Application note | 003867

Pharma and biopharma

Characterization of four saturated fatty acids using gradient HPLC-CAD highlighting optimized evaporation temperature control features

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Keywords

Charged aerosol detector (CAD), Vanquish Flex Quaternary UHPLC system, saturated fatty acids, evaporation tube temperature, Temperature Coupling Mode

Application benefits

- Optimized method parameter settings for fatty acid analysis
- Enhanced temperature control providing improved measurement of semi-volatile species
- 24-hour study of signal-to-noise ratios and resulting standard deviations verifying method robustness

Goal

Investigating the effects of improved evaporation temperature control features of the Thermo™ Scientific™ Vanquish™ Charged Aerosol Detector P series, such as the Temperature Coupling Mode, on the analysis of four common saturated fatty acids. This highlights the HPLC-CAD application robustness including improved response for semi-volatile analytes.

Introduction

Polysorbates (PS) are non-ionic surfactants that are used in pharmaceutical and biopharmaceutical formulations as emulsifiers and solubilizers for poorly soluble drug substances. They are a crucial component of protein formulations because they prevent protein unfolding and aggregation and help maintain the protein in its active form.[1]

Commercially available PS are complex heterogeneous mixtures, varying in composition across suppliers and even between lots from the same manufacturer. During the manufacturing process, PS can be contaminated with impurities such as fatty acids (FAs).[2] Moreover, during storage or transit, PS in formulations can degrade also forming FAs. This is a major problem as FAs can form insoluble particles that can seriously compromise product quality, efficacy, and patient safety.[3] Thus, the ability to measure FA in both raw materials and formulations is of the upmost importance.

FAs do not contain a suitable chromophore, so they cannot be detected without derivatization by traditional liquid chromatographic methods such as HPLC-UV. Rather, researchers now use HPLC with charged aerosol detection for routine measurement of FA.[4] The charged aerosol detector (CAD) is sensitive, has a wide linear and dynamic range, and shows uniform response for all non-volatile and many semi-volatile compounds present in a sample, including FAs.

Charged aerosol detection involves pneumatic nebulization of the mobile phase eluting from the analytical column to form an aerosol. Aerosol droplets enter a heated evaporation tube and are de-solvated to form particles. In the charging detection module (CDM) particles are charged, and the charge is then measured using an electrometer. The amount of charge is directly proportional to the quantity of the analyte present. The temperature of the evaporation tube (EvapT) significantly influences detector performance. For example, higher EvapT can reduce noise (improving signal-to-noise ratio (S/N)) but may lead to poor sensitivity for semi-volatile species. Conversely, a lower EvapT may improve response uniformity for semi-volatile species, but the S/N may be adversely impacted.[5,6] In previous CAD models, while the EvapT could be adjusted, the temperature of the CDM, is fixed at 40 °C. This could adversely affect CAD measurement of semi-volatile compounds that require a low EvapT. The Vanquish CAD P series has a feature where both EvapT and CDM temperature can be coupled by switching on the Temperature Coupling Mode, which can be used for method optimization like the improved measurement of semi-volatile species such as the FAs.

In this work, a gradient LC method[4] for the determination of four FAs was used to evaluate the improved temperature control design (EvapT can now be adjusted in 0.1 °C increments) and Temperature Coupling Mode feature of the Vanquish Charged Aerosol Detector HP (Figure 1). The recommended EvapT to maximize S/N is presented, and the S/N stability over a 24-hour period was studied. Finally, the positive effect on the response of especially semi-volatile species with the activated Temperature Coupling Mode is demonstrated.

Experimental

Chemicals

- Deionized water, 18.2 MΩ.cm, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification (P/N 50136149)
- Fisher Chemical™ Acetonitrile, Optima™ LC/MS grade (P/N A955)
- Fisher Chemical™ Methanol, Optima™ LC/MS grade (P/N A456)
- Fisher Chemical™ Formic acid, Optima™ LC/MS grade (P/N A117)
- Myristic acid, Lauric acid, Palmitic acid, and Stearic acid, purchased from a reputable vendor

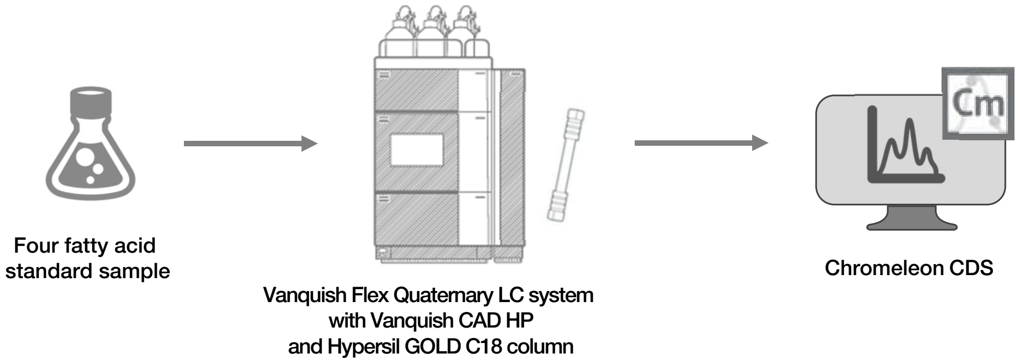


Figure 1. Schematic workflow for the analysis of four common saturated FAs. The workflow uses a Vanquish Flex Quaternary UHPLC system with a Vanquish Charged Aerosol Detector HP and Hypersil GOLD C18 UHPLC column. Chromeleon Chromatography Data System is used for data acquisition and processing.

Sample handling

- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100–1000 µL (P/N 4641100N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 10–100 µL (P/N 4641070N)
- Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 1–10 µL (P/N 4641030N)
- Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ Orion™ 3-Star pH Benchtop Meter (P/N 13-644-928)
- Thermo Scientific™ SureSTART™ 2 mL Amber Glass Short Thread Screw Top Vials, 100/pack, Level 2 (P/N 6ASV9-2P)
- Thermo Scientific™ SureSTART™ Blue Polypropylene 9 mm AVCS™ Screw Caps with Soft Blue Silicone/Clear PTFE Septa, 100/pack, Level 3 (P/N 6PSC9ST101)

The Fisher Scientific product codes can be unique to different countries, the codes given above should be compatible across EU and USA.

Instrumentation

- Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system consisting of:
  - Vanquish System Base Horizon/Flex (P/N VF-S01-A-02)
  - Vanquish Quaternary Pump F (P/N VF-P20-A-01)
  - Vanquish Split Sampler FT (P/N VF-A10-A-02)
  - Vanquish Column Compartment H (P/N VH-C10-A-03)
  - Vanquish Charged Aerosol Detector HP (P/N VH-D21-A-01)

Sample preparation

Stock solutions of the four saturated FAs were prepared as follows:

1. Prepare the diluent. The diluent consists of 15 mL acetonitrile and 5 mL water.
2. Create an individual stock of each FA (lauric acid, myristic acid, palmitic acid, stearic acid). 0.25 mg/mL of each individual FA was added in separate vials by weighing 5.0 mg FA and then adding 20 mL methanol. Sonicate for 2 minutes to dissolve.
3. Create one working solution with all four FAs at a concentration of 50 µg/mL. Pipette 200 µL of each 0.25 mg/mL FA stock solution and add 800 µL diluent.

Chromatographic conditions

Table 1. Chromatographic conditions

Parameter	Value	
Column	Thermo Scientific™ Hypersil GOLD™ C18, 50 x 2.1 mm; 1.9 µm (P/N 25002-052130)	
Solvent A	0.05% formic acid in water (v/v)	
Solvent B	0.05% formic acid in acetonitrile (v/v)	
Gradient	Time [min]	B [%]
	0	75
	1	75
	3	85
	3.5	85
	4	75
	5	75
Column temp.	25 °C	
Active pre-heater temp.	Off	
Autosampler temp.	6 °C	
Needle wash solution	10/90 water/isopropanol (v/v)	
Needle wash mode	After draw	
Injection volume	10 µL	
CAD settings	Data collection rate: 10 Hz Filter setting: 5.0 s PV settings: PV 1.5, PV 1.8 Evaporation temperature: Variable (25–40 °C) Temperature Coupling Mode: On & Off	

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.3.2 was used for data acquisition and processing.

Results and discussion

The reversed phase UHPLC-CAD FA method developed by Schilling *et al.* was used to help define optimal application parameters and to evaluate the EvapT and the Temperature Coupling Mode.[4]

The background noise was compared at various EvapT values. It was observed that the EvapT had little effect on the noise (Figure 2). As shown in Figure 3, the S/N for each of the FAs decreased as EvapT increased as analytes became more volatile. Represented FAs are lauric acid > myristic acid > palmitic acid > stearic acid in order of their volatility, where lauric acid is the most volatile. From the S/N comparison at the respective temperature for the FAs, it was determined that an evaporation temperature of 25.0 °C was optimal (Figure 3).

This study was repeated over a 24-hour period to ensure method robustness. The highest relative standard deviation was observed as less than 10%. Except for lauric acid, its S/N was 10x less than the other FAs and at 40 °C was even close to the limit of quantitation (S/N = 10).

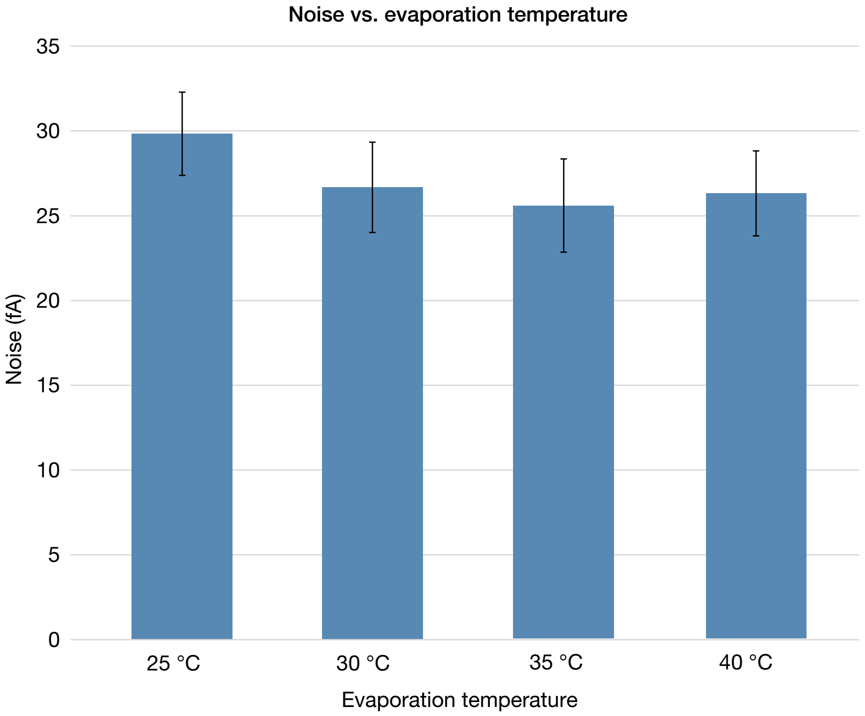


Figure 2. Changes in the evaporation temperature showed insignificant influence on the noise. The noise was calculated by averaging the noise over the time of the gradient (0.5–3.0 minutes) in a blank run with the standard deviation (SD, errors bars) derived from n=3.

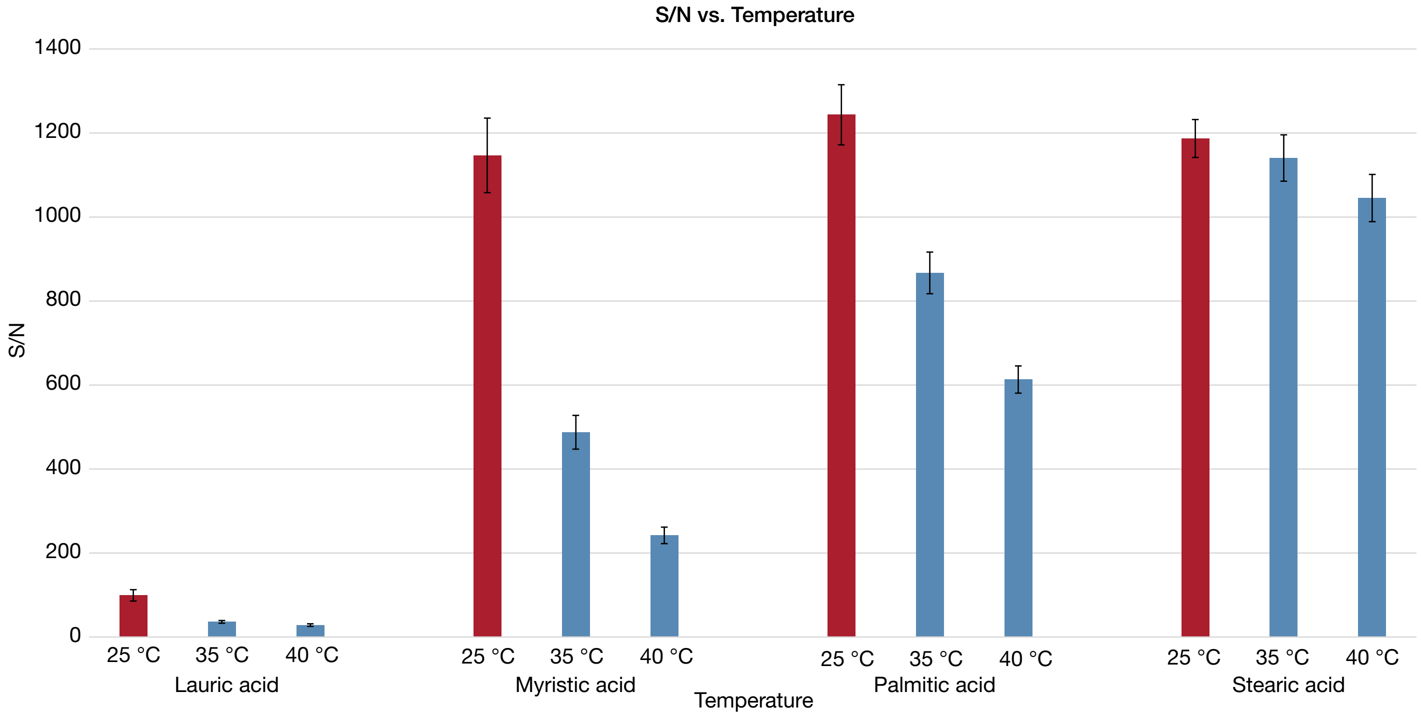
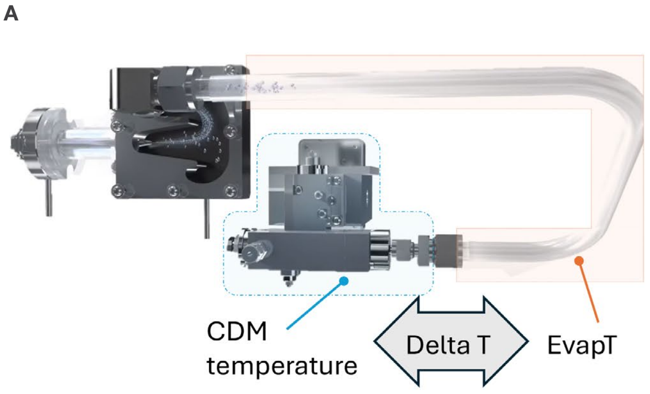


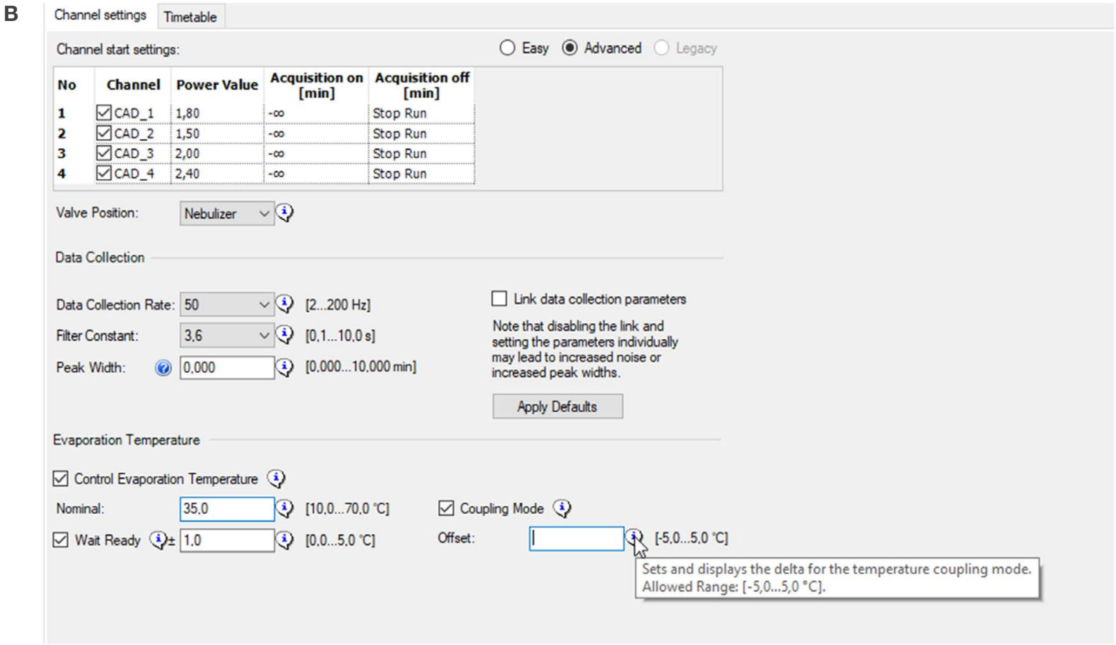
Figure 3. Effects of EvapT on the S/N for the four FAs in the working solution. The optimal EvapT of 25 °C is highlighted in red. It is representative of the average S/N over the entire 24-hour sequence (n=18). The SD is displayed via the error bars.

The Vanquish CAD P series contains enhanced temperature control for the evaporation tube and the CDM. In the Vanquish CAD HP, as in previous versions of CADs, the CDM temperature is always accurately stabilized to 40 °C by default, regardless of the EvapT used. The resulting temperature difference between the EvapT and CDM can adversely affect analyte response—especially for semi-volatile species requiring a low EvapT. This potential issue is now addressed in the Vanquish CAD HP through a feature called Temperature Coupling Mode (Figure 4). In this mode, the CDM temperature is coupled to the evaporation tube temperature by choosing the delta temperature in a range between -5 °C to +5 °C in the advanced mode of the instrument method wizard (Figure 4B). The default setting is a delta temperature of +5 °C, resulting in an CDM temperature elevated by 5 °C compared to the EvapT. For example, if the EvapT is set to 40 °C and the Temperature Coupling Mode is set to on and no changes of delta temperature are made, then the CDM will be thermostatted at 45 °C. Conversely for applications requiring high EvapT (e.g., for EvapTs above the default CDM temperature of 40 °C), the ability to maintain the CDM temperature +5 °C above the EvapT when the Temperature Coupling Mode is set to on minimizes the possibility for compromising measurements due to condensation in the CDM.*

*It is recommended that the EvapT is set to at least 5 °C above ambient temperature. With an anticipated lab environment of 25 °C, the minimum achievable CMD temperature would be 20 °C by setting a delta of “-5 °C” in the coupling mode parameter setting of the instrument method. The maximum allowed CDM temperature is 75 °C (70 °C EvapT +5 °C).



Temperature Coupling Mode	CDM temperature	Delta T (EvapT-CDM temperature)	EvapT
Off (default, method transfer)	40 °C (fixed)	Not settable	Settable
On (method optimization)	EvapT –5 °C up to EvapT +5 °C	-5 °C to +5 °C (settable)	Settable
Example:			
On (for semi-volatiles)	30 °C Avoids removal of semi-volatiles in CDM by evaporation	+5 °C	25 °C



Channel start settings: ○ Easy ● Advanced ○ Legacy

No	Channel	Power Value	Acquisition on [min]	Acquisition off [min]
1	☑ CAD_1	1,80	-∞	Stop Run
2	☑ CAD_2	1,50	-∞	Stop Run
3	☑ CAD_3	2,00	-∞	Stop Run
4	☑ CAD_4	2,40	-∞	Stop Run

Valve Position: Nebulizer

Data Collection

Data Collection Rate: 50 [2...200 Hz]

Filter Constant: 3,6 [0,1...10,0 s]

Peak Width: 0,000 [0,000...10,000 min]

☐ Link data collection parameters

Note that disabling the link and setting the parameters individually may lead to increased noise or increased peak widths.

Evaporation Temperature

☑ Control Evaporation Temperature

Nominal: 35,0 [10,0...70,0 °C]

☑ Wait Ready ± 1,0 [0,0...5,0 °C]

☑ Coupling Mode

Offset: [-5,0...5,0 °C]

Sets and displays the delta for the temperature coupling mode. Allowed Range: [-5,0...5,0 °C].

Figure 4. (A) Relationship between EvapT, CDM temperature, and Temperature Coupling Mode in the Vanquish CAD HP showing how the measurement of semi-volatiles can be improved; (B) Screenshot of instrument method wizard showing the advanced mode with the option to adjust the temperature delta (Offset) between EvapT and CDM temperature in a range of -5 °C to +5 °C.

For this application, the recommended EvapT is 25.0 °C (Figure 3). FA response was then measured with the Temperature Coupling Mode on and off at the EvapT of 25.0 °C (Figure 5). As presented in Table 2, responses for all FAs could be improved when the Temperature Coupling Mode was switched on. Interestingly, the improvement in S/N matched the analyte's semi-volatility with lauric acid (lowest MW and the most semi-volatile) showing the greatest improvement, and stearic acid (highest MW and less semi-volatile) showing the least.

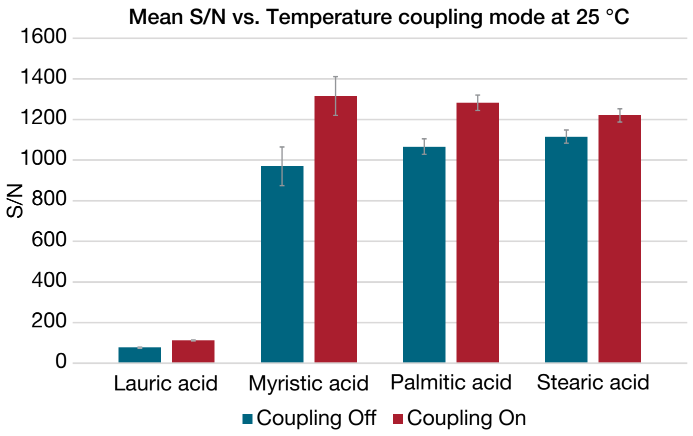


Figure 5. The mean S/Ns of the FAs were improved when the temperature coupling mode was turned on (SD calculated for n=6).

Table 2. The % increase in S/N when the temperature coupling mode is turned on

Compound	% increase in S/N
Lauric acid	+46.8%
Myristic acid	+35.7%
Palmitic acid	+20.3%
Stearic acid	+9.4%

Conclusion

In this work, it was demonstrated that better evaporation temperature control alongside the Temperature Coupling Mode provided improvements to the analysis of the four saturated FAs.

- An EvapT of 25.0 °C resulted in the best S/N for the four FAs.
- The setting of Temperature Coupling Mode On improved the overall S/N for the four FAs at the optimum 25 °C EvapT.
- The temperature control of the CDM via the Temperature Coupling Mode has a positive effect on the response, especially for semi-volatile species when turned on.
- Experiments showed a robust application performance over a 24-hour period.

References

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