

# Evaluating the Isolation and Quantification of Sterols in Seed Oils by Solid-Phase Extraction and Capillary Gas–Liquid Chromatography



Traditional methods of sterol fraction analysis involve saponification of the seed oil, solvent extraction of the unsaponifiable compounds, and a second isolation of the sterols by thin-layer chromatography (TLC). Solvent extraction of the sterols from the TLC medium is followed by a derivatization step to reduce peak tailing caused by some gas–liquid chromatography column stationary phases. If a capillary column is used, as was the case in this study, the free sterols can be analyzed directly without derivatization. The method reported in this article circumvents TLC, along with its time-consuming multiple steps, and incorporates solid-phase extraction (SPE) columns, which enable quicker and easier sterol isolation and cleanup, thus greatly reducing the number of steps and sample preparation time. The authors describe two tests performed using SPE. The first test involved comparing the SPE method for sterol isolation and cleanup of four refined seed oils with the established TLC method according to American Oil Chemists Society official method Ch 6-91. The second test analyzed six crude seed oils using the SPE approach and compared the results with literature values.

**A**n important use of sterol fraction analysis is determining the authenticity of olive oils. Specific limits have been assigned to the sterol fraction in olive oil, and if the analysis indicates that the sterol fraction of an oil sample exceeds or fails to meet the limits, the olive oil may have been adulterated with a less expensive oil (1,2). Most edible seed oils — except pumpkin seed oil, which has a unique set of sterols (see Table I) — have as many as eight common sterols that typically can be found in measurable amounts. These sterols are cholesterol, brassicasterol, campesterol, stigmasterol,  $\beta$ -sitosterol,  $\Delta^5$ -avenasterol,  $\Delta^7$ -stigmasterol, and  $\Delta^7$ -avenasterol. Figure 1 illustrates the structures of four common seed oil sterols, which show small structural variations. The other sterols have the same basic structural design as the four illustrated in the figure.

A faster and more efficient way of isolating and subsequently cleaning up the sterol fraction obtained from seed oils was developed based on an existing established method that used thin-layer chromatography (TLC) to isolate the sterol fraction (3). The newly developed method used solid-phase extraction (SPE) to separate the sterol fraction from other compounds found in the unsaponifiable portion of the seed oils. Toivo and co-workers (4) developed a slightly different method that incorporated the same approach, used SPE rather than TLC, and resulted in excellent recovery results. However, their work investigated only two types of oils and provided no comparative data to existing, established methods.

In our study, we performed two tests using the SPE approach for sample isolation and cleanup. The first test compared sterol results obtained through SPE with those of

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the established TLC method according to American Oil Chemists Society (AOCS) official method Ch 6-91 for four refined seed oil sterol fractions: coconut, corn, olive, and sunflower. In the second set of tests, we

obtained sterol fractions for six crude seed oils — corn, canola, peanut, pumpkin, safflower, and soybean — and compared them with literature values (2).

## Experimental Procedures

**Chemicals:** We used high performance liquid chromatography (HPLC)-grade acetonitrile from EM Science (Gibbstown, New Jersey). The water was HPLC grade from AlliedSignal Inc. (Muskegon, Michigan). We purchased American Chemical Society (ACS)-grade acetone from EM Science and ACS-grade chloroform from Fisher Scientific (Pittsburgh, Pennsylvania). The petroleum ether was Optima grade (low impurities for gas chromatography [GC] work) from Fisher Scientific, and the ethyl alcohol was 95% reagent grade denatured with 5% isopropyl alcohol from VWR Scientific Products (West Chester, Pennsylvania).

We obtained individual cholesterol, stigmasterol, campesterol, and  $\beta$ -sitosterol standards from Aldrich (St. Louis, Missouri). Sunflower seed oil sterols were used to locate and identify  $\Delta^7$ -stigmasterol and  $\Delta^7$ -avenasterol. Coconut oil sterols were used to locate and identify  $\Delta^5$ -avenasterol. The  $\Delta^7$ -stigmasterol and  $\Delta^7$ -avenasterol ranges stated in the literature (see Table I) for sunflower seed oil were 20 and 3–6.5%, respectively, and the  $\Delta^5$ -avenasterol range of 20.0–40.7% stated for coconut oil indicates that these sterols are always present in the oils, which gave us confidence in using these oils as sterol reference materials (2). These three sterols are not readily available commercially; therefore, we used mixtures of oils in established methods to help identify the location of these three sterols.

**Unsaponifiable matter preparation:** To obtain the sterols from the seed oil, we saponified 2.5 g of sample for the SPE method and 5.0 g of sample for the TLC method, in accordance with AOCS official method Ca 6a-40. The sample was weighed into a 250-mL Erlenmeyer flask, and 30 mL of 95% ethyl alcohol and 5 mL of 26.73 M aqueous potassium hydroxide (60 g of potassium hydroxide in 40 mL of water) were added to the flask along with boiling chips. The mixture was refluxed for 1 h, cooled to room temperature, and transferred to an extraction cylinder. The flask was washed into the cylinder with two 10-mL portions of hot distilled water and two 10-mL portions of room-temperature distilled water until we collected 80 mL. The 80-mL mixture was transferred to a 250-mL separatory funnel and extracted five times with 50-mL portions of petroleum ether. Each 50-mL portion was collected into a 500-mL separatory funnel. The combined extracts were washed with four 30-mL portions of a 10% ethyl alcohol solution. The washed ether

**Table I: Sterol fraction results comparing literature values with the SPE method**

Sample and Sterols	Reference Value (% of Total)	SPE (% of Total)
<b>Corn Oil</b>		
Cholesterol	0.2–0.6	0.2
Brassicasterol	0.0–0.2	0.1
Campesterol	18.6–24.1	24.3
Stigmasterol	4.3–7.7	7.7
$\beta$ -Sitosterol	54.8–66.6	61.6
$\Delta^5$ -Avenasterol	4.2–8.2	3.8
$\Delta^7$ -Stigmasterol	1.0–4.2	0.7
$\Delta^7$ -Avenasterol	0.7–2.7	0.8
Others	0.0–2.4	0.8
<b>Canola oil</b>		
Cholesterol	0.5–1.3	0.2
Brassicasterol	5.0–13.0	7.7
Campesterol	24.7–38.6	31.8
Stigmasterol	0.0–0.7	0.0
$\beta$ -Sitosterol	45.1–57.9	57.4
$\Delta^5$ -Avenasterol	3.1–6.6	1.8
$\Delta^7$ -Stigmasterol	0.0–1.3	0.2
$\Delta^7$ -Avenasterol	0.0–0.8	0.8
<b>Peanut oil</b>		
Cholesterol	0.0–3.8	0.2
Brassicasterol	0.0–0.2	0.4
Campesterol	12.0–19.8	13.4
Stigmasterol	5.4–13.2	9.5
$\beta$ -Sitosterol	47.4–67.7	62.2
$\Delta^5$ -Avenasterol	8.3–18.8	7.2
$\Delta^7$ -Stigmasterol	0.0–5.1	4.2
$\Delta^7$ -Avenasterol	0.0–1.4	1.9
<b>Pumpkin seed oil</b>		
Stigmasterol	1	0.9
$\Delta^7$ -Stigmasterol	4	1.4
$\Delta^7$ -Avenasterol	10	13.6
24-Methyl-cholest-7-enol	6	5.6
$\Delta^{7,22,25}$ -Stigmastatrienol	29	35.1
$\alpha$ -Spinasterol	27	23.3
$\Delta^{7,25}$ -Stigmastadienol	22	18.6
<b>Safflower oil</b>		
Cholesterol	0.0–0.5	0.0
Brassicasterol	0.0	0.0
Campesterol	9.2–13.0	8.4
Stigmasterol	6.5–9.6	7.1
$\beta$ -Sitosterol	40.2–49.8	41.9
$\Delta^5$ -Avenasterol	2.1–4.0	4.8
$\Delta^7$ -Stigmasterol	15.7–22.4	23.8
$\Delta^7$ -Avenasterol	2.9–5.3	5.6
Others	0.5–2.8	8.3
<b>Soybean oil</b>		
Cholesterol	0.6–1.4	0.3
Brassicasterol	0.0–0.3	0.0
Campesterol	15.8–24.2	21.8
Stigmasterol	15.9–19.1	15.9
$\beta$ -Sitosterol	51.7–57.6	50.6
$\Delta^5$ -Avenasterol	1.9–3.7	2.3
$\Delta^7$ -Stigmasterol	1.4–5.2	3.5
$\Delta^7$ -Avenasterol	1.0–4.6	5.0
Others	0.0–1.8	0.5

extract was transferred to a 250-mL flat-bottomed boiling flask, and the ether was evaporated on a steam bath under a fume hood. In preparation for the SPE method, the ether was completely removed to dryness. In preparation for the TLC method, the ether was reduced to approximately 1 mL.

#### Isolation and recovery of the sterols by

**SPE:** Complete removal of the ether from the flask left only the unsaponifiable fraction that contained the sterols. The sterols were reconstituted in 20 mL of acetonitrile with slight heating to ensure complete dissolution. We conditioned two C18 SPE tubes by passing 5 mL of acetonitrile through them and stacked them using a coupling adapter for 6-mL columns (VWR Scientific Products). The acetonitrile sterol mixture was then loaded onto the stack of two 6-mL, 1-g, C18 SPE tubes (Supel-clean LC-18 tubes, Supelco, Bellefonte, Pennsylvania) by use of a Baker-10 extraction system (J.T. Baker Chemical Co., Phillipsburg, New Jersey) with a vacuum pressure of 5 in. of Hg (0.17 atm). We first washed the tubes with 25 mL of distilled water using a vacuum pressure of 15 in. of Hg and then with 25 mL of acetonitrile using a vacuum pressure of 5 in. of Hg to remove water-soluble compounds and any extraneous organic compounds that were extracted into the petroleum ether. The sterols were eluted with 25 mL of a 3:1 acetonitrile–toluene mixture at a vacuum pressure of 5 in. of Hg. The eluent was dried by heating and nitrogen purging. The sterol crystals were recon-

stituted in 3 mL of acetone and injected directly into the gas chromatograph.

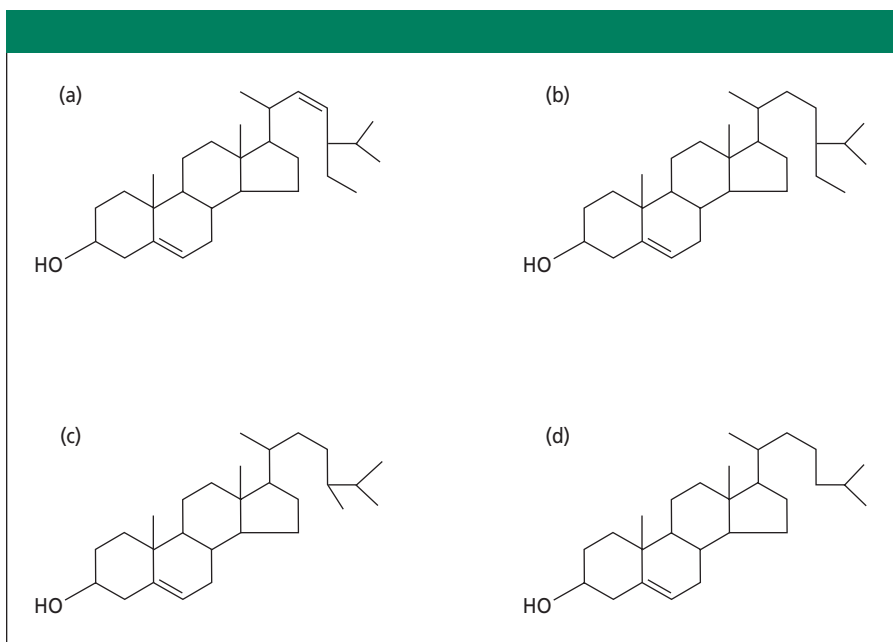
**Isolation and recovery of the sterols by TLC:** We washed the ether sterol extract with a 10% ethyl alcohol solution and then evaporated it on a steam bath until approximately 1 mL of solution was left. Throughout the tests, we used commercially prepared, 20 cm × 20 cm, 60-Å pore diameter, Whatman K6 silica gel TLC plates (Whatman Inc., Clifton, New Jersey) with a 250-μm layer thickness. We placed two 50-μL spots of a 100 g/L cholesterol solution in chloroform 10 mm from the left-hand and right-hand edges of the plate. The unsaponifiable compounds were spotted on the plate in a continuous line of 50-μL spots between the two cholesterol reference spots.

We placed the plate in a developing tank containing chloroform as the developing solvent. The plate was left in the tank until the solvent reached a point of 10 mm from the upper edge. Then we removed the plate and allowed it to dry. The sterol fraction was identified by the two cholesterol reference spots on each edge. The sterols were scraped from the plate into a 25-mL conical flask. To extract the sterols, we placed 5 mL of ethyl ether in the flask and refluxed it gently for 15 min. The ether was filtered through 9.0-cm circles of Whatman number 5 paper into another 25-mL conical flask. The original flask then was washed by the addition of another 5 mL of ether and refluxing. This process was repeated three times. The solvent was removed from the combined filtrates under a stream of nitro-

gen. The sterol crystals were dissolved in a minimal amount of acetone (approximately 300 μL) and injected into the gas chromatograph for sterol composition analysis.

**Sterol analysis by gas-liquid chromatography:** We analyzed the sterols using an Agilent 5890 series gas-liquid chromatograph coupled to an Agilent 3396A integrator (both from Agilent Technologies, Wilmington, Delaware). We used a 30 m × 0.25 mm, 0.25-μm *d<sub>f</sub>* SAC-5 fused-silica capillary column (Supelco). The injection temperature was 260 °C; the flame ionization detector temperature was 250 °C; and the oven temperature was held at 250 °C for 80 min. We used a split ratio of 27:1 with a 2.2-mL/min column flow rate of helium. The injection volume was 3 μL.

**Calculation of results:** The individual sterols reported in Tables I and II are calculated as a percentage by mass of the total



**Figure 1:** Structures of (a) stigmasterol, (b) β-sitosterol, (c) campesterol, and (d) cholesterol.

**Table II: Sterol fraction results of SPE and TLC methods**

Sample and Sterols	SPE (% of Total)	TLC (% of Total)
<b>Coconut</b>		
Cholesterol	0.0	0.0
Brassicasterol	0.0	0.0
Campesterol	7.1	6.9
Stigmasterol	12.9	12.4
β-Sitosterol	51.0	54.5
Δ <sup>5</sup> -Avenasterol	29.0	26.2
Δ <sup>7</sup> -Stigmasterol	0.0	0.0
Δ <sup>7</sup> -Avenasterol	0.0	0.0
<b>Corn</b>		
Cholesterol	0.0	0.0
Brassicasterol	0.0	0.0
Campesterol	20.5	20.8
Stigmasterol	6.3	6.5
β-Sitosterol	65.6	65.7
Δ <sup>5</sup> -Avenasterol	5.2	4.9
Δ <sup>7</sup> -Stigmasterol	0.3	0.5
Δ <sup>7</sup> -Avenasterol	2.1	1.5
<b>Olive</b>		
Cholesterol	0.0	0.0
Brassicasterol	0.0	0.0
Campesterol	2.6	2.5
Stigmasterol	1.2	1.0
β-Sitosterol	92.3	92.0
Δ <sup>5</sup> -Avenasterol	3.2	3.7
Δ <sup>7</sup> -Stigmasterol	0.7	0.8
Δ <sup>7</sup> -Avenasterol	0.0	0.0
<b>Sunflower</b>		
Cholesterol	0.0	0.0
Brassicasterol	0.0	0.0
Campesterol	6.6	7.7
Stigmasterol	9.6	10.6
β-Sitosterol	71.7	70.5
Δ <sup>5</sup> -Avenasterol	2.7	2.0
Δ <sup>7</sup> -Stigmasterol	8.5	8.4
Δ <sup>7</sup> -Avenasterol	0.9	0.8

sterols present. The equation used to calculate the individual sterol content ( $n$ ) was

$$n = \left( \frac{A_n}{\sum A} \right) 100 \quad [1]$$

where  $A_n$  is the area of the peak corresponding to sterol  $n$  and  $\sum A$  is the sum of the total sterol peak areas.

## Results and Discussion

In the course of performing sterol analyses by the traditional TLC method, we determined it was desirable to find a quicker and more straightforward method of isolating and cleaning up the sterol fraction from the unsaponifiable compounds rather than going through all the steps involved with TLC. The TLC method — plate spotting and development, sterol band removal from the plate, and solvent extraction — required approximately 2–3 h of work per sample. The SPE method takes approximately 30 min to pass through the columns, remove the solvent, and be ready for the gas-liquid

chromatography step of the analysis. The SPE method also uses considerably less solvent in the sterol analysis compared with the TLC method.

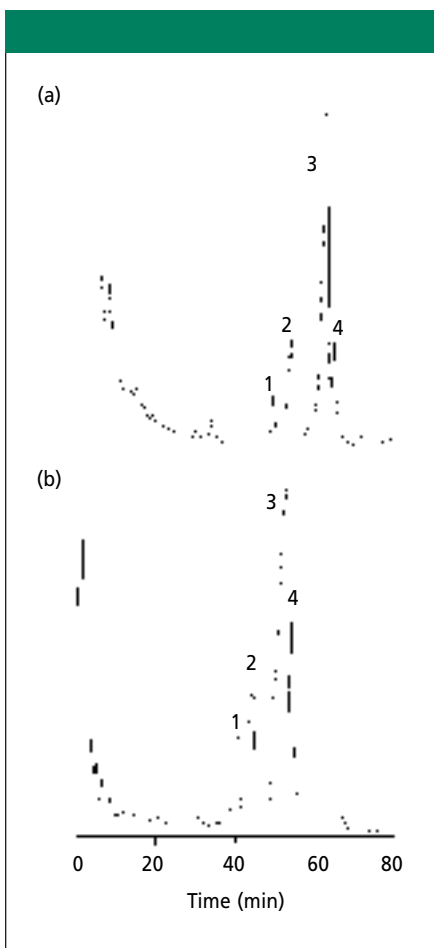
Through experience with HPLC, we knew that the sterols remain strongly bound to reversed-phase LC-18 columns when mobile phases such as aqueous buffers, acetonitrile, and methanol are used. Sterol elution requires the use of stronger solvents such as 99.9:0.1 hexane-isopropyl alcohol, which is a technique known as nonaqueous reversed-phase HPLC (5). Therefore, we felt that an SPE LC-18 tube would retain the sterols, and the other interferences could be washed off of the packing with water and acetonitrile.

To test this hypothesis, we selected four refined seed oils and tested for total sterol content using the new SPE approach and the established TLC method of AOCS official method Ch 6-91. Table II lists the results of this comparison. Cholesterol and brassicasterol were not detected in the sterol fractions, as shown in Table II. This outcome can be attributed to the use of refined

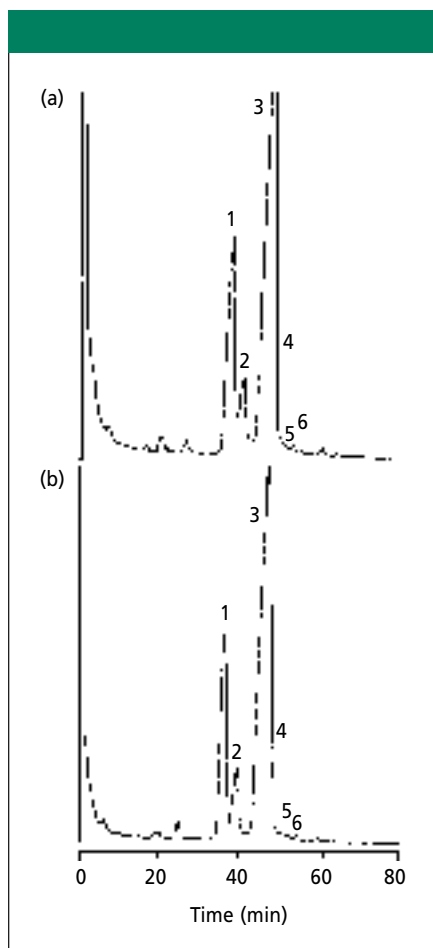
oils, in which some sterol loss can occur in the deodorization step of the refining process (6), in the first study versus crude oils in the second study.

Figures 2–5 illustrate comparative chromatograms obtained from the SPE and TLC methods whose results are listed in Table II. In Figures 2a and 4a, the retention times are shifted slightly longer because of an oven temperature of 240 °C for 80 min during optimization of the GC method. The optimized settings for the gas chromatograph as the new column equilibrated were determined to be 250 °C for 80 min, which yielded relatively good separation of all the sterol peaks analyzed.

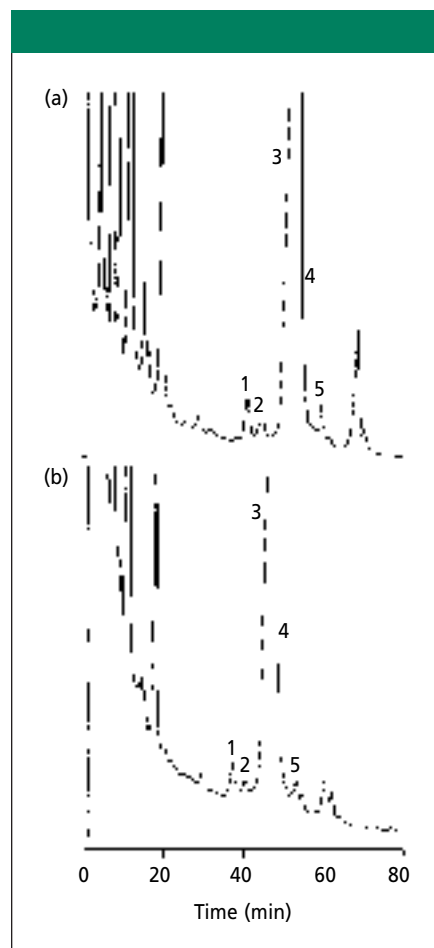
We used the chromatograms to calculate the individual sterol contents as a percentage of the total sterol mass amounts, which were normalized to 100%. This calculation was performed to compare the sterol compositions obtained from the two method approaches, not to test the efficiency of the percentage of the sterols extracted quantitatively based on the amount of oil saponified and extracted. The determination of quan-



**Figure 2:** GC-FID chromatograms of coconut oil sterols isolated using (a) SPE and (b) TLC. Peaks: 1 = campesterol, 2 = stigmasterol, 3 =  $\beta$ -sitosterol, 4 =  $\Delta^5$ -avenasterol.



**Figure 3:** GC-FID chromatograms of corn oil sterols isolated using (a) SPE and (b) TLC. Peaks: 1 = campesterol, 2 = stigmasterol, 3 =  $\beta$ -sitosterol, 4 =  $\Delta^5$ -avenasterol, 5 =  $\Delta^5$ -stigmasterol, 6 =  $\Delta^7$ -avenasterol.



**Figure 4:** GC-FID chromatograms of olive oil sterols isolated using (a) SPE and (b) TLC. Peaks: 1 = campesterol, 2 = stigmasterol, 3 =  $\beta$ -sitosterol, 4 =  $\Delta^5$ -avenasterol, 5 =  $\Delta^5$ -stigmasterol.

titative extraction efficiencies for the two methods was unnecessary because the quantitative (or complete) extraction of the sterols was assumed with the established TLC method and already has been demonstrated and reported for the SPE approach by Toivo and colleagues (4). Incomplete extraction would have manifested itself in the SPE–TLC comparative results by showing substantial disagreement between the two, which we did not observe. Therefore, the agreement between the two methods concerning the main sterol compounds demonstrated that using SPE in place of TLC is viable for a variety of seed oils.

In the second study, we saponified six crude seed oil samples, passed them through the SPE columns, and analyzed them for total sterols. The recovered sterols were compared with published values as illustrated in Table I. Some of the experimental values were outside the stated literature ranges, which probably occurred because the reference values are derived from published results of studies performed on the

various seed oils. As with the pumpkin seed oil, only single values have been reported, but there is always variation from crop batch to crop batch, as illustrated in the results. This situation is the same with other seed oils: variations in the sterol compositions occur that may be outside the stated literature ranges or values. The relative agreement between the two methods (SPE versus TLC, Table II) demonstrates that the SPE method is an accurate approach, as opposed to indicating it is inaccurate because of natural variations in seed oil sterol content that may be outside of the stated literature values. Different sterol values used to categorize different oils, or to determine if adulteration has occurred, have established limits, and the study results have not violated these limits (for example, the olive oil limits [1]). These results strongly suggest that the SPE approach is an accurate and viable one.

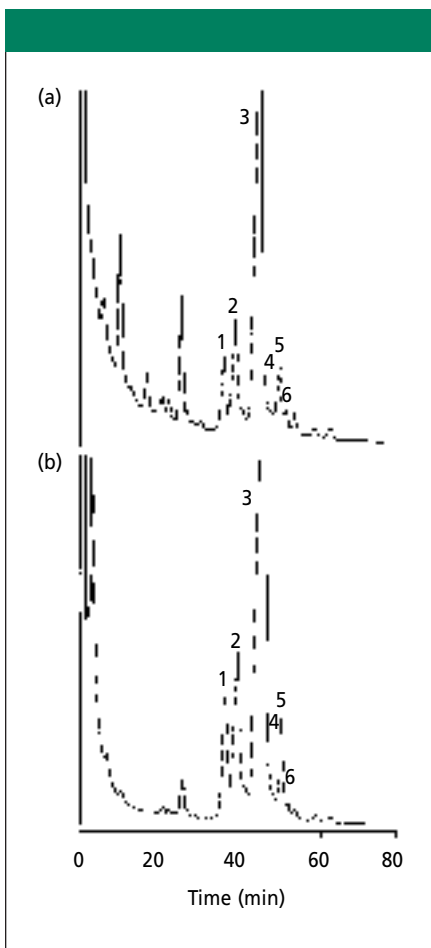
### Conclusion

We found the use of C18 reversed-phase SPE columns to be an acceptable alternative to the traditional TLC approach. The amount of solvent needed is greatly reduced, as is the time involved in separating the unsaponifiable compounds from the sterol fraction. The sterols recovered by SPE were free of interfering peaks in all samples tested.

In conclusion, the two tests performed — the comparison of SPE with an established TLC method and the comparison of the six seed oil sterols obtained by SPE with literature values — both demonstrated that using SPE in place of TLC is a convenient, accurate, time-saving, and cost-effective alternative to the traditional method for a wide variety of seed oil sterol fractions.

### References

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**Figure 5:** GC-FID chromatograms of sunflower oil sterols isolated using (a) SPE and (b) TLC. Peaks: 1 = campesterol, 2 = stigmasterol, 3 =  $\beta$ -sitosterol, 4 =  $\Delta^5$ -avenasterol, 5 =  $\Delta^5$ -stigmasterol, 6 =  $\Delta^7$ -avenasterol.

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