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Development of a design of experiments optimized method for quantification of polysorbate 80 based on oleic acid using UHPLC-MS

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ABSTRACT

The aim was to develop a straightforward UHPLC-MS quantification method for polysorbate 80 using oleic acid as surrogate marker, which was the commonest substance within the emulsifier. However, hydrolysis of polysorbate 80 and subsequent analysis of fatty acids revealed a co-elution of oleic acid and an isomer while all the other fatty acids were successfully separated by varying retention times and mass-to-charge ratios. For identification and separation of the isomer a derivatization method was evaluated. Oxidation to the corresponding dihydroxystearic acids with potassium permanganate resulted in peak separation of *cis*/*trans* and structural isomers of the 18:1 fatty acids. Hydrolyzed and derivatized polysorbate 80 was quantified indirectly in the range of 0.046–5.83 μg/mL ($R^2 > 0.997$) with a limit of detection of 11.4 ng/mL. Quantification of polysorbate 80 using oleic acid as a surrogate marker showed good reproducibility and linearity. As all isomers of the 18:1 fatty acids were successfully separated, the previously co-eluting peak was identified as elaidic acid and was found as a component in the mixture of the emulsifier polysorbate 80. Additionally, *cis*-vaccenic acid was separated as a second co-eluting isomer. Therefore, derivatization led to successful chromatographical separation of *cis*/*trans* and structural 18:1 fatty acid isomers.

1. Introduction

The emulsifier polysorbate 80 is often used in a wide range of pharmaceutical formulations such as protein formulations and emulsions. In water containing systems, polysorbate 80 is added to increase the solubility of active pharmaceutical ingredients (API) in the aqueous phase [1], typically in a concentration dependent manner. As the critical micelle concentration (CMC) is 13 μg/mL [2], quantification of polysorbate 80 is required at concentrations below the CMC for calculations of the impact of micelle-free solutions on API partitioning. In previous studies, various properties (CMC, cloud point, micellar molecular weight) of polysorbate 80 from different suppliers were compared [3]. The byproducts of the synthesis such as polyethylene glycol have significant impact on those properties. Therefore, quantification of emulsifiers in aqueous phases is an important aspect for understanding interactions within formulations.

So far, the composition of polysorbate 80 regarding the fatty acids is mostly measured via gas chromatography of the methylated fatty acids [4,5], which demands the hydrolysis and derivatization of the emulsifier. To check the usage of state-of-the-art techniques for analytical methods, they should undergo continuous life-cycle management to ensure the application of the best method for the task [6]. Keeping this in mind, various methods for polysorbate 80 analysis have been compared. A simple quantification of polysorbate 80 hydrolyzed to oleic acid is performed with a HPLC and a spectrometric detector [7] but needs 6 h for hydrolysis. Polysorbate 80 can also be quantified by the ethylene oxide chain content as those chains react with cobalt thiocyanate to a blue complex,

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which can be detected spectrometrically in concentrations above 10 μg/mL [8]. As polyethylene glycol is frequently used within pharmaceutical formulations, this method is often not applicable without further calculations as the added polyethylene glycol would undergo the reaction as well. The combinations HPLC-CAD (charged aerosol detector) or HPLC-ELSD (evaporative light scattering detector) also allow quantification of polysorbate 80 as a single peak at low concentrations above 5 μg/mL [9–12]. The variation of analysis methods (fluorescence micelle assay, mixed-mode HPLC coupled to CAD or ELSD) shows comparable results regarding the content of polysorbate 80 but varies in the detection of degradation products [13].

As the mentioned methods are not sufficiently sensitive for most formulations with rather low polysorbate 80 concentrations, a new method shall be evaluated with a UHPLC coupled to a single mass spectrometer (QDa) using a surrogate marker. Polysorbate 80 consists of mixtures of mono- and diesters of fatty acids and polyethylene glycol chains of different lengths [14–16], resulting in a difficult quantification of the whole molecule as the composition varies from batch to batch [17]. The European Pharmacopeia (Ph. Eur.) demands a concentration of more than 58% oleic acid [4], therefore, oleic acid is chosen as the marker substance. Within chromatographic analysis, it is important to include a washing step with high organic phase concentrations to prevent changes in retention properties of columns [18]. As the European Pharmacopeia also describes the presence of further fatty acids (myristic acid, palmitic acid, palmitoleic acid, stearic acid, linoleic acid, linolenic acid) to a certain degree [4], the separation of those shall be obtained by using the mass spectrometer to selectively detect them by their mass-to-charge (*m/z*) ratios.

During the method development a co-eluting peak with the same *m/z* as oleic acid was found. For the separation of the proposed isomers, silver-ion thin-layer chromatography or HPLC followed by gas chromatography was applied in earlier studies [19–23]. As not all isomers were fractionated with this method, the DoE (Design of Experiments) approach with UHPLC-MS should be followed. The separation of various *cis*-, *trans*- and the structural 18:1 fatty acid isomers should be performed within one run. Therefore, the derivatization to UV-detectable substances and separation of structural isomers according to [24–26] is not considered being sufficient. Derivatization through oxidation of oleic acid results in mono- and dihydroxystearic acids using varying oxidizing agents such as ozone [27], selenium dioxide/*tert*-butylhydroperoxide [28], hydrogen peroxide [29]. For the method development in this study, a long-known method for derivatization of double bonds with potassium permanganate according to Lapworth et al. [30] is chosen. As freezing and heating of fatty acids result in an isomerization from the *cis*- into the *trans*-form according to [31,32], the reaction is performed at room temperature to avoid further transformations. In previous investigations, oleic acid reacted with potassium permanganate under alkaline conditions to diols of stearic acid [33–35], which could be analyzed with UHPLC-MS as identity of dihydroxystearic acid has already been proposed with LC–MS/MS (*m/z* 315) [36]. Therefore, the derivatives should be separated and quantified.

As method transfers are crucial, the separation is also optimized chromatographically. The Design of Experiments software Drylab®, which has already been applied for life-cycle management of methods of the European Pharmacopeia [37], is used for method development and robustness testing to obtain the best chromatographic resolution. As retention times and other factors vary from apparatus to apparatus [38,39], a tolerance range and a robust area shall be calculated.

Consequently, polysorbate 80 is hydrolyzed to measure the concentration of oleic acid. The same polysorbate 80 batch is used as reference for quantification in known concentrations to minimize the influence of the varying compositions and esterification grades.

2. Material and methods

2.1. Material

Polysorbate 80 Ph. Eur. quality was supplied by Croda GmbH (Nettetal, Germany, batches: 2607TD1683, 2503TP490, 1403UP2005). Acetonitrile LC–MS grade and acetone HPLC grade were from Th. Geyer (Renningen, Germany). Ammonium acetate in LC–MS quality was ordered from Promochem (Wesel, Germany). Potassium permanganate and sodium hydroxide were ordered from Merck KGaA (Darmstadt, Germany) in Ph. Eur. quality.

Citrate buffer 0.1 M pH 5 was prepared with citric acid monohydrate Ph. Eur. quality (Merck KGaA, Darmstadt, Germany), sodium citrate dihydrate Ph. Eur. quality (Biesterfeld AG, Hamburg, Germany) and purified water Ph. Eur. (DEWA Engineering und Anlagenbau GmbH, Vienenburg, Germany). Purified water in the quality of water for chromatography R (Ph. Eur.) for UHPLC analysis was obtained with a Maxima-apparatus from ELGA LabWater (Celle, Germany) and purified water in LC–MS quality was ordered from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

The following fatty acids were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany): linoleic acid >99%, palmitic acid >99%, stearic acid >99%, myristic acid >98%, oleic acid ((Z)-octadec-9-enoic acid) >99%, palmitoleic acid >98.5%, linolenic acid >99%, elaidic acid ((E)-octadec-9-enoic acid) >99%, petroselinic acid ((Z)-octadec-6-enoic acid) >99%, *cis*-vaccenic acid ((Z)-octadec-11-enoic acid) >97%, *trans*-vaccenic acid ((E)-octadec-11-enoic acid) >99%. Petroselaidic acid ((E)-octadec-6-enoic acid) >99% was ordered from Larodan AB (Solna, Sweden).

2.2. Chromatographic conditions for UHPLC analysis with QDa detection

The analysis was performed with a UHPLC system (ACQUITY UPLC™ system, Waters GmbH, Eschborn, Germany) using a BEH™ 1.7 μm, 2.1 × 50 mm column (Waters GmbH, Eschborn, Germany). Eluents were acetonitrile (B) and aqueous ammonium acetate 10 mM (A) at a flowrate of 0.8 mL/min. Injection volume was 10 μL and injections were performed in triplicate. Peaks were detected with a single mass spectrometer (QDa, Waters GmbH, Eschborn, Germany, mass range 30 to 1250 *m/z*). Negative scan mode with a cone voltage of 15 V was used. The sampling rate was 10 points per second. The substances were measured in single ion mode. Capillary voltage was set to -0.5 kV. The probe temperature was held at 600 °C. The gas flows were standardized by Waters GmbH, the consumption rate of nitrogen was 1200 L/h. The mentioned *m/z* values represented the loss of a proton $[M-H]^-$. The *m/z* values were: *m/z* 227.2 (myristic acid), *m/z* 253.2 (palmitoleic acid), *m/z* 255.2 (palmitic acid), *m/z* 277.2 (linolenic acid), *m/z* 279.2 (linoleic acid), *m/z* 281.2 (oleic acid), *m/z* 283.3 (stearic acid) and *m/z* 315.3 (oxidized 18:1 fatty acids). Evaluation and interpretation of the data was accomplished with the software Empower 2 (Waters Corporation, Milford, USA).

2.3. Chromatographic method optimization using DoE software Drylab®

The DoE software Drylab®4 (Molnár-Institute for applied chromatography, Berlin, Germany) was used to calculate optimized and robust methods for peak separation with a full factorial design. The steady and varied input parameters can be found in Table 1. Four test runs were performed with varying column temperatures (30 °C and 60 °C) and gradient times (5 min and 15 min) for calibration of the model. With those four chromatograms, contour plots of the gradient time versus column temperature were generated. The color code in those diagrams represented the peak resolution

Table 1
Input parameters for DoE software Drylab® for the calibration of the model to calculate an optimized method.

Steady parameters of analytical method		
Flow rate	0.8 mL/min	
Gradient	5–95 % acetonitrile	
Varied parameters of analytical method		
Column temperature	30 °C	60 °C
Gradient time	5 min	15 min

(Rs) between the closest peaks, the so-called critical peaks (blue: nearly no separation, red: peak resolution ≥ 1.5). An optimized method can be found in the red area. Robustness testing of this optimized method could be carried out *in silico* by variation of five parameters (gradient composition at start and end ±1%, column temperature ± 1 °C, gradient time ± 1 min, flow rate ± 0.1 mL/min) to check whether the critical peaks kept a resolution above 1.5 within the selected range. By varying the mentioned factors, 243 experiments were performed *in silico* by the software to obtain the robust area for every optimized method. A frequency distribution (N) gave insight into the distribution of the resulting critical peak resolutions within those 243 experiments.

2.4. Sample preparation and optimized chromatographic methods

2.4.1. Fatty acids of polysorbate 80

As polysorbate 80 should be quantified using the oleic acid peak, a separation of the incorporated fatty acids according to the monograph in the Ph. Eur. [4] should be achieved. Therefore, the four basic test runs were performed with solutions of 2 μg/mL fatty acids (myristic acid, linolenic acid, palmitoleic acid, linoleic acid, palmitic acid, oleic acid, stearic acid) in a mixture of 20% acetonitrile and 80% water (v/v). The DoE optimized method was calculated with a column temperature of 60 °C and gradient of 35–75% acetonitrile in 5 min followed by a washing step with organic medium (Fig. 1a). Retention times of the experimentally tested fatty acids were (Fig. 1b): 2.60 min (myristic acid), 2.72 min (linolenic acid), 2.94 min (palmitoleic acid), 3.32 min (linoleic acid), 3.73 min (palmitic acid), 4.03 min (oleic acid) and 4.82 min (stearic acid).

Afterwards, a solution of polysorbate 80 (29.2 μg/mL) was hydrolyzed with sodium hydroxide solution (1 mol/L) to obtain free fatty acids and analyzed with the calculated optimized method (final polysorbate 80 concentration 5.83 μg/mL).

2.4.2. 18:1 fatty acid isomers

The hydrolysis of polysorbate 80 to oleic acid revealed a co-elution with the same *m/z*. Therefore, quantification was put on hold while possible 18:1 fatty acid isomers (*cis*-vaccenic acid, oleic acid, *trans*-vaccenic acid, petroselinic acid, elaidic acid, petroselaidic acid) were analyzed. Table 2 summarizes those fatty acids which were generated using [40]. Method development was performed with the DoE software Drylab®4. Gradient elution of 50–60% acetonitrile in 5 min, followed by a washing step with organic eluent and a column temperature set to 33 °C was found to be the theoretically best method. The experimentally determined retention times of the 18:1 fatty acids were (Fig. 3): 4.19 min (*cis*-vaccenic acid), 4.34 min (oleic acid), 4.60 min (*trans*-vaccenic acid), 4.62 min (petroselinic acid), 4.71 min (elaidic acid) and 4.91 min (petroselaidic acid).

2.4.3. 18:1 fatty acid derivatives

In the case of insufficient separation of accompanying 18:1 fatty acids, it was necessary to derivatize them which resulted in oxidized 18:1 fatty acid isomers. All six 18:1 fatty acids were dissolved separately at a concentration of 5 μg/mL in acetonitrile containing 0.25% (v/v) acetone. At first, 200 μL fatty acid solution was mixed with 100 μL citrate buffer pH 5 (0.1 mol/L) and 75 μL sodium hydroxide solution (1 mol/L) in Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and 450 μL aqueous potassium permanganate solution (0.4%, w/v) was added. 15 min later, 100 μL acetonic water solution (20%, v/v) was added. Another 15 min later, the tubes were centrifuged (Fresco 21 Centrifuge from Thermo Electron LED GmbH, Langenselbord, Germany) and 800 μL of the supernatant were mixed with 75 μL citric acid (1 mol/L) and analyzed. The optimized method was calculated with the following parameters: the column temperature was set to 40 °C and a gradient elution (0–5 min: 25–40% acetonitrile, v/v) followed by a washing step with organic medium was performed. Experimentally determined retention times of the dihydroxystearic acid derivatives of the 18:1 fatty acids (Fig. 4) were: 2.73 min (*cis*-vaccenic acid), 3.06 min (*trans*-vaccenic acid), 3.18 min (oleic acid), 3.49 min (elaidic acid), 4.42 min (petroselinic acid) and 4.62 min (petroselaidic acid).

As peak separation was achieved using Drylab®4, 200 μL of a hydrolyzed polysorbate 80 solution (29.2 μg/mL) was treated the same way as the 18:1 fatty acids. Briefly, the solution was hydrolyzed for an hour, derivatized in 30 min, centrifuged, the supernatant neutralized and analyzed. As reference polysorbate 80 from the same batch was used in 8 known concentrations for calibration. Calibration of polysorbate 80 using the oleic acid derivative as surrogate marker was performed in the range of 0.046–5.83 μg/mL (R^2>0.997). The limit of quantification was set to the lowest standard value (46 ng/mL), while the limit of detection was 11.4 ng/mL polysorbate 80.

3. Results and discussion

3.1. Analysis of fatty acids

Separation of those fatty acids mentioned in the Ph. Eur. monograph of polysorbate 80 [4], was achieved chromatographically with the DoE software Drylab® and additionally with their varying *m/z* values (Fig. 1a).

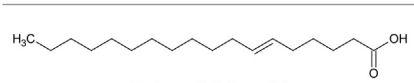
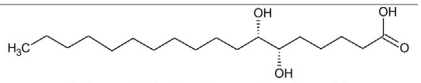
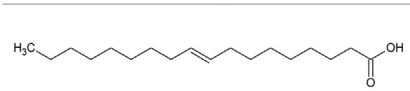
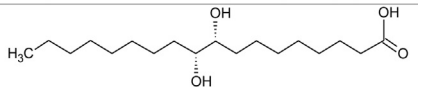
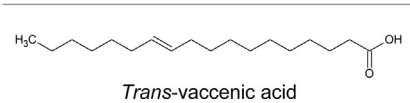
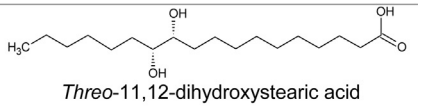
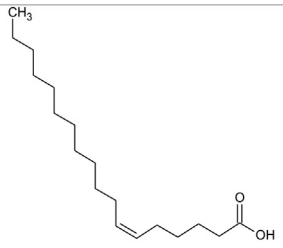
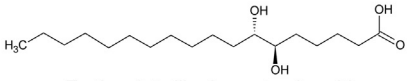
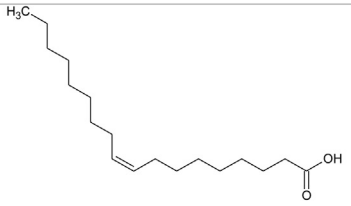
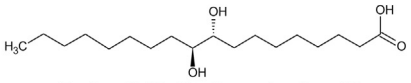
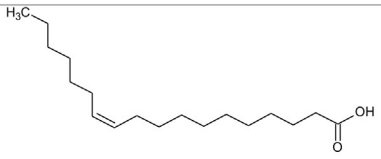
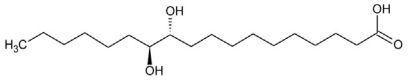
The chosen optimized method allowed a peak resolution of 2.75 between the critical peaks (myristic and linolenic acid, Fig. 1b). The calculated model was practically tested and a very good correlation between theoretical and experimental retention times was found (R^2 = 0.9996; slope 1.0185; intercept −0.2325; Fig. 1).

The *in silico* robustness testing of the optimized method revealed (Fig. 2) that even with a variation of the parameters all experiments passed the criterion (Rs≥1.5). The lowest peak resolution was 1.91 which was found with a column temperature of 59 °C, gradient time of 4 min, flowrate of 0.7 mL/min, start gradient of 36% and end gradient of 76% organic eluent. The highest resolution (3.09) was achieved when the factors were varied in the opposite direction (column temperature of 61 °C, gradient time of 6 min, flowrate of 0.9 mL/min, start gradient of 34% and end gradient of 74% organic eluent). The gradient time had the highest impact on the peak resolution in comparison to the other factors. As an increased gradient time would only prolong the analysis and not improve it significantly, it was not adapted to higher values.

In comparison to the fatty acid method in the Ph. Eur. [4] this method was significantly faster as the samples were only hydrolyzed and not derivatized and the total run-time of the analysis lasted only 7 min including the washing and equilibration step. Additionally, it was more sensitive as the used concentration of polysorbate 80 was less than 6 μg/mL.

After hydrolysis of polysorbate 80, a co-elution (retention time 4.16 min) with the oleic acid peak (retention time 4.03 min) occurred (Fig. 1c). As it had the same mass-to-charge ratio, an

Table 2
Structures of 18:1 fatty acid isomers and their exemplary derivatization products after oxidation.

Isomer	Exemplary derivative
 Petroselaidic acid	 *Threo*-6,7-dihydroxystearic acid
 Elaidic acid	 *Threo*-9,10-dihydroxystearic acid
 *Trans*-vaccenic acid	 *Threo*-11,12-dihydroxystearic acid
 Petroselinic acid	 *Erythro*-6,7-dihydroxystearic acid
 Oleic acid	 *Erythro*-9,10-dihydroxystearic acid
 *Cis*-vaccenic acid	 *Erythro*-11,12-dihydroxystearic acid

isomer of the 18:1 fatty acid was proposed, which might be petroselinic acid according to previous investigations by Ilko et al. [17]. Furthermore, oleic acid is a known contamination material in mass spectrometry [41], which might lead to interactions with the calibration. Also, stearic and palmitic acid are known contaminants, therefore the analysis with mass spectrometry should always be carried out using the background subtraction with blanks to minimize false calculations.

3.2. Analysis of 18:1 fatty acids

To identify the origin of the co-eluting isomer with the same *m/z* value, various 18:1 fatty acids (*cis*-vaccenic acid, *trans*-vaccenic acid, oleic acid, elaidic acid, petroselinic acid, petroselaidic acid) were analyzed with the calculated fatty acid method. As initially no peak separation was achieved chromatographically, a new DoE model was set up using Drylab®. The resolution map only showed a narrow stable robust area with a critical peak resolution of about 0.35 (Fig. 3a). Consequently, a successful separation of all peaks was not achieved with the optimized method as an overlay of the peaks of *trans*-vaccenic, petroselinic and elaidic acid occurred, while a separation of the *cis*- and *trans*-isomers was possible (Fig. 3 b).

Additionally, the analysis of hydrolyzed polysorbate 80 showed no peak separation with the new method as the peaks were still too close. Therefore, it was investigated if the separation could be improved by means of derivatization of oleic acid and its isomers.

3.3. Analysis of derivatized 18:1 fatty acids and quantification of polysorbate 80

The 18:1 fatty acids were oxidized with potassium permanganate to the corresponding dihydroxystearic acids showing a color reaction of the manganese salts from violet over green to brown and a clear supernatant (exemplary formulae see Table 2). Upon addition of acetone, the oxidation was accelerated as it is oxidized by potassium permanganate as well, which was shown earlier [42]. In previous studies, hydrolysis kinetics of polysorbate 80 were concentration-dependent, above the CMC the reaction was slower due to changes in the micellar structure with increasing concentration [43]. Also, oleic acid itself might act as an emulsifier, therefore its concentration could have an impact on the reaction kinetics [44]. However, as in the present study the concentration of polysorbate 80 was below the CMC, no impact on the reaction kinetics was assumed. For far higher concentrations other detection methods

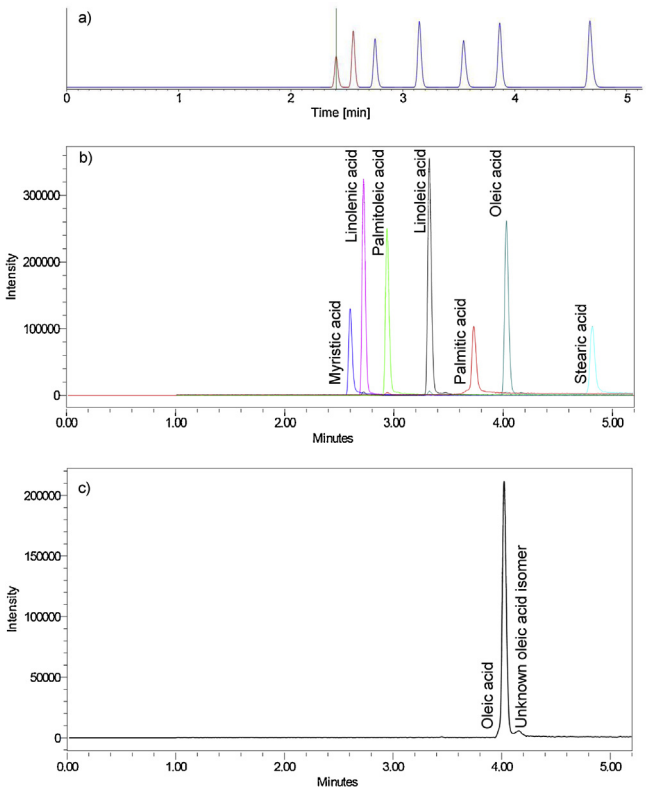


Fig. 1. Chromatogram of the polysorbate 80 fatty acids with Drylab® optimized method (a): calculated method, b): measured chromatogram, c): chromatogram of oleic acid derived from polysorbate 80 by hydrolysis at *m/z* 281.2).

should be preferred as the mass spectrometer might show signal suppression resulting in lower concentrations than actually present.

Optimized peak separation of the 18:1 derivatives was calculated with the DoE software (Fig. 4). The calculated model was practically tested and a very good correlation between theoretical and experimental retention times was found (R^2 = 0.9998; slope 0.9842; intercept −0.1705). The derivatives showed good peak separation with a resolution of 2.72 for *trans*-vaccenic acid and oleic acid which were the critical peaks (Fig. 5a). Consequently, the method was tested for robustness *in silico* (Fig. 5b). All the calculated 243 experiments passed the pre-defined criteria as the peak resolutions were above 2.09. The lowest peak resolution was found at a column temperature of 41 °C, gradient time of 4 min, flowrate of 0.9 mL/min, start gradient of 24% and end gradient of 41% organic eluent. The highest resolution (3.15) was achieved when the factors were varied in the opposite direction (column temperature of 39 °C, gradient time of 6 min, flowrate of 0.7 mL/min, start gradient of 26% and end gradient of 39% organic eluent). The gradient time had the greatest influence on the peak resolution.

As this method was demonstrated to be robust, it was decided to hydrolyze and derivatize polysorbate 80. Quantitative transformation of the emulsifier to its oleic acid derivative was achieved within the given time frame which can be supported by good linearity (R^2 > 0.997) and reproducibility of the reference standards within 0.046–5.83 μg/mL.

In previous studies, degradation of polysorbate 80 in an oxidative environment led to hydroxyl- or keto-derivatives of the oleic acid chain [45,46], which was negligible for this experiment as the calibration was performed with the same substance and therefore the impact of the degradation was the same for the analyzed sample (derived from a formulation) and the standard solutions. Also, the mentioned varying esterification grades of polysorbate 80 [14–16] had no influence as the calibration was undertaken with the same batch.

As peak separation was achieved, the unknown co-eluting 18:1 fatty acid isomer in the polysorbate 80 samples (Fig. 1c) was identified as the derivative of elaidic acid (Fig. 6). Additionally, the isomer *cis*-vaccenic acid was separated while it co-eluted with oleic acid as well within the first fatty acid method (retention time of 3.98 min). *Trans*-vaccenic acid, which is mostly formed in animal tissue [47], was not found, as the used oleic acid for synthesis of the emulsifier was extracted from olive oil. In contrast to [17], petroselinic acid was not found, which might be due to different batches used between the studies.

Consequently, with the additional derivatization a *trans*- and a structural isomer of oleic acid could be separated which has not been achieved before.

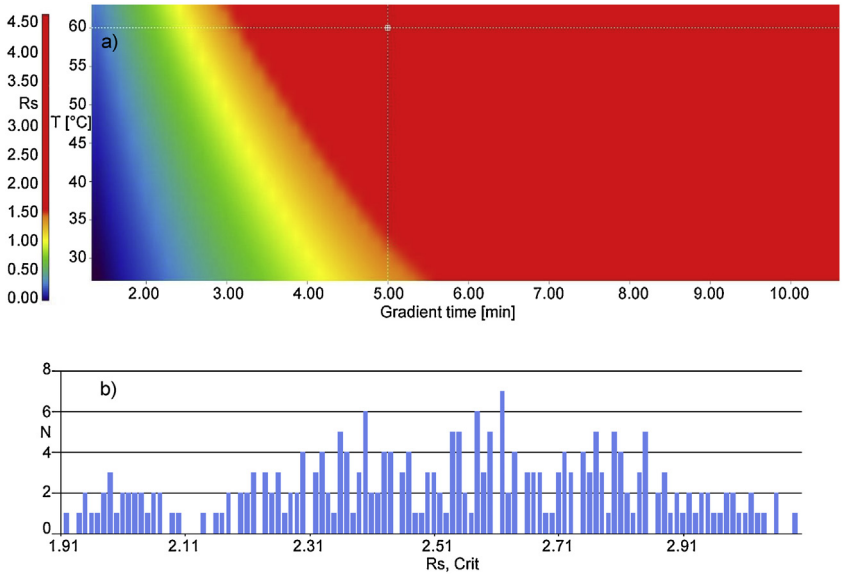


Fig. 2. Robustness displayed as the resolution map (a) and the critical peak resolution (Rs, Crit) by a frequency distribution (N) over the 243 experiments for fatty acid method (b).

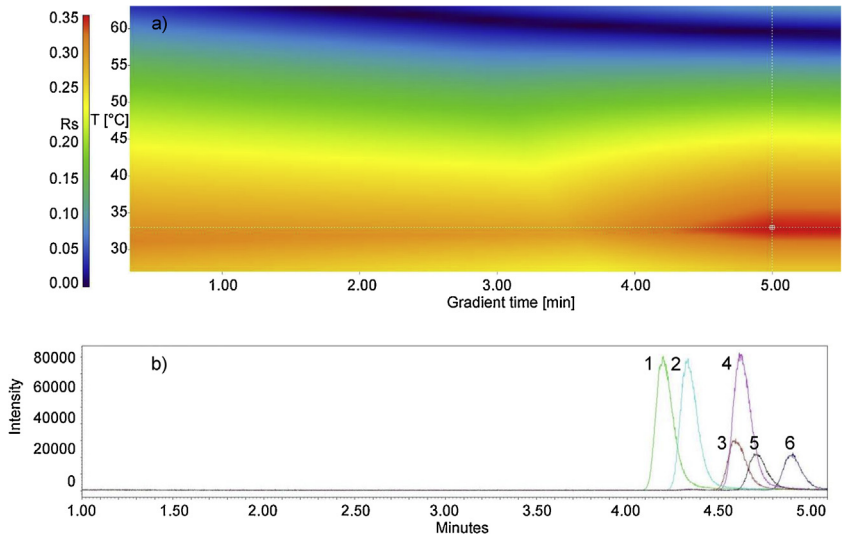


Fig. 3. Resolution map of optimized 18:1 fatty acid method (a) and chromatogram of 18:1 fatty acid isomers with new 18:1 method (b: cis-vaccenic acid (1), oleic acid (2), rans-vaccenic acid (3), petroselinic acid (4), elaidic acid (5), petroselaidic acid (6), chromatogram in time frame of 1–5 min).

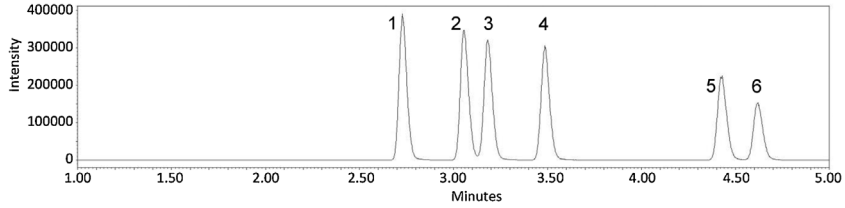


Fig. 4. Chromatogram of derivatized 18:1 fatty acids using oxidation (cis-vaccenic acid (1), trans-vaccenic acid (2), oleic acid (3), elaidic acid (4), petroselinic acid (5), petroselaidic acid (6), chromatogram in time frame of 1–5 min).

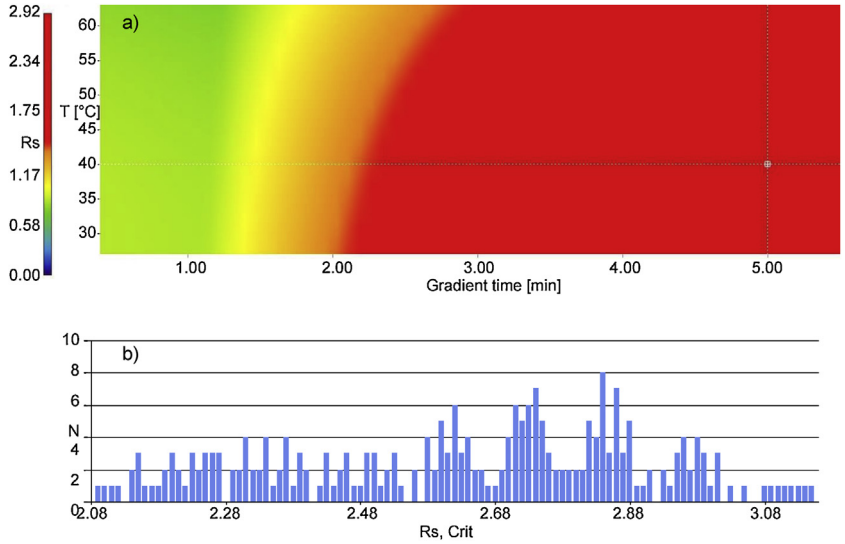


Fig. 5. Robustness displayed as the resolution map (a) and the critical peak resolution (Rs, Crit) by a frequency distribution (N) over the 243 experiments of the derivatized 18:1 fatty acid isomer method (b).

4. Conclusions

A straightforward method was developed for separation of all fatty acids in polysorbate 80 which enables indirect quantification of polysorbate 80 using the oleic acid derivative as the surrogate marker and allowed determinations at low concentrations.

Furthermore, the derivatization of oleic acid in the wet chemical laboratory also minimized the impact of contaminants within the mass spectrometer as they might influence the peak purity and result in low linearities within the calibration.

Additionally, the methods for separation of fatty acids and 18:1 fatty acid isomers showed various advantages. They were highly sensitive, robust and less interference-prone (separation by chromatography and *m/z* values; avoidance of known MS-contaminants after derivatization). The presence of water was allowed, which should be avoided with gas chromatography. This method is sig-

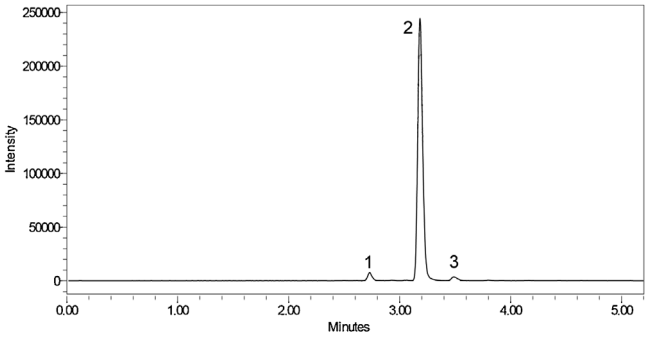


Fig. 6. Chromatogram of hydrolyzed and derivatized polysorbate 80 (derivatives of cis-vaccenic acid (1), oleic acid (2), elaidic acid (3)).

nificantly faster than gas chromatographic determinations in terms of sample preparation and analysis. Consequently, the developed method is an overall-alternative for separation of *cis/trans* and structural isomers.

In further studies, the methods could be used for the correlation of free and bound fatty acids which might be helpful for stability studies of the emulsifier as the samples can be analyzed before hydrolysis resulting in the free acids and after hydrolysis measuring the total fatty acid concentration. By subtraction of the concentration of the free fatty acids, the esterified fraction could be calculated.

Further investigations for adaption of this method at higher polysorbate 80 concentrations are recommended to increase the area of application. As the DoE calculated chromatographic method is not bound to the used detector it could be easily transferred to other systems and detectors when the samples can be detected accordingly.

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