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# Fatty Acid Analysis in Polysorbate 80 by UHPLC-CAD

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## ABSTRACT

**Purpose:** Impurity analysis of Polysorbate 80 is critical to determine suitability of a particular batch for use in pharmaceutical formulations. Commercial Polysorbate 80 is a complex mixture containing fatty acid impurities and other components, most of which do not yield a UV detector signal because they lack chromophores. Instead of a UV detector, a charged aerosol detector was used because it provide more consistent response for nonvolatile and semivolatile analytes than other HPLC detection techniques. In this work, several strategies for analyzing polysorbates by charged aerosol detection (CAD) are discussed. An UHPLC-CAD method with optimized CAD detector settings is presented. Optimization strategies for semi-volatile substances are proposed. The practical implementation of the power function value (PFV), an instrumental setting that helps "linearize" the signal output of CAD, was studied.

**Methods:** A reverse phase gradient method was run on a core-shell C18 column. The response of the charged aerosol detector was optimized using the CAD parameters evaporation temperature, PFV and digital filter. Linearity was evaluated using percent deviation of data points from the linear fit (residual curves).

**Results:** The PFV was found to be a strong tool for the optimization of linearity of response. But the optimal PFV depended on analyte volatility and PFV optimization for each analyte took time. The default, un-optimized PFV of 1.0, in combination with a double-logarithmic transformation, also yielded satisfactory universal results over a range of two orders of magnitude for every homologue fatty acid from $C_{14}$ to $C_{18}$. The new method shows better sensitivity than a method developed for an older generation CAD, as well as time and eluent savings. No pretreatment of the sample is necessary and eleven fatty acids are analyzed in under five minutes including re-equilibration.

## INTRODUCTION

Polysorbate 80, also known as Tween® 80, is a nonionic surfactant commonly used in pharmaceutical formulations, foods and cosmetics. In biopharmaceutical formulations, it prevents surface adsorption and stabilizes proteins against stress-induced aggregation, such as agitation and shear.

## MATERIALS AND METHODS

**Instrumentation**

Thermo Scientific™ Vanquish™ Charged Aerosol Detector/Thermo Scientific™ Corona Veo™ RS detector

Thermo Scientific™ Vanquish™ Flex Binary Pump

Thermo Scientific™ Vanquish™ Split Autosampler

Thermostatted Column Compartment

**Data Analysis**

Thermo Scientific™ Chromeleon™ 7.2.6 Chromatography Data System

**Conditions**

Column: core-shell C18 Kinetex (100 x 2.1 mm, 2.6 µm, 100 Å) (Phenomenex)

Column Compartment Temp.: 25 ˚C , still air mode

Injection volume: 10 µL

Gradient: see table at right. Flow 1.5 mL/min

| Time (min) | %B |
|---|---|
| 0 | 75 |
| 0.8 | 75 |
| 2.5 | 85 |
| 3.0 | 85 |
| 3.5 | 75 |
| 4.5 | 75 |

Eluents: A, 0.05% (v/v) formic acid in water.
B, 0.05% (v/v) formic acid in acetonitrile

Optimized CAD Settings: Evaporation temp. 30 ˚C, power function value 1.0, Filter constant 1 s, Data collection rate 10 Hz, gas inlet pressure 56.4 psi.

**Sample Preparation**

*Sample Solutions for fatty acid composition*

A 15 mg portion of polysorbate was dissolved in 1 M potassium hydroxide containing 10% (v/v) methanol, made up to 10.0 mL, and saponified at 40 ˚C over ≥ 6 hours. A 50 µL portion of neat formic acid was added to 250 µL of the saponified solution in a glass centrifuge tube (VWR International, Darmstadt, Germany). A 500 µL portion of MTBE was added and the mixture was vortexed and centrifuged at 2700 rpm (EBA 20 centrifuge, Hettich, Tuttlingen, Germany) for 5 min. The organic phase was collected, dried under an $N_2$ stream and reconstituted in 1000 µL of acetonitrile 75% / water 25% (v/v).

*Sample and Reference Solutions for free fatty acid determination*

To a 100 mg portion of polysorbate in a 10.0 mL volumetric flask was added 500 µL of a 1 mg/mL methanolic margaric acid stock solution as internal standard (internal standard added to about 0.5% (m/m), exact concentration corrected based on sample weight). The analyte was then dissolved and made up to 10.0 mL with water. A 100 µL portion of 100% formic acid was added to 1000 µL of the polysorbate and internal standard solution in a glass centrifuge tube. After addition of 1000 µL of MTBE the mixture was vortexed and centrifuged at 2700 rpm for 45 min. 500 µL of the organic phase was collected, dried under an $N_2$ stream and reconstituted in 500 µL of acetonitrile 75%/water 25% (v/v). The reference solution consisted of 50 µg/mL of each of margaric acid and oleic acid. It was obtained by diluting the respective stock solutions with a mixture of acetonitrile 75%/water 25% (v/v).

## RESULTS

Figure 1 shows the flow rate optimization for the UHPLC method that yielded a final flow-rate of 1.5 mL/min and a total run time of 4.5 min, which is less than 75% of the 19 min run time for the HPLC method of Ilko et al. [1] and consumed merely 40% of the eluent required by the larger column. Variations in gradient steps, gradient levels, reequilibration time, and injection volume were also examined.

**Figure 1. Chromatogram of 10 µL injection of the reference solution (50 µg/mL margaric acid and 50 µg/mL oleic acid of 65-88% purity). Flow rate was varied between 0.6 and 1.5 mL/min and the hold and gradient steps were adjusted accordingly. Elution order is: 1) linoleic acid; 2) palmitic acid; 3) oleic acid; 4) petroselinic acid; 5) margaric acid; and 6) steric acid**

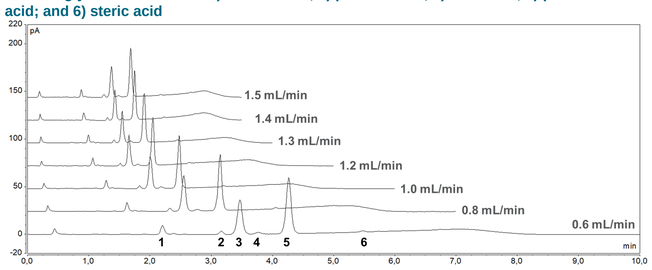



Figure 2 shows that the detector response to each particular fatty acid, listed in order of elution, decreases as evaporation temperature (EvapT) increases. Conversely, Figure 3 shows that noise can also decrease with increasing EvapT. Users must optimize EvapT by raising it until the noise levels are acceptable, but by keeping it as low as possible so as not to affect the signal for semi-volatiles in the sample. Especially for the more volatile lauric acid and myristic acid, temperatures higher than 50 ˚C do not give acceptable results. An EvapT of 30 ˚C yielded maximal S/N ratios for most of the analytes, injected at low concentrations slightly above the original method's LOQs. Experimental LOQs were determined by S/N according to the ICH guideline and injecting 1 ng, 5 ng and 10 ng on column. The comparison of the new UHPLC method's LOQs with those of the original HPLC method and the "older" CAD [1] (Table 1) clearly shows the superiority of detection for every analyte with the exception of the most volatile, myristic acid. The better LOQ for myristic acid in the previous method is due to the lower, ambient, temperature of older CAD's evaporation tube (EvapT could not be controlled). Myristic acid showed improved S/N-ratios at lower EvapT in our tests as well (see Figure 3).

In Figure 2, the signal at a given EvapT increases from left to right across the plot according to elution order. CAD response is sensitive to the gradient composition. Signal generally increases for analytes eluted in solvents that are less viscous and/or have lower surface tension. An inverse gradient added via a second pump can regulate the gradient composition and eliminate this effect.

**Figure 2. Peak height as a function of evaporation temperature. The injected amount on the column was 10 ng.**

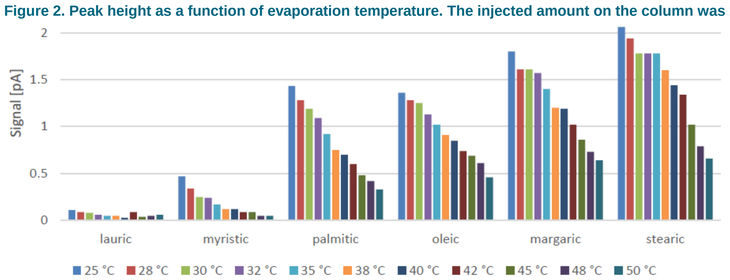



**Figure 3. Signal-to-noise ratio as a function of EvapT. Amount on column: 10 ng each fatty acid.**

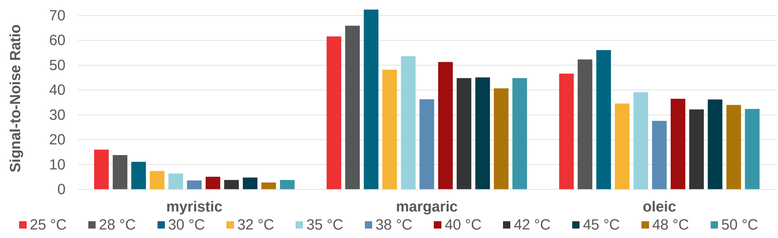
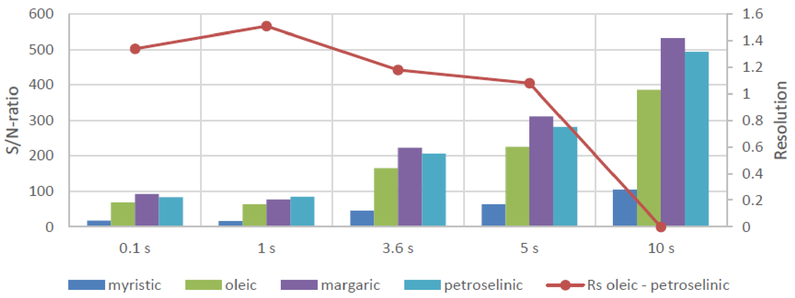



**Table 1. Estimated LOQs for the previously existing HPLC method of Ilko, *et al.* compared with those for the transferred UHPLC-CAD method.**

| Analyte | LOQ by HPLC-CAD (ng on column) | LOQ by UHPLC-CAD (ng on column) |
|---|---|---|
| Myristic acid ($C_{14}H_{28}O_2$) | 6.1 | 8.1 |
| Palmitic acid ($C_{16}H_{32}O_2$) | 4.0 | 2.2 |
| Stearic acid ($C_{18}H_{36}O_2$) | 3.4 | 1.3 |
| Linoleic acid ($C_{18}H_{32}O_2$ (C18:2, ω-6)) | 3.0 | 1.8 |
| Oleic acid ($C_{18}H_{34}O_2$ (C18:1, ω-9)) | 3.9 | 2.1 |
| Petroselinic acid ($C_{18}H_{34}O_2$ (C18:1, ω-12)) | 3.2 | 1.4 |

Figure 4 shows the impact of the CAD filter setting on noise and its importance to the sensitivity limits of a method. The filter is applied to the output current of the detector and affects the collection of the raw data together with the data collection rate. Generally, a higher filter constant results in smoothed baseline, whereas a lower filter constant does not remove a lot or any baseline noise at all [2]. Although a filter setting of 10 shows the highest S/N-ratio, this filter setting was unacceptable from a chromatographic point of view because the broader peaks led to lower resolution. A filter constant of 1 s was chosen because it yielded the best resolution.

**Figure 4. Signal-to-noise ratio as a function of CAD filter setting. The sample amount on the column was 10 ng of each fatty acid.**




Similar to all aerosol-based detectors, the CAD is a non-linear detector and response can be described by a power law function equation [3] as shown in the equation:

$$A = a(m_{inj})^b$$

When b equals 1.0, the curve is linear and the sensitivity coefficient *a* is the slope of the ratio of peak area/mass injected. With b > 1, the shape of the response curve is supralinear and with b < 1, sublinear. Although CAD response is typically quasi-linear over about two orders of magnitude [3], it is advisable to have a closer look at the curve fit especially for the lower calibration levels, as a coefficient of determination close to 1 alone does not necessarily indicate good linearity over the whole range investigated [4]. Calibration curves were established covering concentration levels of 1 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL at power function values (PFVs) ranging from 0.8 to 1.6 and EvapT of 30 ˚C, 35 ˚C and 40 ˚C. The $R^2$-values were established by means of linear regression (Table 2).

**Table 2. Coefficients of determination for linear and log-log linear calibration curves measured with PFV between 0.8 and 1.6 at an evaporation temperature of 30 ˚C.**

| Power Function Value | myristic acid | palmitic acid | margaric acid | stearic acid | oleic acid | petroselinic acid | linoleic acid | alpha-linolenic acid |
|---|---|---|---|---|---|---|---|---|
| **0.8** | 0.9999 | 0.9909 | 0.9891 | 0.9994 | 0.9876 | 0.9836 | 0.9994 | 0.9994 |
| **0.9** | 0.9605 | 0.9935 | 0.9897 | 0.9883 | 0.9891 | 0.9861 | 0.9873 | 0.9955 |
| **1.0** | 0.9981 | 0.9972 | 0.9947 | 0.9914 | 0.9937 | 0.9922 | 0.9929 | 0.9988 |
| **1.1** | 0.997 | 0.999 | 0.9977 | 0.9979 | 0.9996 | 0.9973 | 0.9978 | 0.9975 |
| **1.2** | 0.9938 | 0.9983 | 0.9994 | 0.9993 | 0.9993 | 0.9991 | 0.9994 | 0.9985 |
| **1.3** | 0.9902 | 0.9983 | 0.9994 | 0.9996 | 0.9992 | 0.9999 | 0.9997 | 0.9942 |
| **1.4** | 0.9804 | 0.995 | 0.9976 | 0.9979 | 0.9981 | 0.9994 | 0.9985 | 0.9936 |
| **1.5** | 0.9782 | 0.9928 | 0.9947 | 0.9921 | 0.9953 | 0.9976 | 0.9949 | 0.9876 |
| **1.6** | 0.9664 | 0.9878 | 0.9921 | 0.9906 | 0.9914 | 0.9949 | 0.9919 | 0.9843 |
| **1.0 log-log** | 0.9998 | 0.9998 | 0.9995 | 0.9993 | 0.9994 | 0.9993 | 0.9995 | 0.9995 |

For a better estimation of linearity, the response factor (peak area/mass injected) was plotted against the respective concentration level (Fig. 5). Response linearity is represented by the slope of the resulting regression line. The optimal PFV would then have a slope of zero [5]. The obtained regression lines either show a negative slope indicating sublinear response, or a positive slope indicating supralinear response.

The optimal PFV was determined by comparing the relative standard deviation of the response factors of each analyte for every power function (0.8 to 1.6 in steps of 0.1 units) at 30 ˚C (Figure 6), 35 ˚C (Figure 7) and 40 ˚C (data not shown). The optimal PFV for each analyte was identified using the principle that the lowest RSD indicates the best response linearity [5].

Although the optimal PFV was not the same for all fatty acids, we chose a PFV of 1.1 as a compromise. The most volatile fatty acid, myristic acid showed optimal results at PFV = 0.8, which was expected because semivolatiles often give best results at a PFV <1.0 [6].

**Figure 5. Response factor (peak area / concentration) as a function of analyte concentration for palmitic acid at PFV = 0.8, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 and EvapT = 30 ˚C.**

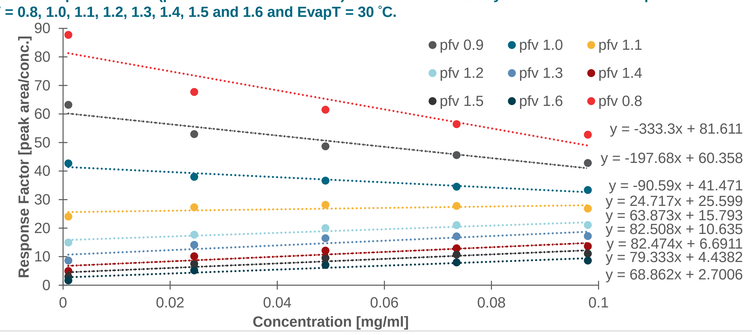



**Figure 6. %RSD of response factors at 30 ˚C EvapT.**

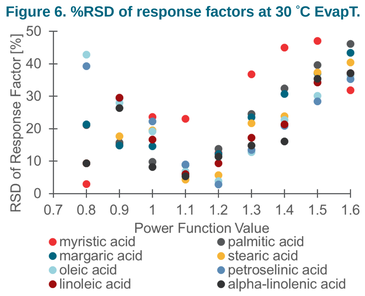
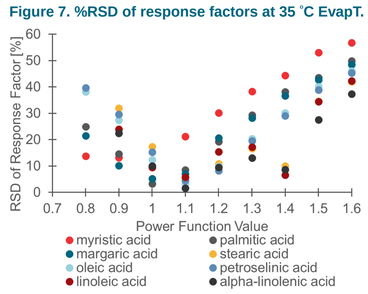



**Figure 7. %RSD of response factors at 35 ˚C EvapT.**




To identify the optimal EvapT at a given PFV, response factor versus concentration plots were obtained for evaporation temperatures of 30 ˚C, 35 ˚C and 40 ˚C. The optimal evaporation temperature for a given PFV was determined by comparison of the slopes of the regression lines as well as of the relative standard deviations of the corresponding response factors (Figure 8). It was shown that linearity of response at 30 ˚C and 35 ˚C were comparable to each other at a PFV of 1.1 (median RSD 5.21 % to 6.50 %), whereas a PFV of 1.1 was not the optimum for 40 ˚C (median RSD 15.37 %). These results lead us to conclude that the PFV may need reoptimization at each EvapT when dealing with semivolatiles such shorter chain fatty acids (Figure 8). As an alternative to PFV optimization at each EvapT, other models for fitting the data at PFV 1.0 were considered. The log-log linear fit, termed "Power" in the software, delivered an $R^2 > 0.999$ (coefficient of determination) for the calibration data collected on every analyte. Furthermore, the obtained residuals for each data point showed very satisfying results, even at low concentrations (Figure 9). Residuals, especially at the lowest concentrations, were found to vary drastically with changes in PFV. This shows that a linear fit to response for a mixture of analytes can be achieved with the default PFV of 1.0 and a log-log transformation, rather than applying various PFVs. In many applications, the goal is to obtain satisfactory and low LOQs. Thus, for partially volatile analytes, it seems most appropriate to evaluate the optimal evaporation temperature before determining the best power function value to receive an appropriate fit since for semi-volatiles the best PFV changes when altering evaporation temperature (Fig. 8) and the evaporation temperature affects sensitivity strongly (Fig.3). This is in contrast to the common approach of determining PFV before evaporation temperature for non-volatiles [3].

**Figure 9. Residual plot of relative amount deviation of each point from the linear log-log line. EvapT = 30 ˚C and PFV = 1.0.**

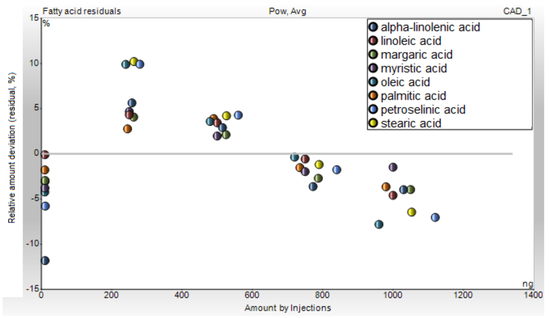
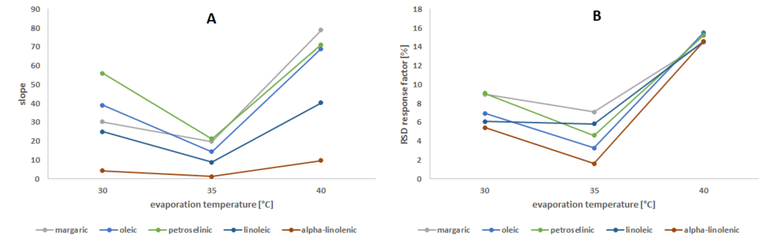



**Figure 8. (A) Slope of the line fitting the plot of response factors as a function of temperature for PFV = 1.1. (B) Percent RSD of the response factors versus EvapT plot for PFV = 1.1.**




## CONCLUSIONS

A HPLC-CAD method for the analysis of polysorbate 80 was successfully transferred to an UHPLC system including the newest generation CAD resulting in:

- Time saving of over 75%
- Eluent consumption saving of over 40%
- LOQs decreased by 40 to 61% except for myristic acid, which increased by 33% due to chosen EvapT

Transfer of the method to a current-generation CAD offered:

- Straightforward method optimization using evaporation temperature, power function value, and filter parameters
- Improved sensitivity and S/N ratio due to optimization of evaporation temperature
- A new method to optimize response linearity and LOQ with PFV and EvapT based on application goal and analyte volatility

For the investigated two-order concentration range of fatty acids, including semi-volatiles, a double-logarithmic transformation proved to be superior and less time consuming than optimization of the PFV for each analyte and evaporation temperature.

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## ACKNOWLEDGEMENTS

Special thanks are due to Christiane Theiss and her assistance with her expert knowledge of fatty acids and their properties as well as Oliver Wahl (both University of Wuerzburg) for insightful discussions and advices, as well as Paul Gamache (Thermo Fisher Scientific, Chelmsford, Massachusetts, USA) and Frank Steiner (Thermo Fisher Scientific, Germering, Germany) for project coordination and crucial ideas, reviews and discussions.

## TRADEMARKS/LICENSING



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