Lipid applications, CAD

Thermo Fisher SCIENTIFIC

Best practices for liposome analysis with the Charged Aerosol Detector

Susanne Fabel[1], Katherine Lovejoy[1], Yan Yang[2], Sissi White[3], Stephanie Koczur[3]

[1]Thermo Fisher Scientific, Germany; [2]Thermo Fisher Scientific, China; [3]Thermo Fisher Scientific, USA

Abstract

Purpose: We present application benefits and practical strategies for lipid quantitation and lipid ratio determination with the new ASTM standard E3297-21 for LC-CAD.

Methods: Lipids were quantified in liposomal formulations with calibration curves ranging from 0.2 to 300 µg/g. The suitability of various columns and system configurations was tested at four sites and with 12 system configurations, all of which passed the system suitability test. Three fully porous C18 columns of 3 or 3.5 µm particle size were tested.

Results: The quality criteria for the method, including resolution, reproducibility of peak area, and quality of calibration curves, were met in all test scenarios.

Introduction

Liposome formulations containing cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), and hydrogenated soy phosphatidylcholine (HSPC) are common drug carriers for cancer therapeutics, for example in liposomal doxorubicin (Doxil®) and liposomal irinotecan (Onivyde®). The relative ratios are important for controlling drug potency. ASTM method E3297-21 (1) is a standard method for quantitation of these lipids using the charged aerosol detector (CAD). The CAD is well-suited for this analysis because these lipids show excellent signals with the CAD, but weak or nonexistent signals in UV detectors. HPLC systems with quaternary and binary pumps were used at four different sites in three different countries in a comparison test. A separate rigorous round robin test was conducted by the ASTM to evaluate the general HPLC method and those results are not published here. The results below suggest additional suitable columns for this method and confirm system suitability of Thermo Scientific™ Vanquish™ Flex and Core HPLC systems for the method.

Materials and methods

For complete method requirements, refer to the new ASTM standard test method for lipid quantitation in liposomal formulations, ASTM E3297-21.(1)

Sample and eluent preparation:

Calibration standards and system suitability (SST) standards were prepared in methanol using two stock solutions of each substance of 10 mg in 10 mL methanol and 4 mg in 10 mL methanol. Stocks were stored at -20 ˚C in 20 mL amber glass vials. Samples were sonicated and vortexed at room temperature before use to prevent sample loss due to association with glass walls.

Solvent bottles were rinsed three times with water and once with solvent. The eluents were degassed in an ultrasonic bath to reduce noise in the baseline.

System preparation:

The system was prepared according to Technical Guide 73914 (2). If the baseline increased by more than 0.8 pA after the 16-minute mark of the gradient (Table 1), the column was removed and system was washed with 1 mL/min of water, isopropanol and acetonitrile, switching between the solvents every 10 minutes for at least two hours while holding the evaporation tube temperature as high as possible. If a baseline shift persisted, a new acetonitrile bottle was used.

Data analysis

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software was used for data acquisition and analysis. Key processing steps included the “peak group” function to combine the HSPC peak areas and the “Power” fit function to produce the log-log linear equation.

Table 1. Chromatographic conditions and gradient

Parameter	Value
Recommended column	Thermo Scientific™ Hypersil GOLD™ C18 150 x 3mm, 3 µm (P/N 25003-153030)
Eluent A	Acetonitrile/water (90/10 v/v) with 5 mM ammonium acetate
Eluent B	100% Methanol with 5 mM ammonium acetate
Flow rate	0.7 mL/min
Column temp.	35 ˚C, still air mode heating
Preheater temp.	35 ˚C
Autosampler temp.	10 ˚C
Injection	5 µL, sample in methanol
Rear seal wash	10% methanol in water
Needle wash	methanol
CAD evaporation tube temp.	35 ˚C
CAD power function value	1.0 or 1.2 (see text)
Gradient ([time, %B])	[0.0 min, 40%], [6.0, 40%], [6.1, 90%], [16.0, 90%], [16.1, 40], [21.0, 40%]

Chromatographic conditions are shown in Table 1. For complete chromatographic conditions, refer to the ASTM standard E3297-21. Proper column temperature control is crucial for this method because the cholesterol and DSPE-PEG 2000 peaks can swap elution order with improper temperature control (3). The gradient is a step gradient that begins at 40% B and increases to 90% B after six minutes (Table 1). Thermo Scientific™ Vanquish™ Flex UHPLC and Thermo Scientific™ Vanquish™ Core HPLC systems with quaternary and binary pumps were used (Table 2) and three columns were tested (Table 3).

Table 2. System specifications.

Module	System 1	System 2	System 3
System base	Vanquish system base		
Pump	Quaternary Pump F (VF-P20-A)	Quaternary Pump C (VC-P20-A)	Binary Pump C (VC-P10-A)
Autosampler	VF-A10-A	VC-A12-A	
Column compartment	VH-C10-A	VC-C10-A	
Charged aerosol detector	VH-D20 or VF-D20-A or Thermo Scientific™ Corona™ Veo™ CAD		

Table 3. Columns tested

	Description
1	Thermo Scientific™ Hypersil GOLD™ C18, 150 x 3mm, 3 µm particle size (PN 25003-153030)
2	fully porous ethylene-bridged hybrid particle C18, 150 x 3mm, 3.5 µm
3	fully porous silica particle C18, 150 x 3mm, 3 µm

Results

Quality criterion 1 of 3: resolution

As required, the resolution of all peak pairs was greater than 1.5 for all columns tested (Figures 1 and 2). Although column 3 met the criteria, resolution could be further improved by reducing the B content of the second gradient step from 90% to 80% or increasing the ammonium acetate concentration from 5 mM to 10 mM.

Figure 1. Chromatograms from a quaternary system for all columns showing elution of cholesterol (peak 1), DSPE-PEG2000 (peak 2), HSPC 1 (peak 3) and HSPC 2 (peak 4). HSPC 1 and 2 are the 16:0 and 18:0 fatty acids.

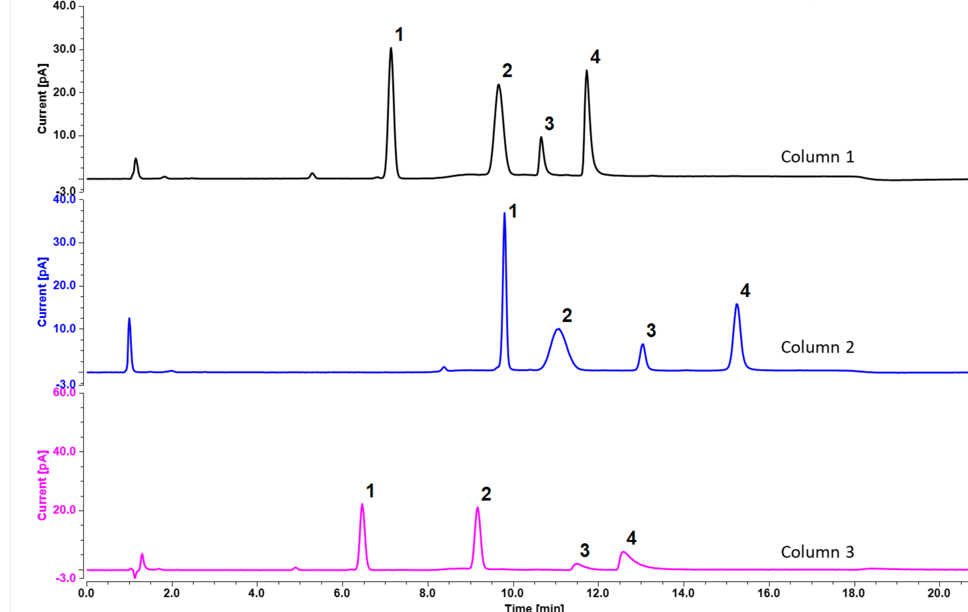
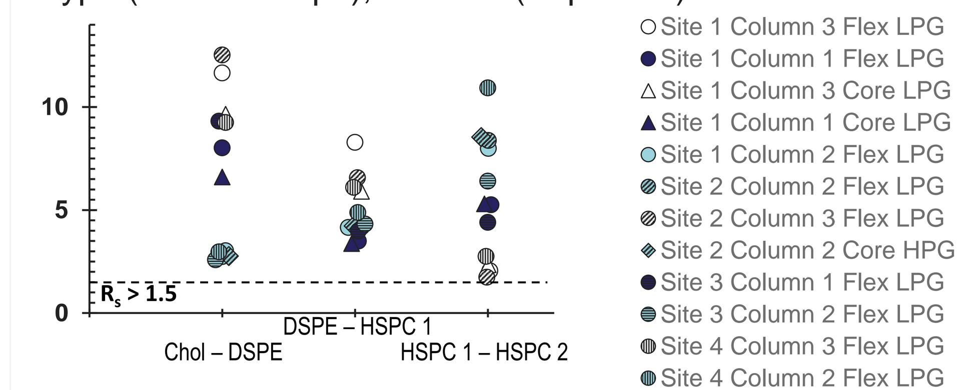


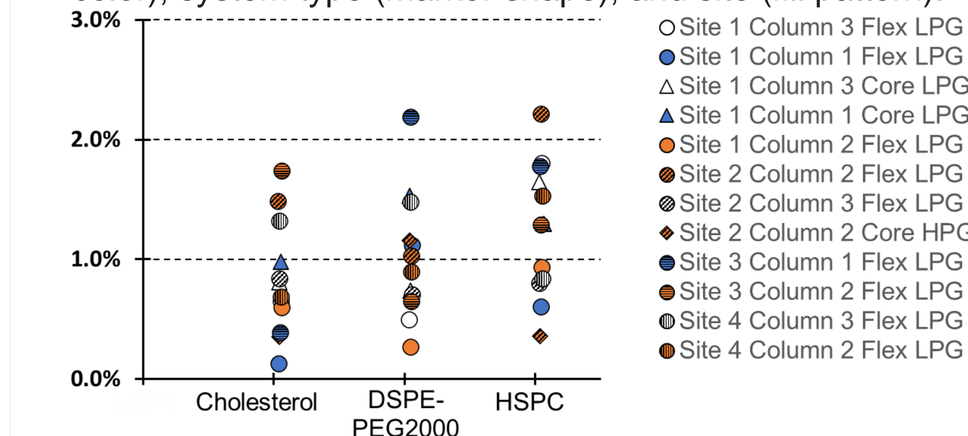
Figure 2. Resolution quality criteria of Rs>1.5 was met in all cases. Shown by column type (marker fill color), system type (marker shape), and site (fill pattern).



Quality criterion 2 of 3: peak area reproducibility

The peak area %RSD for three repeated injections of the 50 µg/g standard was < 3% in all datasets (Figure 3). The peak areas of HSPC 1 and 2 were summed for each injection and then averaged. The quality criteria in the standard method require RSD < 5% and this limit was easily met at all sites and with all columns and systems. No relationships between peak area reproducibility and system type, column type, or site were observed.

Figure 3. %RSD for peak area by column type (marker fill color), system type (marker shape), and site (fill pattern).



Quality criterion 3 of 3: quality of calibration curves

CAD response is approximately linear, over 1.5 - 2 orders of magnitude ($10^{1.5}$–10^2) and non-linear over a wider range. Other nebulizer-based detectors such as ELSD and mass spectrometers are also inherently non-linear. The CAD response function is detailed in ref. 5. The wider range response can be linearized either after data collection with a fitting equation (ref. 6, Fig. 4) or during data collection with the power function method parameter (Ref 6, Fig. 5).

The ASTM method specifies a log-log fitting equation (Fig. 6) after data collection. All log-log fit curves for all system configurations met the quality criteria (R^2 > 0.995) for this method (Fig. 4). The log-log fit is termed “Pow” in Chromeleon CDS. Quadratic and weighted linear fits also met the criteria (Fig. 4). The quadratic fits had the smallest residuals of the three fit functions.

Figure 4. From left to right, quadratic, log-log and weighted linear fits for DSPE-PEG2000 from three injections of each standard onto column 3.

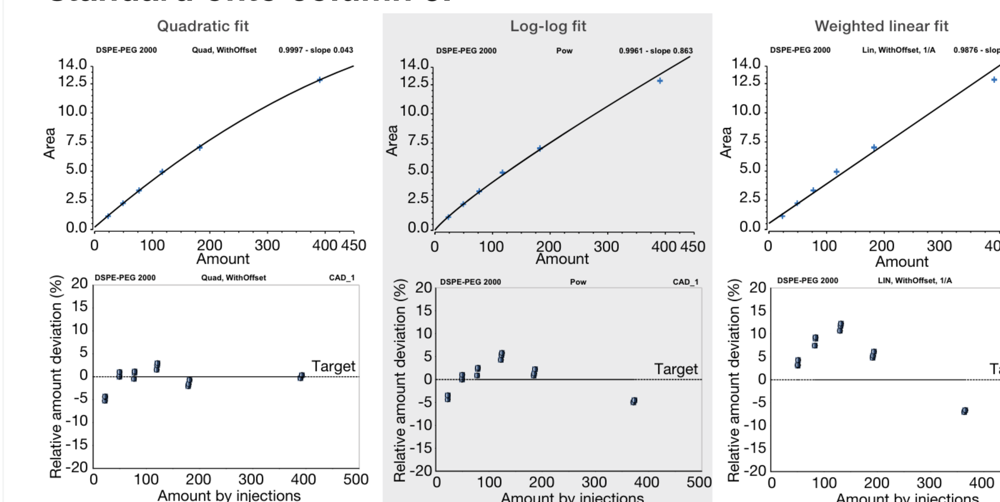
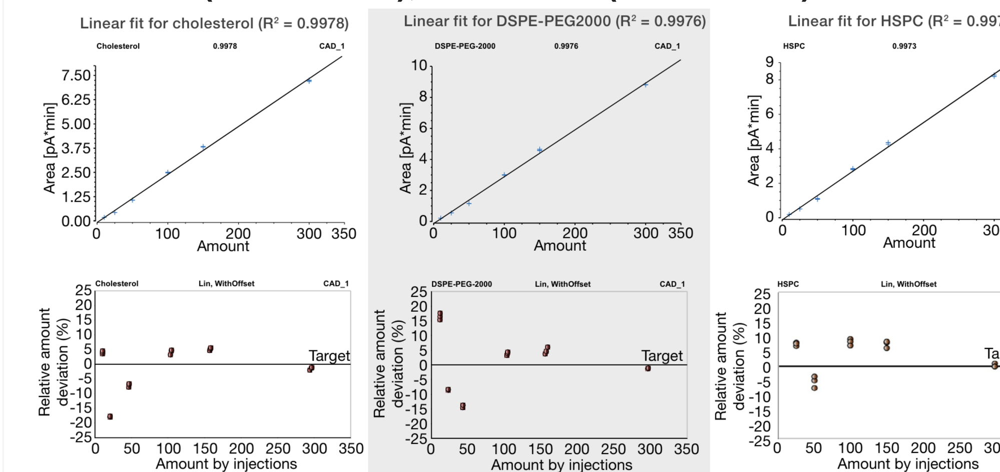
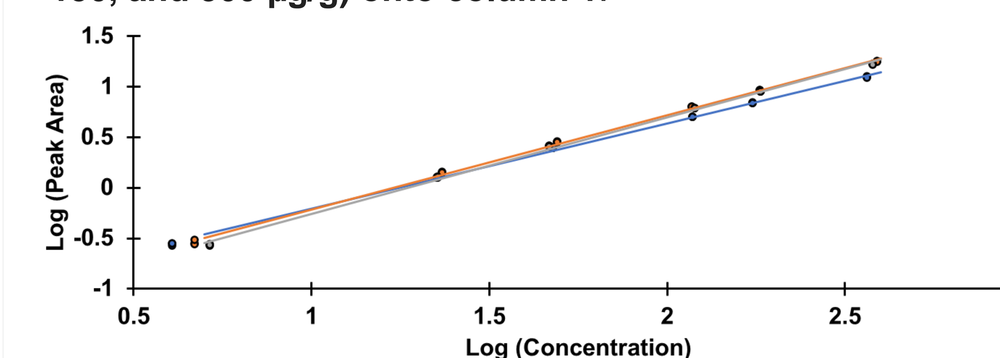


Figure 5. From left to right, dataset collected with PFV = 1.2 showing linear fits for cholesterol (R^2 = 0.9978), DSPE-PEG2000 (R^2 = 0.9976), and HSPC (R^2 = 0.9973).



The CAD’s uniform response for nonvolatile substances allows accurate quantification of unknowns using a surrogate calibration curve, unlike the highly variable response factors found by mass spectrometry (Figure 6). The curves for DSPE-PEG2000 and HSPC overlapped and quantifying both substances with one curve would save analyst time. Any impurity eluting in the later step of the gradient can likewise be quantified with either the DSPE-PEG2000 or the HSPC curves. All three curves would overlap if the inverse gradient were used to compensate the low organic part of the step gradient, where cholesterol elutes (4).

Figure 6. A log-log plot with linear fit for peak areas from three injections of each of six standards (5, 25, 50, 100, 150, and 300 µg/g) onto column 1.



Limit of Quantification (LOQ)

The LOQ values for the three columns are reported in Table 4. The signal-to-noise (S/N) ratios of analyte mixtures at concentrations of 2, 1, 0.5, 0.2 and 0.1 µg/g were evaluated to determine LOD and LOQ. A S/N > 3 was required for LOD and a S/N > 10 was required for LOQ. The PFV 1.2 signal-to-noise values were higher than those of the PFV 1.0 dataset, as expected (for discussion, see TN 73299 (6)). The lowest LOQs were obtained with the optimized PFV of 1.2.

Table 4. LOQ data for power function value (PFV) 1.0 for all columns and PFV 1.2 for column 1.

(µg/g)	Column 1 (PFV 1.0)	Column 1 (PFV 1.2)	Column 2 (PFV 1.0)	Column 3 (PFV 1.0)
Cholesterol	0.5	0.2	0.5	5
DSPE	1	0.2	2	5
HSPC	2	0.5	2	8

Conclusions

- Relative and absolute amounts of cholesterol, DSPE-PEG2000 and HSPC were analyzed with a step-gradient HPLC-CAD method. All 12 experiments at four different sites with three different systems and on 12 individual columns passed the system suitability tests.
- DSPE-PEG2000 and HSPC eluted in the same step of the gradient and could be quantified with the same calibration curve. Similarly, all nonvolatile impurities eluting in this step can also be quantified with the same curve.
- The Hypersil Gold 150 x 3 mm, 3 µm column is an appropriate column for this analysis. All three columns in the test at all sites on all systems passed the system suitability tests.
- Suitably linear concentration areas were found between 10 and 300 µg/g for power function value 1.2 and between 0.5 and 50 µg/g for the default power function value.
- Quadratic fits or log-log fits of the data were suitable for calibration from 0.2 to 300 µg/g for cholesterol and DSPE-PEG2000 and from 0.5 to 300 µg/g for HSPC.

References

1. ASTM International Standard E3297-21. “Standard test method for lipid quantitation in liposomal formulations using high performance liquid chromatography (HPLC) with a charged aerosol detector (CAD). Edition published April 2022, DOI: 10.1520/E3297-21.
2. Thermo Fisher Scientific Technical Guide 73914. “Getting the most out of your charged aerosol detector: Factors influencing charged aerosol detector performance.” https://assets.thermofisher.com/TFS-Assets/CMD/brochures/tg-73914-hplc-cad-factors-influencing-performance-tg73914-en.pdf
3. Thermo Fisher Scientific White Paper 72711. “An instrument parameter guide for successful (U)HPLC method transfer.” https://assets.thermofisher.com/TFS-Assets/CMD/Reference-Materials/wp-72711-lc-method-transfer-guide-wp72711-en.pdf
4. Thermo Fisher Scientific Technical Note 73449. “Why use charged aerosol detection with inverse gradient?” https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-73449-cad-inverse-gradient-tn73449-en.pdf
5. P.H. Gamache, ed., Charged aerosol detection for liquid chromatography and related separation techniques, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2017. https://doi.org/10.1002/9781119390725
6. Thermo Fisher Scientific Technical Note 73299. “Charged aerosol detection – use of the power function and robust calibration practices to achieve the best quantitative results.” https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-73299-lc-cad-calibration-tn73299-en.pdf

Trademarks/licensing

© 2024 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries unless otherwise specified. Doxil is a trademark of Baxter Healthcare Corporation. Onivyde is a registered trademark of Merrimack Pharmaceuticals, Inc. ASTM international granted permission to cite ASTM International Standard E3297-21. This information is not intended to encourage use of these products in any manner that might infringe the intellectual property rights of others.

Learn more at thermofisher.com/CAD