separation of linear and branched ethoxylates without hydrophobic groups (such as PEG, their mono- and dimethyl ethers, PEG macromonomers, ethoxylated glycerol, etc.) according to the number of hydroxy groups can be achieved without any influence of the number of EO units. Hence we have tried to apply this concept also to amphiphilic molecules.

2. Experimental

2.1. Materials

Polysorbates were purchased from Sigma–Aldrich (Vienna, Austria), fatty acids and PEGs from FLUKA (Buchs, Switzerland). Fatty acid ethoxylates were provided by the Institute of Heavy Organic Synthesis (ICSO), Kedzierzyn-Kozle (Poland).

2.2. Methanolysis of polysorbates

Methanolysis of polysorbates was performed in 2–5 ml (filling volume) mirowave process vials (Biotage AB), which were oven dried under vacuum prior to use. The samples were dissolved in the 20-fold volume of methanol, to which 0.0005 g sodium had been added [22]. These solutions were added to the vials which were subsequently sealed with an aluminum crimp top and Teflon septum. The vials were then evacuated and purged with argon (3 cycles). The sealed microwave process vials were subsequently introduced into the cavity of the single-mode microwave reactor (Biotage Initiator Eight EXP 2.0, absorbance level: high). The reaction temperature was 100 °C. The reaction time required for complete methanolysis (as confirmed by LCCC) was 10 min [22].

2.3. Synthesis of esters

Mono and diesters of PEG and higher esters of polysorbates were synthesized by conventional heating in toluene with p-toluene sulphonic acid as catalyst. Water was removed by physical means such as distillation as low boiling azeotropes with toluene using a Dean–Stark apparatus. For synthesis of mono and diesters of PEG, molar ratio of alcohol and fatty acid was approximately 1:2, respectively. The reaction was completed in 5–6 h at 150 °C. Both mono and diesters are obtained in reasonable amounts. Higher esters of polysorbates were synthesized in the same way with the corresponding molar ratio.

2.4. Chromatography

These investigations were performed using the following instrumentation.

System A was an isocratic HPLC system Ultimate 3000, consisting of a pump ISO-3100A, column thermostat TCC-3000, autosampler WPS-3000SL, an RI detector RI 101, all from Dionex (Germerink, Germany). Mobile phases were mixed by mass and vacuum degassed, their composition was controlled by density measurement using a DMA 60 density meter equipped with a measuring cell DMA 602M (A. Paar, Graz, Austria).

System B was a gradient system Ultimate 3000, consisting of a pump DGP-3600A, solvent degasser SRD-3600, column thermostat TCC-3000, autosampler WPS-3000SL, all from Dionex (Germerink, Germany), an evaporative light scattering detector PL-ELS 2100 (Polymer Laboratories/Varian, Church Stretton, Shropshire, UK). One-dimensional separations were performed at a flow rate of 0.5 ml/min. The mobile phases were mixed by the gradient pump, so their compositions are given in volume percent. Data acquisition and processing was performed using the software Chromeleon (Dionex, Germerink, Germany).

Comprehensive two-dimensional liquid chromatography was performed using the 10-port valve in the TCC-3000, which was equipped with two 200 μl loops. The flow rate in the first dimension was always 0.1 ml/min, in the second dimension 2.0 or 2.5 ml/min, depending on the column used.

The following columns were used in this study (specifications given by the producer):

- Luna HILIC 5 μm (Phenomenex, Torrance, CA, USA; 250 mm × 4.6 mm; particle diameter = 5 μm; pore size = 200 Å, carbon load: 5.7%)
- Jupiter 5 μm C18 300 Å (from Phenomenex, Torrance, CA, USA: silica-based octadecyl phase; 250 mm × 4.6 mm; particle diameter = 5 μm; pore size = 300 Å, carbon load: 13.34%)
- Jupiter 3 μm C18 300 Å (from Phenomenex, Torrance, CA, USA: silica-based octadecyl phase; 150 mm × 4.6 mm; particle diameter = 3 μm; pore size = 300 Å, carbon load: 13.34%)

The solvents (acetonitrile, acetone and water, all HPLC grade) were purchased from Roth (Karlsruhe, Germany). Polyethylene glycols (PEG), fatty acids and polysorbates were purchased from FLUKA (Buchs, Switzerland).

In the comprehensive separations, we have used two different setups:

Setup 1: First dimension: Luna HILIC (250 mm × 4.6 mm), 95% acetone, 0.1 ml/min
Second dimension: Jupiter 3 μm C18 (150 mm × 4.6 mm), 93% acetone, 2.5 ml/min

Setup 2: First dimension: Jupiter 5 μm C18 (250 mm × 4.6 mm), 90% acetone, 0.1 ml/min
Second dimension: Luna HILIC (250 mm × 4.6 mm), 95% acetone, 2.0 ml/min

3. Results and discussion

As the first step we tried to separate fatty ester ethoxylates on the HILIC column under typical conditions in acetonitrile–water and acetone–water mobile phases.

As can be seen in Fig. 1, very similar results were obtained in both acetonitrile–water and acetone–water mobile phases. Because of the higher price and toxicity of acetonitrile we have, however, preferred acetone in the following measurements. The main advantage of acetone is, however, that on the reversed phase column used in the other dimension there is a second CAP for PEG at 90–95% acetone, while PEG elutes in the SEC mode in mobile phases containing 80% acetonitrile or more. Then we

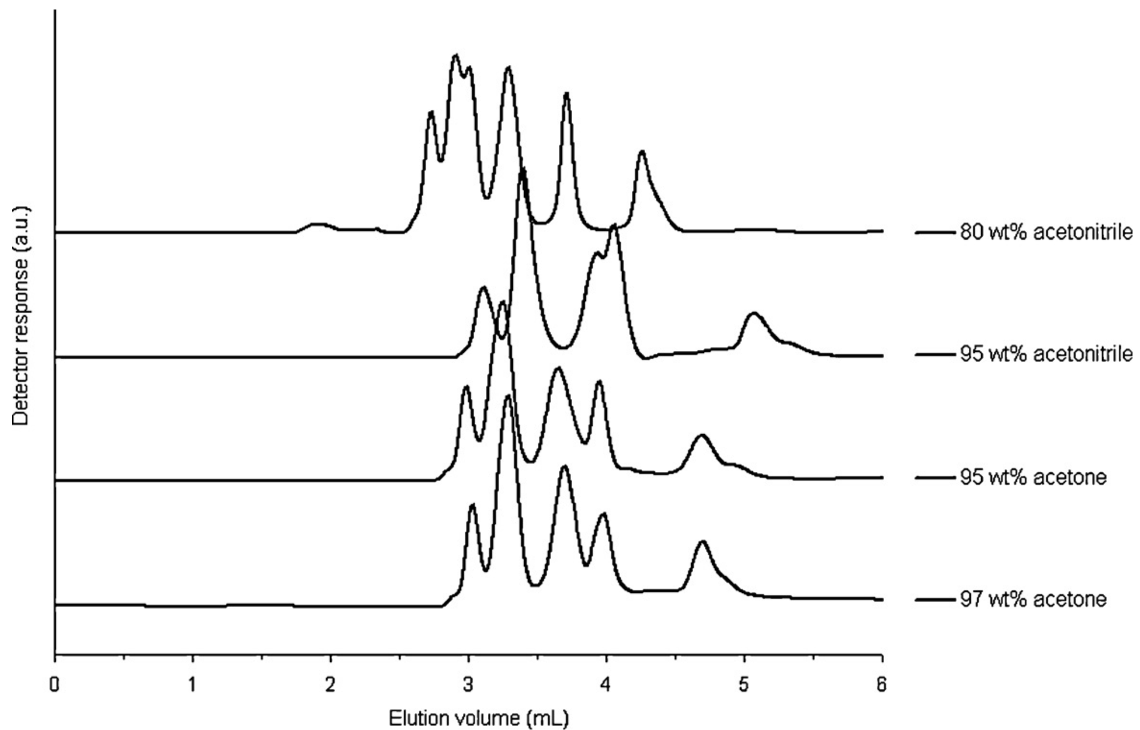

Fig. 1. Separation of Tween 20 on LUNA HILIC 200 Å in different mobile phases, flow rate 0.5 ml/min, and RI detection.

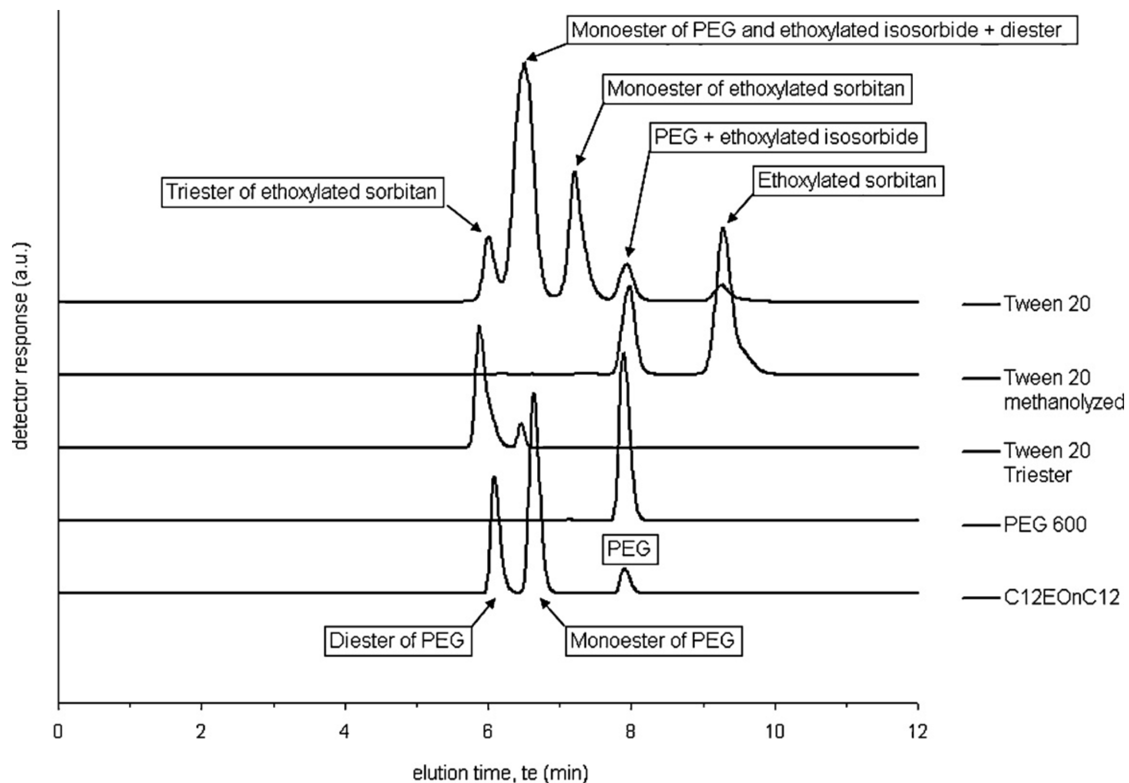

Fig. 2. Separation on LUNA HILIC 200 Å in 97% acetone, flow rate 0.5 ml/min, ELSD.

analyzed several Tweens, the methanolyzed samples, the corresponding triester and PEG 600 under the same conditions The result obtained in 97% acetone with Tween 20 is shown in Fig. 2. Evidently, the methanolyzed samples contain two fractions with different functionality: PEG and the ethoxylated isosorbide elute at about 8 min, the ethoxylated sorbitan at 9.5 min. The triester appears at 6 min, the peaks at 6.5 and 7.5 min should correspond to the di- and monoesters. This could be confirmed by MALDI-TOF-MS, as will be shown in another paper [26]. We have collected fractions from the HILIC column and subjected them to MALDI-TOF-MS, which allowed an identification of the individual series.

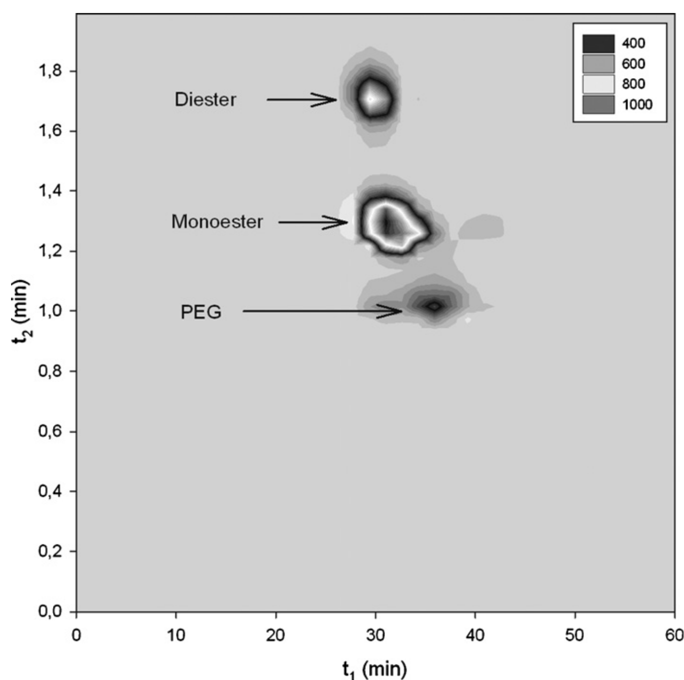

Fig. 3. Comprehensive separation of PEG 600 palmitate (Setup 1).

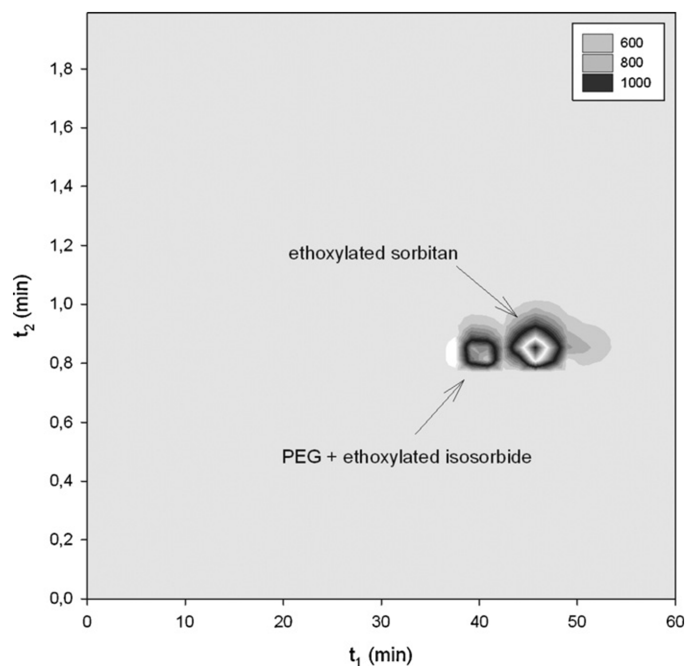

Fig. 5. Comprehensive separation of methanolyzed Tween 40 (Setup 1).

Based on these findings, we have combined this separation with LCCC on a reversed phase column (as has already been described previously [8,22,24]) to a comprehensive separation.

In the second dimension, a 15 cm column had to be used on which the back pressure at the required flow rate of 2.5 ml/min was still in a reasonable range (typically less than 200 bar).

As can be seen in Fig. 3, a clear separation of PEG, mono- and diesters can be achieved for PEG 600 monopalmitate (synthesized as described), and a similar result was found for Tween 40 (Fig. 4).

The peak of the hydrophilic part consists of PEG, ethoxylated isosorbide and ethoxylated sorbitan, as can be seen from Fig. 5, which shows the separation of methanolized Tween 40. Evidently, the peak of the ethoxylated sorbitan appears only in the methanolyzed sample, which is quite reasonable: in the esterification the probability to react is much higher for a tetrafunctional molecule than for difunctional molecules (like PEG or ethoxylated isosorbide). This was confirmed by comparison with the results described in another paper [26], in which fractions from a one-dimensional separation on the HILIC column were subsequently analyzed by MALDI-TOF-MS.

Fig. 6 shows the separation of the triester, which was synthesized by esterification of Tween 40 with palmitic acid. This sample consists mainly of the triester, but it contains also some diester and also the tetraester of ethoxylated sorbitan, which did not elute

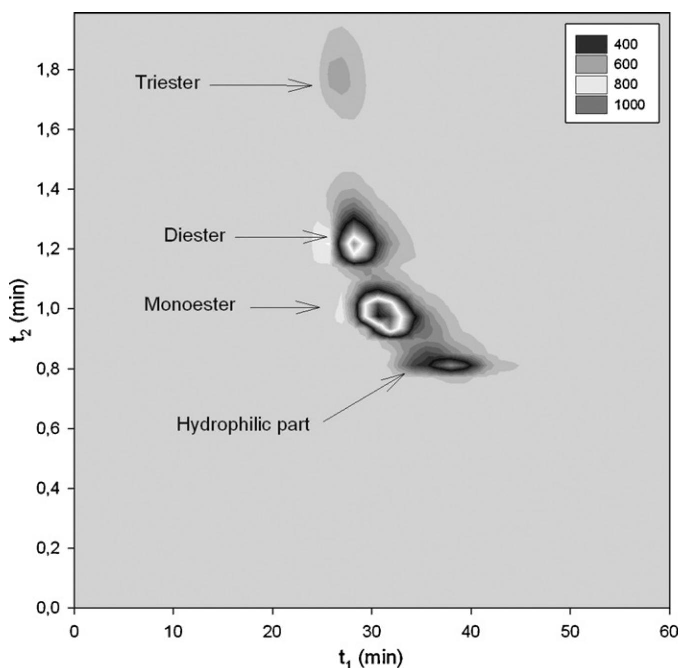

Fig. 4. Comprehensive separation of Tween 40 (Setup 1).

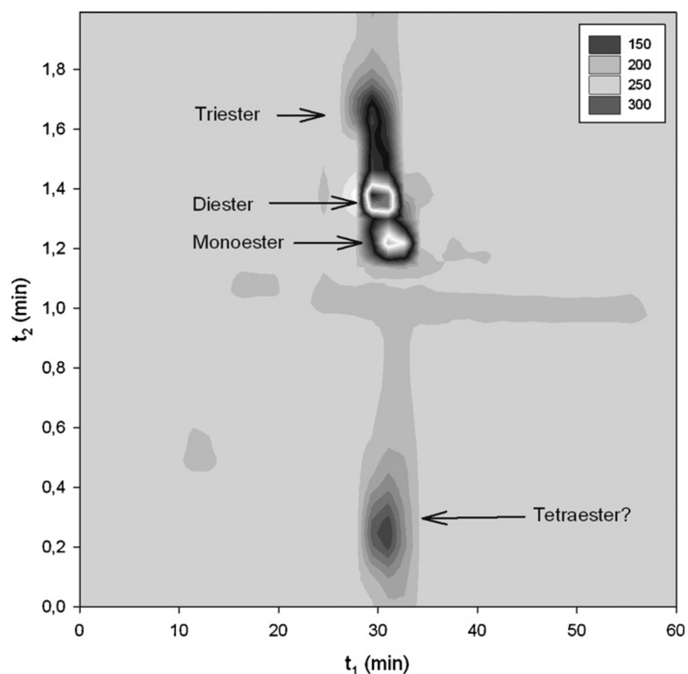

Fig. 6. Comprehensive separation of Tween 40 triester (Setup 1).

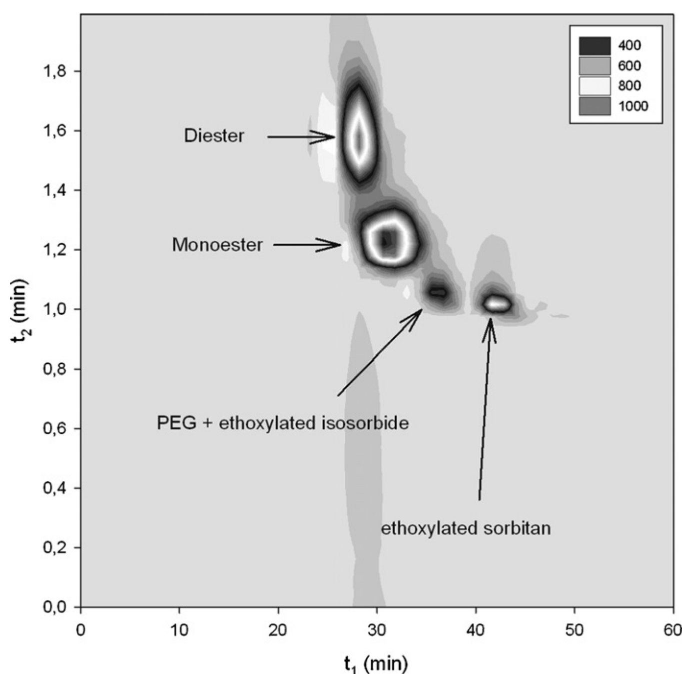

Fig. 7. Comprehensive separation of Tween 60 (Setup 1).

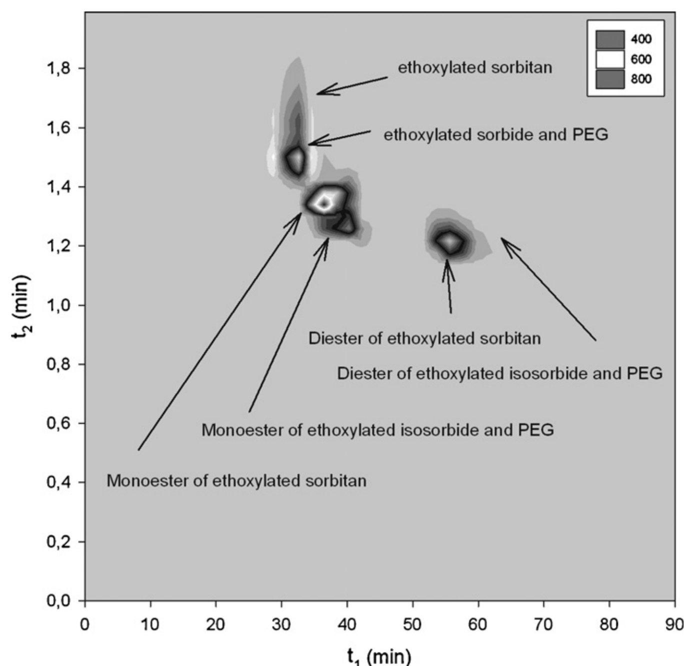

Fig. 9. Comprehensive separation of Tween 40 (Setup 2).

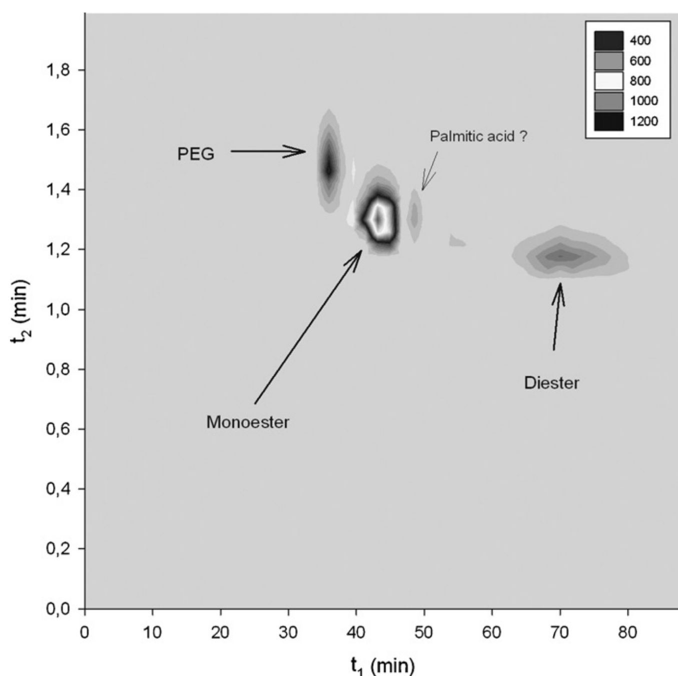

Fig. 8. Comprehensive separation of PEG 600 palmitate (Setup 2).

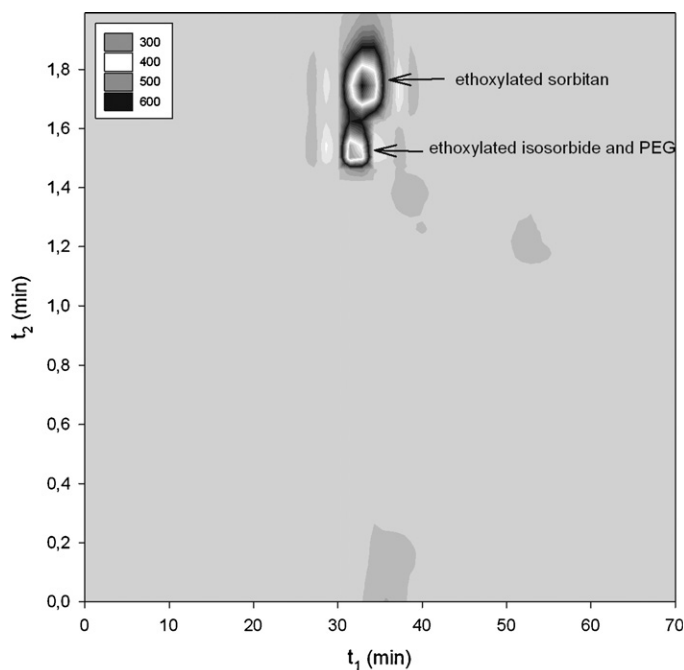

Fig. 10. Comprehensive separation of methanolyzed Tween 40 (Setup 2).

within the cycle time, hence it appears only in the next cycle (at about 0.2 min). Such a wrap-around is also observed for the triesters in Tween 60 (Fig. 7).

The longer the fatty acids are, the higher is the retention on a reversed phase column, which means, that the cycle time of 2 min is not sufficient for such samples. This problem cannot be solved by using higher flow rates in the second dimensions because of the increasing back pressure of the column, and a shorter column would not allow a sufficient resolution. Hence we had to use a different approach.

In LCCC on a reversed phase column the higher esters appear at rather high elution volumes, while the retention of the last peak on the HILIC column (the tetrafunctional sorbitan ethoxylate) elutes still at a reasonable volume (less than 5 ml). On this column, all peaks appear before 2 min at a flow rate of 2.5 ml/min, which could be applied with a moderate back pressure (typically less than 150 bar).

Hence we exchanged the dimensions: now we used the HILIC column in the second dimension, and in the first dimension, where the flow rate is just 0.1 ml/min, a 25 cm Jupiter column. As can be seen in Fig. 8, a very nice separation of PEG, mono- and diesters can be achieved for the palmitate of PEG 600. Of course it is also possible to analyze such samples by one-dimensional LCCC. This separation was only performed for comparison, as it shows, where

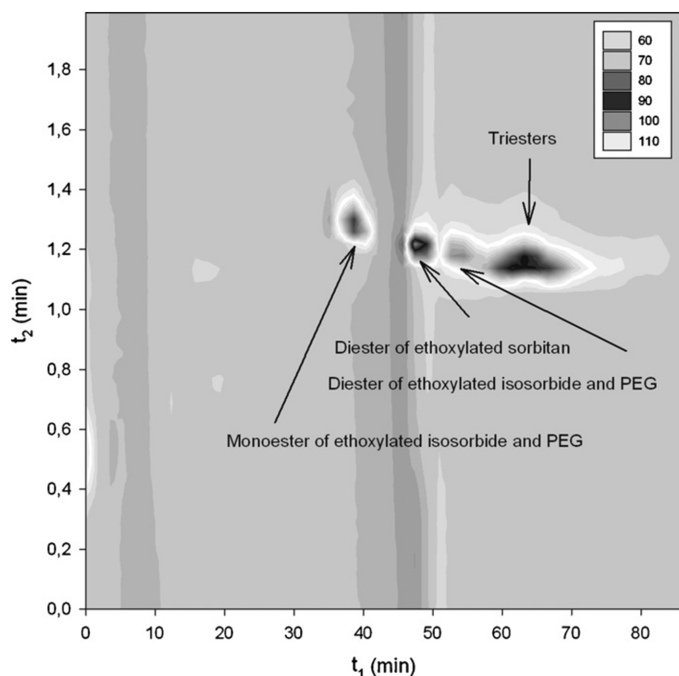

Fig. 11. Comprehensive separation of Tween 40 ester (Setup 2).

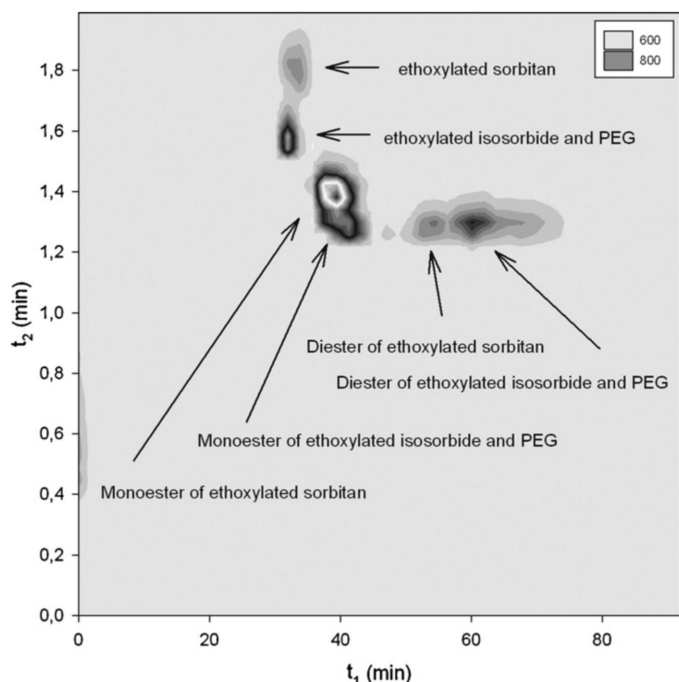

Fig. 12. Comprehensive separation of Tween 60 (Setup 2).

the mono- and dipalmitates appear in the 2D separation of a more complex sample.

Very similar results were obtained with Tween 40 (Fig. 9). In this case even a separation of the monoesters with a different hydrophilic part could be achieved. A comparison with the methanolyzed sample (Fig. 10) shows, that in Tween 40 most of the ethoxylated sorbitan exists as monoesters: evidently, almost no ethoxylated sorbitan (in free form) is present in the original sample, while it is the main component in the methanolyzed product.

Fig. 11 shows a chromatogram of the triester of Tween 40. Evidently, this product contains still some mono- and diesters (which have different architecture).

A quite similar result is obtained for Tween 60 (Fig. 12). In all samples an excellent separation of all series can be achieved in 90 min.

The results obtained with other polysorbates (Tween 20, 21, and 80) are given as supplementary materials.

The analysis time could even be reduced by using shorter columns and smaller sample loops. Further studies with a 10 cm Luna 3 μm HILIC column and 100 μl loops shall show, whether the same results can also be obtained in less than 1 h.

4. Conclusions

Polysorbates can be characterized by comprehensive two-dimensional liquid chromatography with a combination of a reversed phase column and a HILIC column. In principle, the order of dimensions can be changed, but in practice it is favorable to use the reversed phase column in the first dimension, as the higher esters have rather high elution volumes, which would not allow to finish a chromatogram within a reasonable cycle time. On the HILIC column, all peaks appear in a sufficiently short time, which allows cycle times of 2 min. Under such conditions, a full separation of the different functionalities in polysorbates can be performed in 90 min. Further optimization with shorter columns in both dimensions and smaller sample loops should reduce the cycle time to 1 min and the total analysis time to less than 1 h.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.10.118.

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