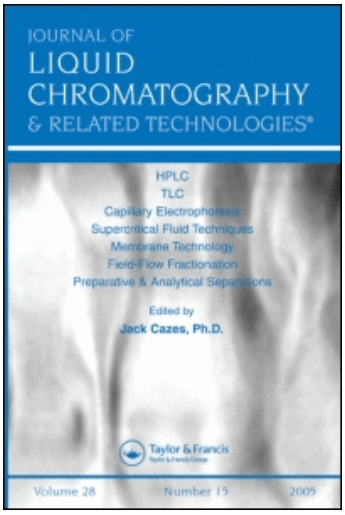

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bitman, Joel and Wood, D. L.(1981) 'Quantitative Densitometry in Situ of Lipids Separated by thin Layer Chromatography', Journal of Liquid Chromatography & Related Technologies, 4: 6, 1023 — 1034
To link to this Article: DOI: 10.1080/01483918108059602
URL: http://dx.doi.org/10.1080/01483918108059602

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(6), 1023-1034 (1981)

QUANTITATIVE DENSITOMETRY IN SITU OF LIPIDS SEPARATED BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Operating parameters are described for a densitometric method to determine in situ eight lipid classes separated by thin layer chromatography. The separated lipids, visualized on the TLC plate by a cupric acetate-phosphoric acid charring method, were quantitatively determined by spectrodensitometry using the Shimadzu CS-910 Dual Wavelength TLC Scanner. Plates were scanned in either a linear scanning mode or in a zigzag scanning mode (flying spot).

Reproducibility of a) sample application (spotting) and b) the lipid separation procedure was determined by scanning. Transmittance measurements yielded response areas that were 2.8 X higher than reflectance measurements. Operating parameters such as scanning direction, wavelength, single and dual-wavelength measurement, scanning speed, and slit geometry were studied. Optimal conditions were established for quantitative densitometry of lipids on thin layer plates.

INTRODUCTION

Lipids are broad groups of diverse materials including waxes, fats and oils which range chemically from simple aliphatic hydrocarbons or cyclic carbon compounds to complex aliphatic compounds containing various functional groups. Thin layer chromatography (TLC) is able to separate the lipids into different classes because of polarity differences conferred upon the lipids by the differing functional groups. After application of a visualization technique to the thin layer plate, the resolved lipids may be measured directly by densitometry. This paper describes the in situ quantitative spectrodensitometry of lipids separated by TLC. Operating parameters for

optimal quantitation were established. The reproducibility of the lipid class separation procedure and of individual elements of in situ quantitation by densitometric scanning were studied.

EXPERIMENTAL

TLC Procedure (Standards, TLC Plates, Sample Application, Development, Visualization)

The TLC technique employed in this study was described previously (1). The lipids studied consisted of the following 8 classes listed in ascending order from the origin: phospholipid (PL), monoglyceride (MG), free fatty acid (FFA), cholesterol (C), 1,2-diglyceride (1,2-DG), 1,3-diglyceride (1,3-DG), triglyceride (TG), and cholesterol ester (CE). The standard mixture was composed of lipids containing equal amounts of C_{18} monounsaturated (oleic) and polyunsaturated (linoleic) fatty acids in the lipid moiety. The separated lipids were visualized by use of a cupric acetate-phosphoric acid reagent with subsequent heating to produce charred spots (2).

Apparatus

The Shimadzu CS-910 Dual-Wavelength TLC scanner (Shimadzu Scientific Instruments, Inc., Columbia, MD 21045) was used for densitometric scanning. The instrument system includes two monochromators and can be used in transmission, reflectance or fluorescence modes. Deuterium and tungsten lamps provide a range of wavelengths in the ultraviolet-visible range (200-800 nm). A xenon lamp provides excitation light for the fluorescence mode. The TLC plates were scanned in either a linear scanning mode in which the plate moves linearly under a rectangular light beam, width 0.3-1.0 mm, length 0-10 mm, or in a zigzag scanning mode (flying spot) in which the plate oscillates in a zigzag manner under a very small light beam, 1.25 x 1.25 mm

RESULTS AND DISCUSSION

Reproducibility

The reproducibility of scanning by the zigzag method (Fig. 1) was compared to the linear method (Fig. 2). A single lane containing 7 µg of the standard for each lipid class was scanned 8 times in the direction of solvent flow

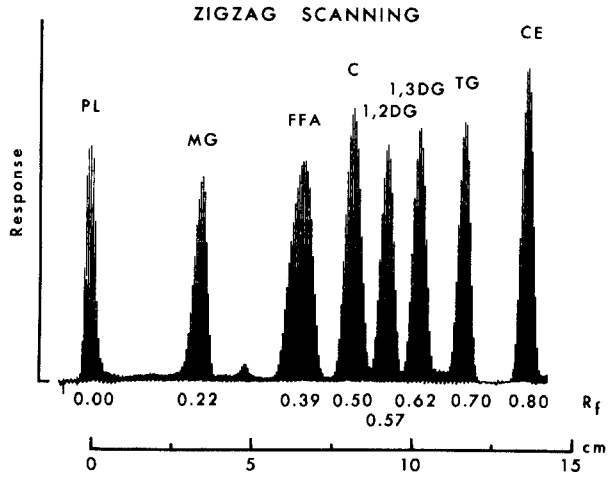

FIGURE 1. Zigzag scan of lipid classes on Whatman K5 silica gel plate obtained with the Shimadzu CS-910 Dual Wavelength TLC scanner.

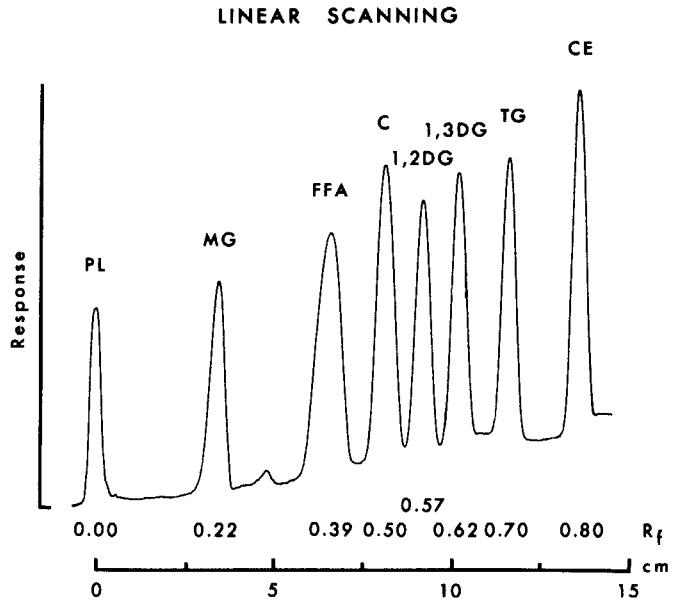

FIGURE 2. Linear scan of lipid classes on Whatman K5 silica gel plate obtained with the Shimadzu CS-910 Dual Wavelength TLC scanner.

(Table 1A). The coefficients of variation observed for linear scanning ($\overline{CV}$ = 0.6%), and for zigzag scanning ($\overline{CV}$ = 0.8%), demonstrated that instrumental variability was low and that both scanning methods were reproducible. Differences between linear and zigzag values (Table 1A) reflect the manner in which the two scanning methods measure irregularly shaped spots. The zigzag method provides more reproducible integrated values of peak area for spots with irregular shapes and sizes, since the unit automatically corrects for background absorption during each pass over the charred spot, thereby minimizing differences in spot geometry.

TABLE 1
Reproducibility of Lipid Quantitation

Lipid Class	Linear Scanning Response μV. sec/10^3 $\bar{X}$	SD	CV %	ZigZag Scanning Response μV. sec/10^3 $\bar{X}$	SD	CV %
A. PL	35.5	0.1	0.4	27.4	0.3	1.0
MG	30.9	0.4	1.5	18.4	0.3	1.5
FFA	110.1	0.2	0.2	104.4	0.7	0.7
C	109.2	0.2	0.2	102.1	0.6	0.6
1,2-DG	63.9	0.2	0.3	58.9	0.5	0.8
1,3-DG	65.3	0.2	0.3	58.2	0.4	0.7
TG	63.3	0.9	1.4	56.7	0.3	0.5
CE	92.7	0.3	0.3	86.5	0.6	0.7
		$\overline{CV}$	0.6		$\overline{CV}$	0.8
B. PL	33.6	3.5	10.5	29.1	3.8	12.9
MG	51.2	6.6	12.9	23.5	4.8	20.6
FFA	149.0	19.0	12.7	116.4	11.4	9.8
C	124.8	7.7	6.2	108.9	6.9	6.3
1,2-DG	72.4	7.0	9.6	61.4	4.8	7.9
1,3-DG	71.6	8.5	11.8	63.9	5.5	8.6
TG	67.7	10.5	15.5	62.1	5.4	8.7
CE	89.9	9.5	10.5	93.6	7.3	7.8
		$\overline{CV}$	11.2		$\overline{CV}$	10.3

Single λ, 350 nm. Linear: Beam, 10.0 x 0.5 mm. Scanning Speed, 80 mm/min. ZigZag: Beam, 1.25 x 1.25 mm. Scanning Speed, 20 mm/min.

A. Reflectance measurements of 8 scans of the same lane.

B. Reflectance measurements of 6 different lanes on the same TLC plate.

The reproducibility of spotting and scanning was determined by scanning the 7 μg standard which had been spotted on 6 different lanes of the same plate (Table 1B). The mean coefficient of variation was 11.2% for linear scanning and 10.3% for zigzag scanning. Since scanning alone had a variation of <1% (Table 1A), 9-10% of the variability must be attributed to differences in spotting, lipid separation, and visualization aspects of the overall lipid class quantitation method.

Quantitation of Lipid Classes: Transmittance vs Reflectance

Transmittance measurements yielded response areas that were 2.8 X higher than reflectance measurements (Table 2). Slope sensitivity values, used to determine background noise, were also 2.2 X higher in the transmission mode. Reflectance measurements were adopted for routine scanning of lipid TLC plates since greater sensitivity could be achieved with the lower slope sensitivity values.

Quantitation of Lipid Classes: Variation in Charring

Cholesterol (C), cholesterol ester (CE), and free fatty acids (FFA), always gave the greatest response for an equal amount of lipid (Response

TABLE 2
Transmittance vs Reflectance Determination of Lipid Classes

Lipid	Response (μV. Sec/10^3) T	R	T/R	Response Ratio (C = 1.00) T	R
PL	73	34	2.1	0.12	0.16
MG	179	58	3.1	0.28	0.27
FFA	589	210	2.8	0.93	0.99
C	632	213	3.0	1.00	1.00
1,2-DG	397	139	2.9	0.63	0.65
1,3-DG	424	147	2.9	0.67	0.69
TG	384	134	2.9	0.61	0.63
CE	420	183	2.3	0.66	0.86
		$\bar{X}$	2.8		
Slope Sensit. μV/min.	10.1	4.6	2.2		

Single λ, 350 nm. Linear Scan: Beam, 10.0 x 0.5 mm. Scanning Speed, 40 mm/min. 7 μg of each lipid.

ratios, 0.86-1.00 with C = 1.00), indicating greater charring with the cupric acetate-phosphoric acid visualization procedure (Table 2). The triglycerides and diglycerides yielded smaller responses (0.63-0.69), whereas the phospholipids and monoglycerides yielded still smaller densitometric responses (0.16 and 0.27). Thus, quantitative differences in the amount of charring were observed for compounds of different structure which contained approximately equivalent amounts of carbon. Privett et al. (3) have discussed the problems involved in charring procedures and the non-quantitative relative yields of carbon for a variety of compounds representative of different lipid classes.

Linearity in Densitometry of the Separated Lipids on the TLC Plate

Curvilinear relationships were observed between the integrated response values and amounts of lipid over a range of 0.125-35 µg measured densitometrically using reflectance (Fig. 3). Linear relationships over such a wide concentration range are desirable but are seldom observed in practice. The calibration curve shown was obtained with linear scanning of the TLC plate using a rectangular light beam. Zigzag scanning, however, also yielded curvilinear calibration curves.

The Shimadzu CS-910 densitometer is equipped with a calibration curve linearizer which can electronically transform the non-linear curve into a linear relationship between absorbance and amount of material. Selection of 7 correcting concentration points on an absorbance calibration curve, and adjustment of 7 variable resistors, can correct for scatter and absorption which occur in the turbid medium (silica gel) of the TLC plate. This programming permits conformation to the theoretical Kubelka-Munk equations for optical transfer in turbid media (4). A linear relationship for all lipid classes over a range of 0.125-10 µg was observed when the linearizer was employed (Fig. 4). Use of the linearizer did not produce a linear relationship between response and the 25 and 35 µg concentrations when scanned in either the linear or zigzag mode.

Scanning Direction

The influence of scanning direction was evaluated by scanning 6 lanes of the 7 µg FFA and TG standards either in the direction of solvent flow (up TLC plate, Y direction) or at a right angle to solvent flow (across TLC plate, X

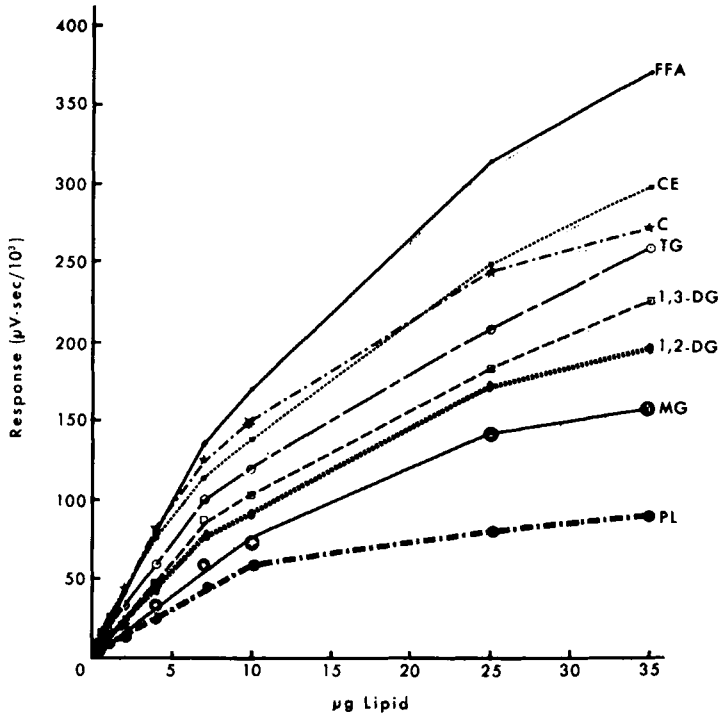

FIGURE 3. Calibration curve showing relationship between response area and concentration of lipid. Scanning conditions: Linear scan, 80 mm/min; Single λ, 350 nm; Reflectance measurement with linearizer function off; Beam, 10 x 0.3 mm.

direction). The results in Table 3 demonstrate that zigzag scanning yielded very close correspondence in response areas when scanned either up or across the TLC plate (deviations: FFA, -3%; TG, -4%). Deviations from the Y-value by measurement in the X-direction were considerably greater when scanned linearly (deviations: FFA, -25%; TG, +7%).

Pollak (5) has explained the superiority and benefits of using a flying spot scanner for measuring separated substances. Errors in integration of areas due to variations in concentration within the spot, in size and shape of the spot, and in differences of scanning direction can be kept small by keeping the area of the zone seen by the photodetector so small that the

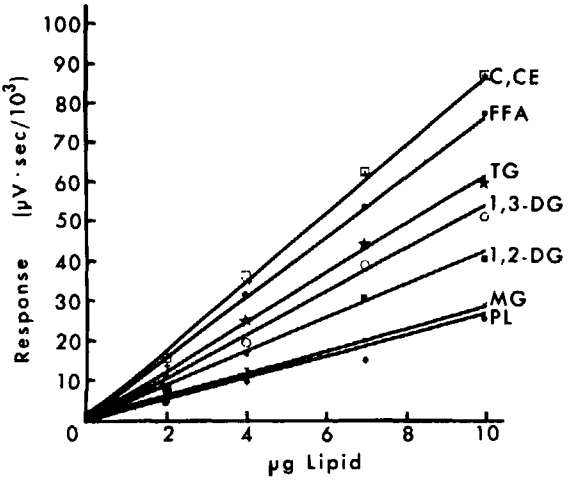

FIGURE 4. Linearized calibration curve showing relationship between response area and concentration of lipid. Scanning conditions: Linear scan, 80 mm/min; Single λ, 350 nm; Reflectance measurement with linearizer on; Beam, 10 x 0.3 mm.

TABLE 3
Influence of Scanning Direction on Response

		LINEAR		Deviation $\left(\frac{X-Y}{Y}\right)100$	ZIGZAG		Deviation $\left(\frac{X-Y}{Y}\right)100$
		Scanning Y	Direction X		Scanning Y	Direction X	
FFA	$\bar{X}$	149.0	112.3	-25	116.4	112.7	-3
	SD	19.0	12.9		11.4	10.6	
	CV	12.7	11.5		9.8	9.4	
TG	$\bar{X}$	67.7	72.7	+7	62.1	59.7	-4
	SD	10.5	6.1		5.4	5.3	
	CV	15.5	8.3		8.7	8.8	

Single λ, 350 nm Reflectance measurement.
Response units, uV.sec/10^3.
Linear: beam, 10.0 x 0.5 mm Scanning speed, 80 mm/min
Zigzag: beam, 1.25 x 1.25 mm Scanning speed, 20 mm/min
Scanning direction: Y = in direction of solvent flow
X = across TLC plate

concentration in this area can always be considered as constant. The 1.25 mm square light beam in the flying spot zigzag mode was only 31% of the 10 x 0.5 mm linear mode beam area.

Selection of Optimum Wavelength for Absorption

A survey was made of the effect of wavelength on absorption in order to select the optimum wavelength for densitometry. All lipid classes were scanned in both the transmission and reflectance modes at wavelengths between 300 and 800 nm. The black, charred spots absorb at all wavelengths but a maximum response was obtained in both measurement modes at 350 nm. Figure 5 shows the absorption curve for cholesterol, as measured by both transmission and reflectance. A comparison was made between reflectance and transmission in dual-wavelength and single-wavelength scanning of the 7 μg standard of the separated lipid classes on a thin-layer chromatogram (Table 4). Single wavelength operation yielded larger responses than dual-wavelength operation in both transmission and reflectance modes. Two light beams of different

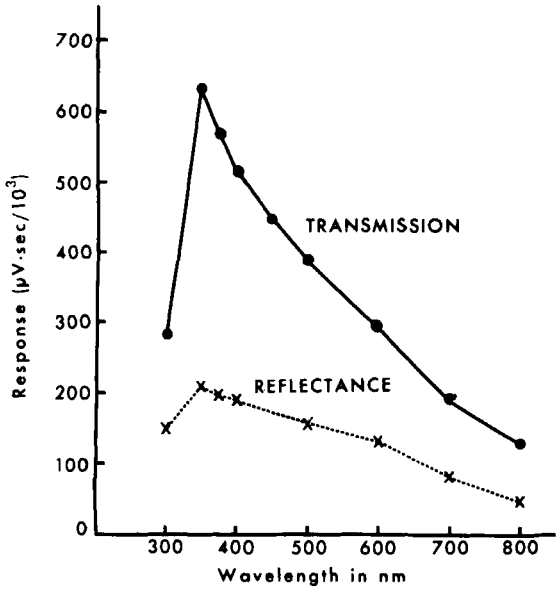

FIGURE 5. Relationship between wavelength and absorbance as measured by transmission or reflectance spectrodensitometric scanning.

TABLE 4
Single Wavelength vs Dual Wavelength Densitometry

Lipid	Response (μV.sec/10^3) Transmission Dual	Transmission Single	Reflectance Dual	Reflectance Single
PL	66	93	30	55
MG	180	222	60	91
FFA	305	429	79	126
C	271	407	63	110
1,2-DG	191	297	47	78
1,3-DG	183	311	46	82
TG	171	286	54	91
CE	185	272	64	116

Linear scanning, 80 mm/min. Beam, 10.0 x 0.5 mm.
7 μg standard.
Single Wavelength: λsample = 350 nm
Dual Wavelength: λsample = 350 nm
λreference = 800 nm

wavelengths alternately illuminate the TLC spots in the dual-wavelength single beam mode. The light transmitted through, or reflected from, the TLC plate is then measured. The response in the dual-wavelength mode is smaller than that observed in the single wavelength mode, essentially because the absorption at 800 nm is subtracted from that at 350 nm.

Influence of Scanning Speed and Slit Geometry on Response in Linear and Zigzag Scanning of TLC Plates

Figure 6 shows the influence of scanning speed on the response obtained with both linear and zigzag scanning. The effects of altering slit geometry in linear scanning were evaluated. A 4 μg MG standard spot which was 6 mm wide was scanned at a constant speed (40 mm/min) but slit height and width were varied (Table 5). Response areas were independent of slit height (Table 5A).

When a window much smaller than the width of the spot (3.25 mm, ca 1/2 X spot width) or much larger (10 mm, ca 2 X spot width) were used, response areas were ca 20% larger or smaller (Table 5B). In the case of the small window, a greater amount of the incident light was absorbed, giving higher values. With the much larger window, a greater proportion of the beam impinged upon non-absorptive background areas of the TLC plate, giving lower absorption readings.

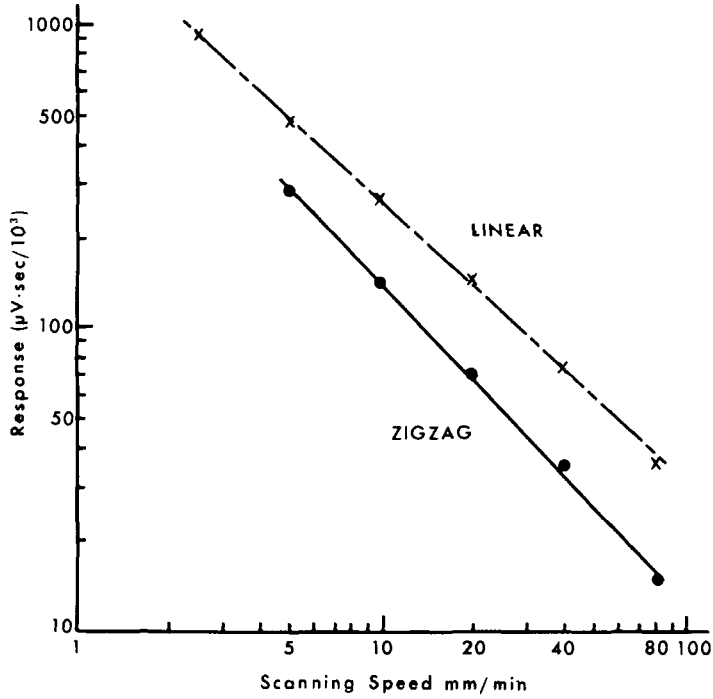

FIGURE 6. Relationship between spectrodensitometric scanning response and scanning speed. Linear: 4 ug FFA standard scanned with 6.25 x 0.3 mm beam. Zigzag: 4 ug FFA standard scanned with 1.25 x 1.25 mm beam.

TABLE 5
Effect of Slit Geometry on Peak Area

A		B	
Slit height mm	Response μV.sec/10³	Slit width mm	Response μV.sec/10³
0.1	71.9	3.25	83.1
0.2	72.2	4.25	80.7
0.3	71.5	5.25	76.5
0.4	71.5	6.25	71.5
0.6	71.6	8.25	64.4
0.8	71.7	10.00	60.5
1.0	72.0		

Reflectance measurement: slit width = 6.25 mm speed, 40 mm/min

Linear scan, λ = 350 nm slit height = 0.3 mm speed, 40 mm/min

CONCLUSIONS

Optimal Operating Parameters for Spectrodensitometric Scanning of Lipid Classes

The results of this study demonstrate that the reproducibility of spotting and scanning was similar using either linear or zigzag densitometric scanning. Although transmission measurements gave higher response areas, reflectance measurements were adopted for routine scanning because of lower noise (background) readings. Single wavelength, double beam operation at 350 nm yielded maximal absorbance values for the lipids. Zigzag scanning was less affected by the direction of plate scanning than linear scanning. Zigzag scanning, however, involved considerably more operator time and attention. By the use of 10 mm scored lanes on the silica gel plates, it is possible to control the spot width dimension. Use of a light beam which covers the width of the lipid spot permitted routine use of the linear scanning mode. Reflectance measurement, using a single wavelength at 350 nm in a linear scanning mode, gave linear responses for 0.125 to 10 μg of all lipid classes.

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