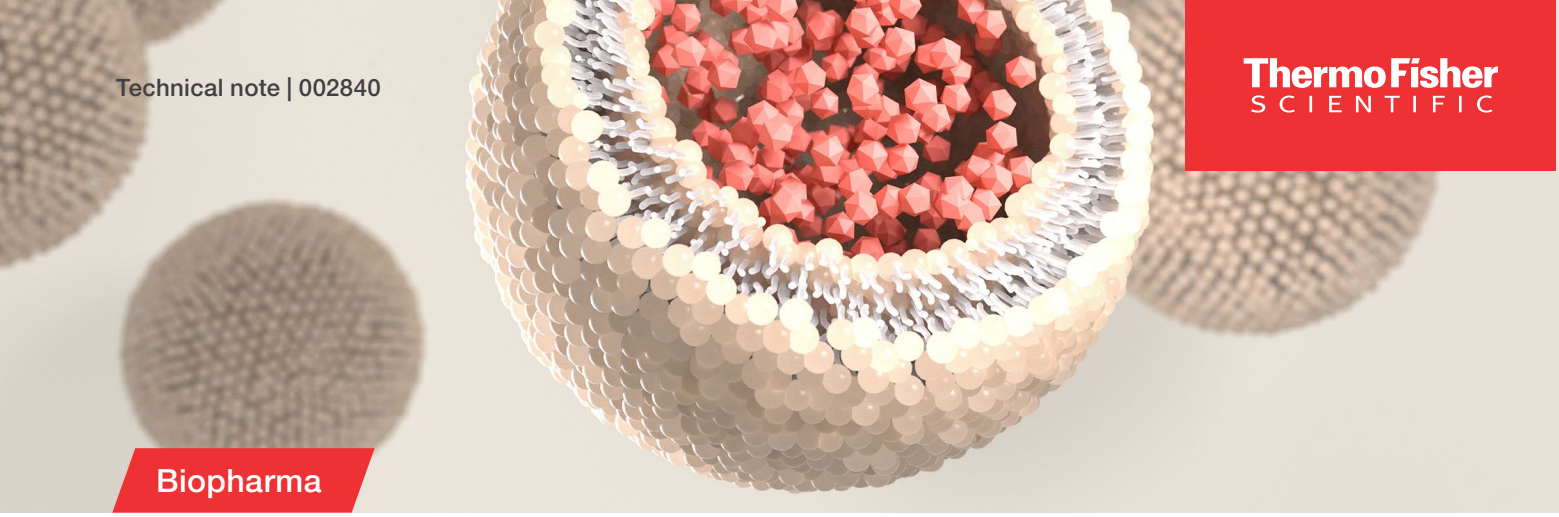
Technical note | 002840



Biopharma

Best practices for liposome analysis with the charged aerosol detector

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Keywords

Liposomes, vaccines, Vanquish Charged Aerosol Detector, uniform response, universal detection, Vanquish Flex UHPLC, Vanquish Core HPLC, cholesterol, DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]), HSPC (hydrogenated soy phosphatidylcholine)

Application benefits

- Using an ASTM method,[1] lipids for liposomal formulations are quantified with calibration curves of each lipid from 0.2 to 300 µg/g with linear, quadratic, or log-log fits.
- In a test comparing columns and system suitability for the method, all four sites and 12 system configurations passed the system suitability test, including sufficient resolution between HSPC 1 and HSPC 2, %RSD for peak area, and calibration curve suitability. All tested columns and all Thermo Scientific™ Vanquish™ Flex and Core systems easily meet the requirements of this robust and versatile method.
- The Thermo Scientific™ Hypersil GOLD™ 150 × 3 mm, 3 µm column is an excellent column for this analysis. All three columns in the test at all sites on all systems passed the system suitability tests.

Goal

Facilitate column choice and system set-up and provide quantitation strategies to save analyst time and improve result accuracy.

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, liposomal doxorubicin (Doxil™) and liposomal irinotecan (Onivyde™) are commonly prescribed formulations with these lipids. The relative ratios are important for controlling drug potency and must be confirmed in the final product using an efficient and simple-to-interpret quality control method. ASTM E3297-21[1] is a standard method that allows separation of these lipids and quantitation using the charged aerosol detector (CAD). The CAD is well-suited for this analysis because all three lipids and their impurities lack strong chromophores. These lipids show

strong signals with the CAD but weak or nonexistent signals in UV detectors. Furthermore, the CAD's uniform response for nonvolatile substances allows accurate quantification of unknowns using a surrogate calibration curve, unlike the highly variable MS (mass spectrometry) response factors. Thermo Scientific™ Vanquish™ Flex UHPLC and Thermo Scientific™ Vanquish™ Core HPLC systems with quaternary and binary pumps were used at four different sites in three different countries to develop implementation strategies for this CAD liposome method in a comparison test. A separate rigorous and much larger round robin test was conducted by the ASTM to evaluate the general HPLC method according to ASTM protocol, and those results are not published here. The results below are presented to suggest additional suitable columns for this method and to confirm general system suitability of Thermo Scientific Vanquish Flex and Core HPLC systems for the method.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Acetonitrile, Optima™ LC/MS grade, Fisher Chemical™ (P/N A955-212)
- Methanol, Optima™ LC/MS grade, Fisher Chemical™ (P/N A456-212)
- Ammonium acetate, Optima™ LC/MS grade, Fisher Chemical™ (P/N A11450)
- Hydrogenated soy phosphatidylcholine (HSPC) containing fatty acids C16:0 (HSPC 1) and C18:0 (HSPC 2), CAS 97281-45-3, Lipoid GmbH (P/N Lipoid S PC-3)
- 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000), CAS 147867-65-0, Lipoid GmbH (P/N Lipoid PE 18:0/18:0 – PEG 2000)
- Cholesterol (Chol), plant-based, Spectrum Chemical (P/N CH135)

Sample handling

- Stirring hotplate, Fisherbrand™ Isotemp™ (P/N S14365)
- Heated sonicator, 6 L capacity, Fisherbrand™ (P/N FB15337418)
- Mini centrifuge, Fisherbrand™ (P/N 2-006-901)
- Mini vortex mixer, Fisherbrand™ (P/N 4-955-152)
- 2 mL Amber glass screw top vials, Level 2, Thermo Scientific™ SureSTART™ (P/N 6ASV9-2P)
- 9 mm Screw caps, Level 2, Thermo Scientific™ SureSTART™ (P/N 6ASC9ST1)
- Class B amber glass threaded 20 mL vials with attached caps, Fisherbrand™ (P/N 14-955-333)
- Volumetric flasks, 10 mL and 1000 mL, Fisherbrand™ (P/N FB40010 and 10-205F)

Instrumentation

System 1 was a Vanquish Flex quaternary (LPG) UHPLC system with 100 µm i.d. tubing. System 2 was a Vanquish Core quaternary (LPG) HPLC system with 130 µm i.d. tubing, and System 3 was a Vanquish Core binary (HPG) HPLC system with 130 µm i.d. tubing (Table 1).

Sample preparation

For complete sample preparation requirements, refer to the new ASTM standard test method for lipid quantitation in liposomal formulations, ASTM E3297-21.[1] The details of this method are not published here for copyright reasons. Calibration standards and system suitability (SST) standards were prepared in methanol with each of the three substances at 0.5, 1, 2, 10, 25, 50, 100, 150, and 300 µg/g (0.63 to 379 µg/mL). Two stock solutions of 10 mg each substance in 10 mL methanol (1.26 mg/g, 1 mg/mL) and 4 mg each substance in 10 mL methanol (500 µg/g, 4 mg/mL) were used. Stock solutions were stored at -20 °C when not in use. Before use, stock solutions in 20 mL glass amber vials were brought to room temperature, sonicated for 1 minute, vortexed for 2 minutes, and sonicated again for 1 minute before transferring to autosampler vials. This procedure prevented sample loss due to association with glass walls.

Table 1. Component specifications for the Vanquish HPLC systems

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 with standard loop	VF-A10-A	VC-A12-A	
Column compartment	VH-C10-A	VC-C10-A	
Charged aerosol detector	VH-D20-A or VF-D20-A or Thermo Scientific™ Corona™ Veo™ CAD		

Eluent preparation

For complete eluent preparation requirements, refer to ASTM E3297-21.[1] Solvent bottles were rinsed three times with water and once with solvent (acetonitrile for eluent A and methanol for eluent B). The eluents were placed in an ultrasonic bath for 15 minutes at atmospheric pressure for degassing and to reduce noise in the baseline. Later, while running the step gradient method (Table 3), if a large increase in the baseline was seen upon switching from acetonitrile to methanol at minute 16 in the method, eluent A was re-prepared using a new bottle of acetonitrile. For this last problem, see also the next section.

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 in the gradient method,[1] the column was removed and the system was washed. During the wash, the evaporation tube temperature was set to 70 or 90 °C and the system was flushed with 1 mL/min of water, isopropanol, and acetonitrile, switching between the solvents every 10 minutes for at least two hours. If a baseline shift persisted, a new bottle of acetonitrile was used.

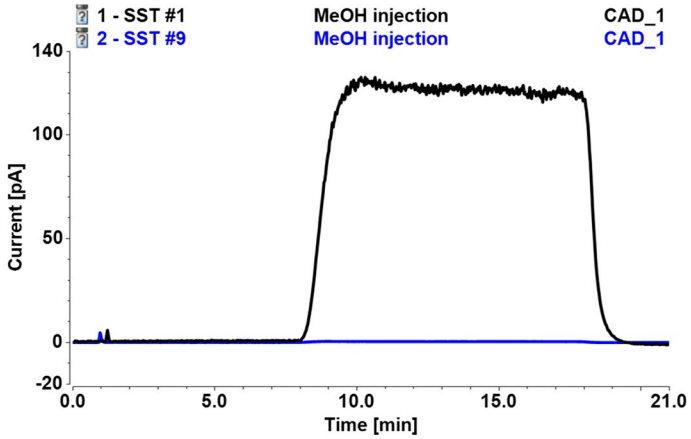

Figure 1. Example baseline of a system not previously used for this application that was flushed for several hours at a high evaporation temperature. The baseline step was 125 pA before cleaning (black) and 0.5 pA after cleaning (blue).

Columns

Three fully porous C18 columns of 3 or 3.5 µm particle size were tested. All columns were 150 mm in length and 3 mm in diameter, as specified in the standard. Column 1 was a Thermo Scientific™ Hypersil GOLD™ C18 column with 3 µm particle size (P/N 25003-153030). Column 2 was a fully porous ethylene-bridged hybrid particle C18 column with 3.5 µm particle size. Column 3 was a fully porous silica particle C18 column with 3 µm particle size.

Chromatographic conditions

For complete chromatographic conditions, refer to ASTM E3297-21.[1]

Proper column temperature control is crucial for this method because the cholesterol and DSPE-PEG 2000 peaks are prone to swapping elution order with improper temperature control. Use of the Vanquish column compartment in still air or forced air mode at 35 °C and an active or passive preheater at 35 °C (Table 2) produces chromatograms with the expected peak order. The relatively low back pressure (<175 bar for all experiments and columns) means that the influence of forced or still air thermostatting modes on the chromatographic separation is negligible.[3]

The gradient is a step gradient that begins at 40% B and increases to 90% B after six minutes (Table 3).

Table 2. Chromatographic conditions

Parameter	Value
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 temperature	35 °C, still air mode heating
Preheater temperature	35 °C
Autosampler temperature	10 °C
Injection details	5 µL, sample in methanol
Rear seal wash	10% methanol in water
Needle wash	Methanol
CAD evaporation tube temperature	35 °C
CAD power function value	1.0 or 1.2 (see text)

Table 3. Gradient conditions

Time (min)	%A	%B
0	60	40
6	60	40
6.1	10	90
16	10	90
16.1	60	40
21	60	40

Chromatography Data System

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

Quantification in Chromeleon CDS: Using peak group to combine HSPC 1 and HSPC 2

HSPC is a natural product and generally contains both 16:0 (HSPC 1) and 18:0 (HSPC 2) fatty acids. The calibration is based on the sum of the areas of these two peaks. This summation is done in Chromeleon CDS with the named peak group function. A named peak group called “HSPC” is created in the “peak group table” tab of the processing method (Figure 2). Then, HSPC 1 and HSPC 2 are assigned to their chromatogram peaks in the component table (Figure 3). Adding the “peak.groupAmount” and “peak.groupArea” functions to the results table allows a readout of the total HSPC area and amount (Figure 4). The “Group Peaks” Chromeleon CDS help page is a useful introduction to the analysis.

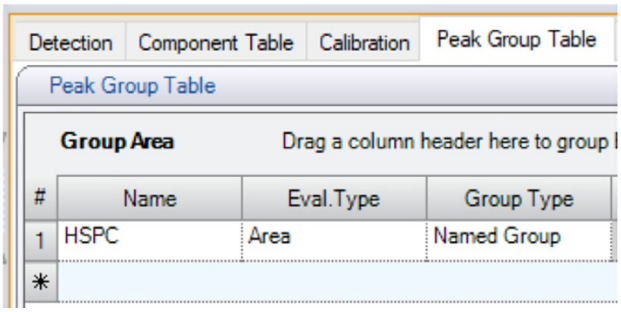

Figure 2. The HSPC peak group is defined as a named group in the peak group table tab.

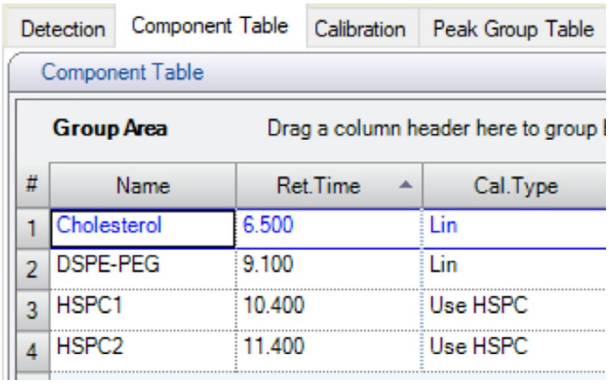

Figure 3. Individual peaks HSPC 1 and HSPC 2 are listed in the component table tab of the processing method, with calibration by the peak group “HSPC.”

Results and discussion

Within the framework of the round robin test, twelve combinations of column, UHPLC system, and laboratory site were evaluated based on whether the method quality criteria[1] were satisfied. The three criteria were resolution > 1.5 for all peak pairs, peak area %RSD < 5% and $R^2 \geq 0.995$ for the log-log fit of the calibration curves for all three substances. All criteria were met in all test scenarios.

Quality criterion 1 of 3: Resolution

Resolution must be greater than 1.5 for all peak pairs to meet quality criteria for this method. The resolution of all peaks in all chromatograms was greater than 1.5 for all columns tested (Figures 5 and 6). Column 1 easily met this requirement. Possible GDV and method adjustments for columns 2 and 3 could be implemented to further increase resolution of critical peak pairs but were not necessary for the tested column batches. These adjustments are discussed below.

Improved resolution of first peak pair with increased GDV

The first peak pair (cholesterol – DSPE-PEG 2000) was the most critical for column 2. DSPE-PEG contains polyethylene glycol, which is not perfectly monodisperse and presents a broad peak in the chromatogram of column 2. Focusing only on column 2, resolution for this peak pair ($R_{1/2}$) was 3.02, 2.74, 2.97, and 2.58 at the four sites, each of which used their own Vanquish Flex UHPLC system and independently purchased columns (Figure 6). If desired, this resolution can be easily manipulated by changing the gradient delay volume because the DSPE-PEG 2000 peak does not elute until the step gradient arrives at the column head. For example, adding 300 µL of mixer volume between the pump and the autosampler delays the elution of DSPE-PEG 2000 by 0.43 min (300 µL at 700 µL/min) and roughly doubles the Chol-DSPE resolution.

	A	B	C	D	E	F	G
1	Inj.	Injection Name	Ret.Time	Amount	Area	Group Area	Group Amount
2	No.	Selected Peak:	min		pA*min	pA*min	
3			CAD_1	CAD_1	CAD_1	CAD_1	CAD_1
4			HSPC 2	HSPC 2	HSPC 2	HSPC 2	HSPC 2
14	10	Injection of Level 1	15.193	3.4867	0.2008	0.267	4.6958
15	11	Injection of Level 1	15.170	3.6200	0.2081	0.269	4.7342
16	12	Injection of Level 1	15.160	3.6124	0.2077	0.264	4.6323
17	13	Injection of Level 1	15.130	3.6142	0.2078	0.266	4.6769
18	14	Injection of Level 1	15.110	3.8309	0.2197	0.277	4.8745
19	15	Injection of Level 1	15.110	3.6456	0.2095	0.272	4.7954

Figure 4. Results table shows total amount calculation for the summed areas of HSPC 1 and HSPC 2 for several injections of HSPC at 5 µg/g.

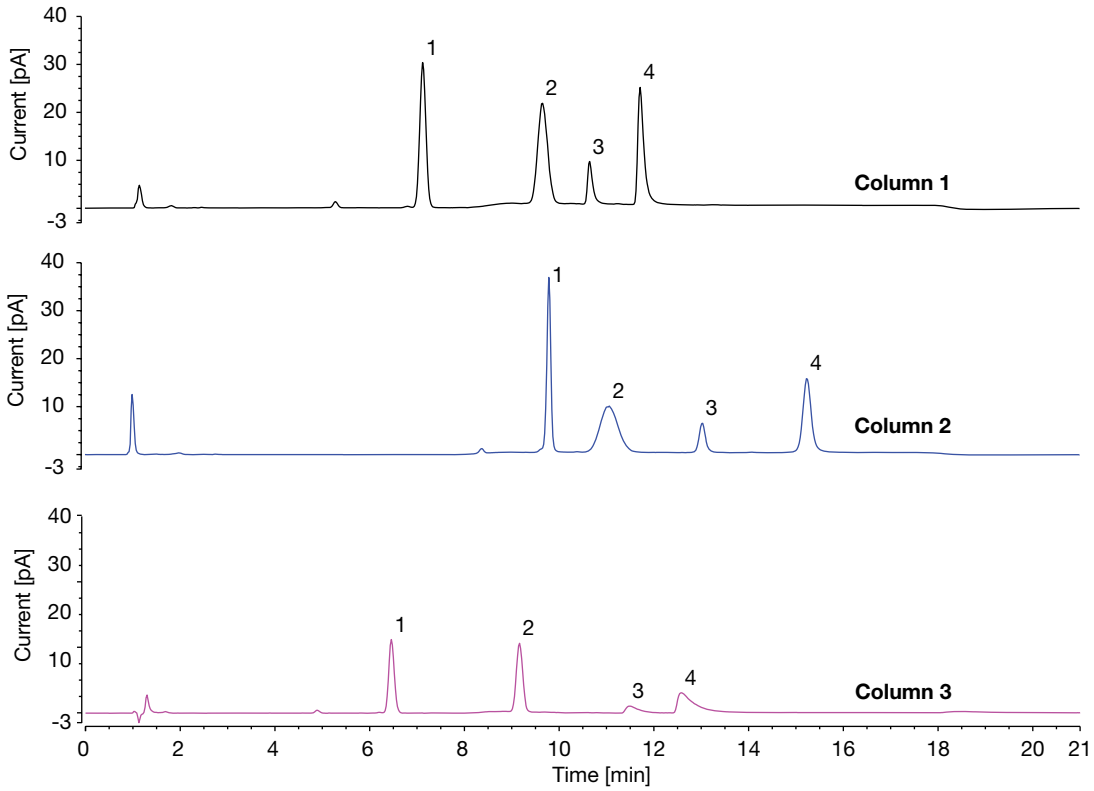

Figure 5. Chromatograms from a quaternary Vanquish Flex system for all three columns showing elution of cholesterol (peak 1), DSPE-PEG2000 (peak 2), HSPC 1 (peak 3), and HSPC 2 (peak 4)

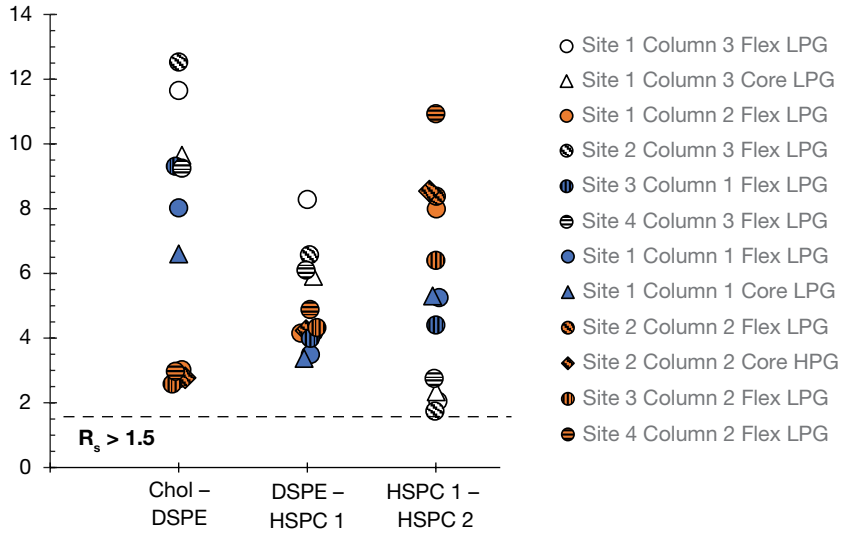

Figure 6. Resolution between all peak pairs by column type (marker fill color), system type (marker shape), and site (fill pattern). The quality criteria for R_s > 1.5 was met for all sites, systems, and columns.

Improved resolution of last peak pair with less eluent B

The resolution of the last peak pair (HSPC 1 – HSPC 2) was close to the critical 1.5 value for column 3, with resolutions of 2.05, 2.12, 1.74, and 2.75 at the four sites. Each of the sites purchased their own column 3 and tested it with their own Vanquish Flex UHPLC system. Reducing the B content of the second step from 90% to 80% led to improved selectivity between the 16:0 and 18:0 fatty acid variants and improvement in the resolution of HSPC 1 and HSPC 2 on column 3 (from 2.12 to 2.53 for the Vanquish Core quaternary pump, Figure 7). There is no ASTM guideline for allowable HPLC method changes comparable to USP General Chapter <621>.

Quality criterion 2 of 3: Reproducibility of peak area

The peak area %RSD for three repeated injections of the 50 µg/g standard is reported for each of the three components in Figure 8. The peak areas of HSPC 1 and 2 were summed for each injection and then averaged. The peak area %RSD was ≤3% in all datasets. 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.

Quality criterion 3 of 3: Quality of calibration curves

The standard method prescribes use of a log-log fitting equation (a plot of log (peak area) versus log (concentration)) after data collection. With the log-log fit, all coefficients of determination for all standards, columns, and system configurations met the quality criteria ($R^2 \geq 0.995$) for this method.

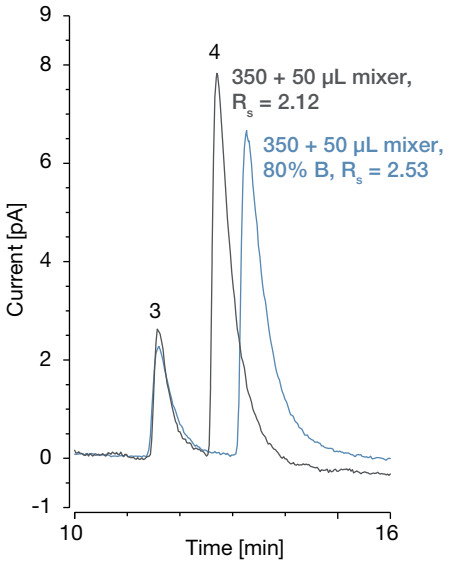

Figure 7. Resolution of HSPC 1 (3) and HSPC 2 (4) on column 3 and the Vanquish Core quaternary HPLC system with the standard 350 + 50 µL mixer system (black) or with the standard mixer system and a second step to 80% B instead of 90% B (blue)

Because DSPE-PEG2000 and HSPC elute under the same high-organic conditions, are non-volatile under the CAD nebulization conditions, and are present in the standards at equimolar concentrations, the calibration curves overlap (Figure 9).

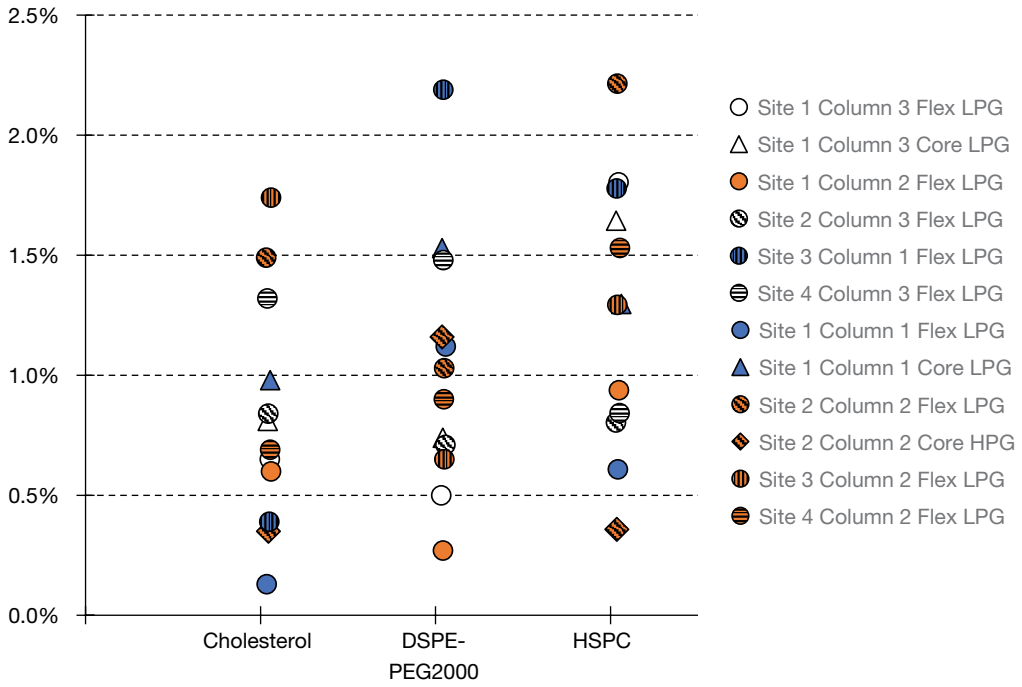

Figure 8. %RSD for peak area for the three components by column type (marker fill color), system type (marker shape), and site (fill pattern) was below 5% in all experiments.

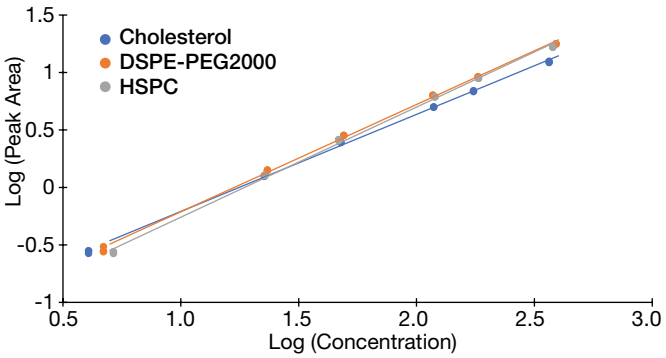

Figure 9. A log-log plot with linear fit for peak areas from three injections of each of six standards (5, 25, 50, 100, 150, and 300 µg/g) onto column 1. The curves for DSPE-PEG2000 and HSPC, the two substances eluting in the high-organic part of the step gradient, are interchangeable because of the CAD's uniform response. All three curves would overlap if the inverse gradient were used to compensate for the low organic part of the step gradient, where cholesterol elutes.

Quantifying both substances with one calibration curve instead of two would save analyst time. All three substances could be quantified with the same calibration curve if the inverse gradient were used to compensate for the low organic part of the gradient, where cholesterol elutes (for more information, see TN73449[4]). Any impurities eluting in the later step can be calibrated using the DSPE-PEG2000 or HSPC calibration curves, provided they are nonvolatile under the conditions of the CAD nebulization process. A nonvolatility factor, calculated as the ratio of the impurity's peak area at 35 °C evaporation temperature to the area at 50 °C evaporation temperature, should be greater or equal to 0.85 if the impurity is nonvolatile.[5,6] Similarly, any nonvolatile impurities eluting in the first step of the gradient can be quantified by the cholesterol calibration curve.

Other calibration curve possibilities: Power function value and curve fit choice

CAD response is commonly described as approximately linear, or quasi-linear, over a range of 1.5 to 2 orders of magnitude ($10^{1.5}$–10^{2}), but non-linear over a wider range. Other nebulizer-based detectors such as ELSD and mass spectrometers are also inherently non-linear. A detailed description of the CAD response function and calibration options is provided in Chapter 1 of *Charged Aerosol Detection for Liquid Chromatography and Related Separation Techniques*.[7] The response can be linearized either after data collection, by mathematically applying a fitting equation[8] or during data collection by setting the power function method parameter.[8] Linearization of already collected data is discussed in the first subsection and linearization of detector response using the power function method parameter is discussed in the second subsection.

Linearization by fit equation

The standard method prescribes application of a log-log fitting equation (a plot of log (peak area) versus log (concentration)) after data collection. The stated rationale for choosing this fit is that CAD response can be modelled empirically with a power law function, and power functions appear as straight lines in a log-log plot. When evaluating CAD data, quadratic linear fits and log-log fits should always be considered because they sometimes produce smaller residuals and more accurate quantitation results.[8] Fitting with a curve weighted by 1/x or $1/x^2$ is also often helpful.

For example, Figure 10 shows a dataset from column 3 that is best fit with a quadratic equation. Although the residuals are smaller and the R^2 is larger for the quadratic fit than the log-log fit, both fits meet the criteria of $R^2 > 0.995$. Weighting by 1/x did not show a benefit in this application.

Residual plots instead of R^2 were evaluated to ensure that all calibration points were relatively evenly distributed above or below the calibration curve and that no points were further away than ~10% relative deviation.[8] Residual plots were used because even a high R^2 value may conceal a poor data fit at some concentrations and lead to bad quantitation results.

The Chromeleon CDS log-log transformation called "Power Function" was used to calculate the fit. This calibration fitting routine is applied after data collection and is different from the detector parameter "Power Function Value" discussed in the next section, which is a setting used during data collection. An index search in Chromeleon CDS of the term "Pow" provides more information about this function. The R^2 used for the log-log plot was the coefficient of determination. The correlation coefficient only indicates the linear dependence and could not be used in the case of this non-linear calibration function.

Linearization by power function value

The calibration curve was linearized during data collection by modifying the instrument setting called the power function value (PFV); the details and equations for this have been reviewed in a recent technical note.[9] Using this capability and the recommendations for choosing a power function value, a linear calibration curve fit showed acceptable residuals for concentrations of 0.5 to 50 with a PFV of 1.0 and from 10 to 300 with a PFV of 1.2 (Figure 11).

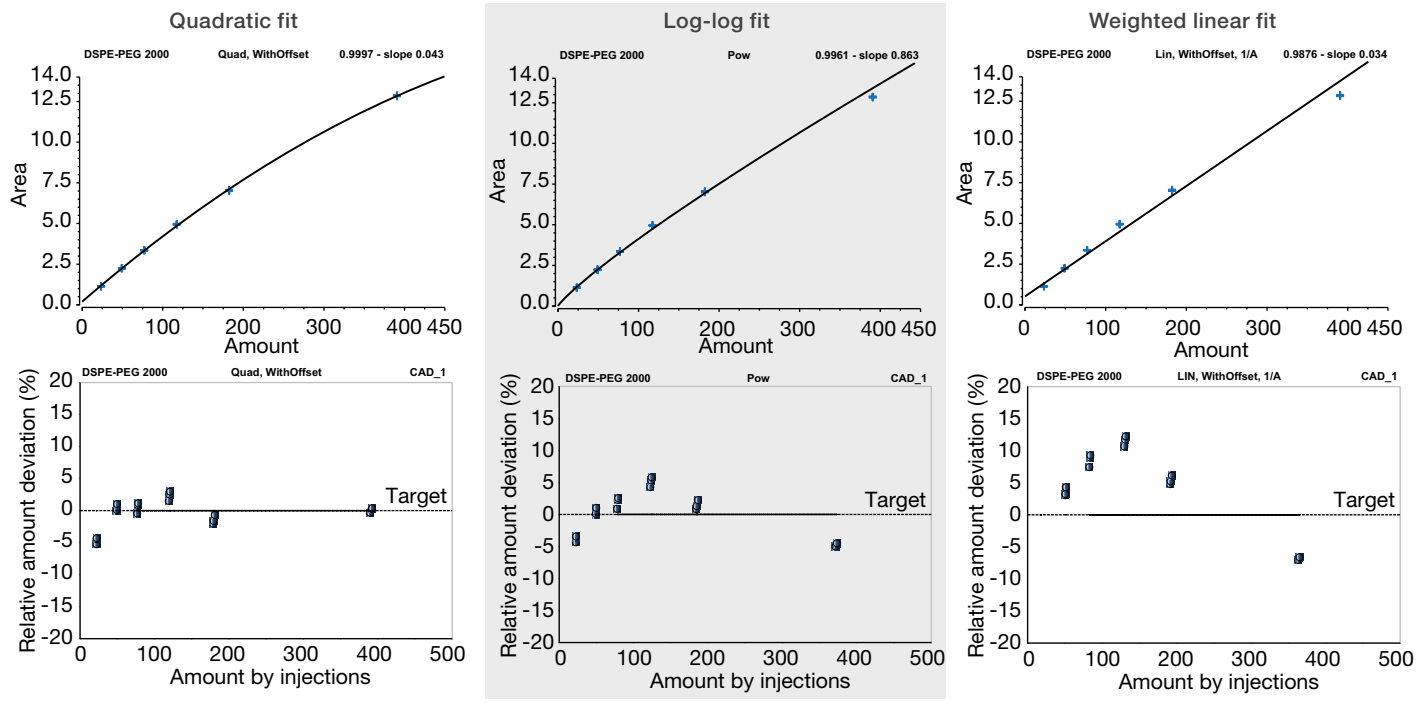

Figure 10. From left to right, quadratic, log-log, and weighted linear fits for DSPE-PEG2000 peak areas from three injections of each standard onto column 3. The quadratic fit has the smallest residuals of the three fits. Both the quadratic and the log-log fit meet the R^2 criteria of the standard method ($R^2 > 0.995$).

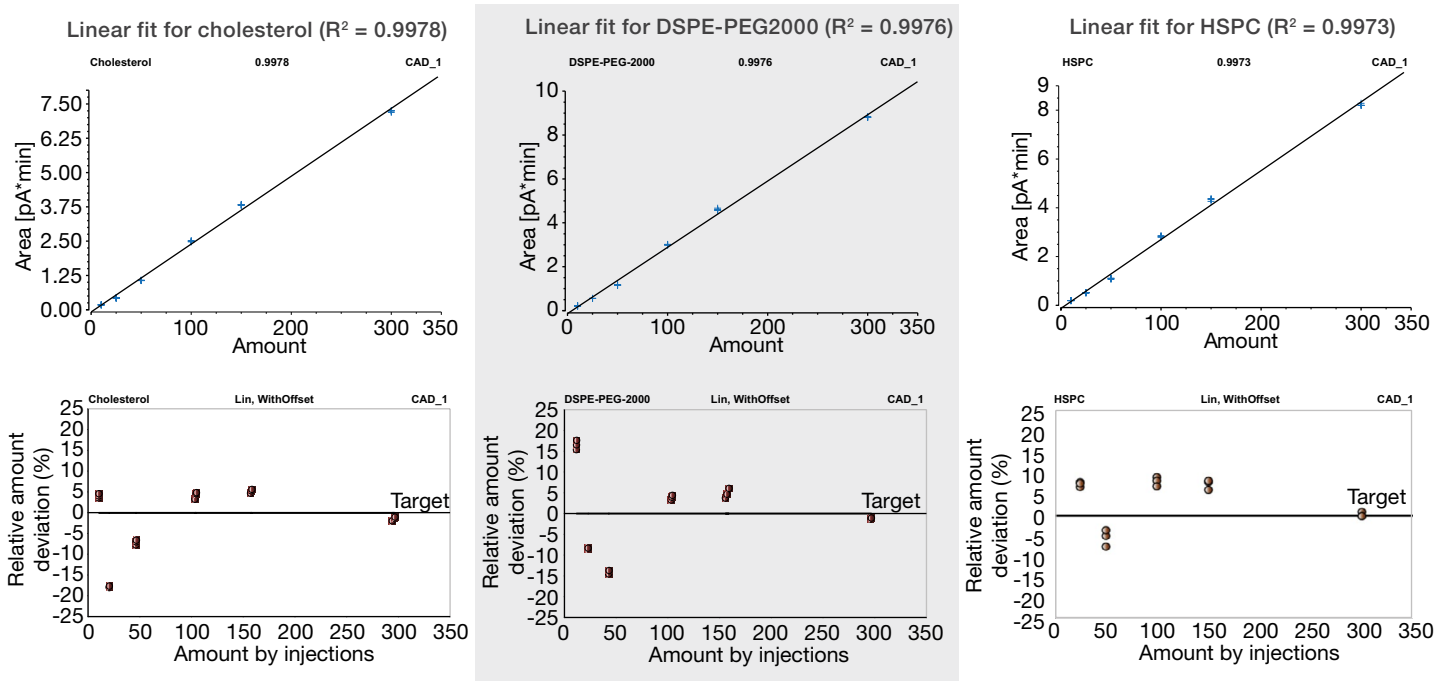

Figure 11. From left to right, dataset collected with PFV = 1.2 showing linear fits for cholesterol ($R^2 = 0.9978$), DSPE-PEG2000 ($R^2 = 0.9976$), and HSPC ($R^2 = 0.9973$) from three injections of 10, 25, 50, 100, 150, and 300 µg/g standard solutions onto column 1

Table 4. LOQ data for at PFV 1.0 and PFV 1.2 for column 1 and at PFV 1.0 for columns 2 and 3

	Column 1 (µg/g)	Column 1 (µg/g)	Column 2 (µg/g)	Column 3 (µg/g)
PFV	1.0	1.2	1.0	1.0
Cholesterol	0.5	0.2	0.5	5
DSPE-PEG2000	1	0.2	2	5
HSPC	2	0.5	2	8

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 S/N values were higher than those of the PFV 1.0 dataset, as expected (for discussion, see TN73299[8]). Customers striving for a lower LOQ may be especially interested in collecting data with the optimized PFV of 1.2. The peak shapes of HSPC 1 and 2 on column 3 required a calibration curve with concentrations starting at 10 µg/g instead of the 5 µg/g suggested in the method because of the higher LOQ for HSPC for this column. Such a change is explicitly allowed in the method.

Conclusion

- Relative and absolute amounts of cholesterol, DSPE-PEG2000, and HSPC were analyzed with a step-gradient HPLC-CAD method, and 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 × 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 appropriate for calibration over a concentration range of 0.2 to 300 µg/g for all three substances.

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