Application Note

Clinical Research

LC/MS/MS Separation of Cholesterol and Related Sterols in Plasma on an Agilent InfinityLab Poroshell 120 EC-C18 Column

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Abstract

Cholesterol and its metabolites, as well as several phytosterols, are separated using Agilent InfinityLab Poroshell 120 columns with LC/MS/MS. Two phases of InfinityLab Poroshell 120, EC-C18 and SB-C18, are compared in terms of the separation of 12 sterols. The InfinityLab Poroshell 120 EC-C18 column has better selectivity for these sterols, and gives better resolution. Two different size particles of InfinityLab Poroshell 120 EC-C18 are also compared. Smaller particle size provides much better resolution, especially for the closely eluting isomers such as lathosterol and cholesterol. LC/MS/MS with APCI provides high analytical sensitivity.

Introduction

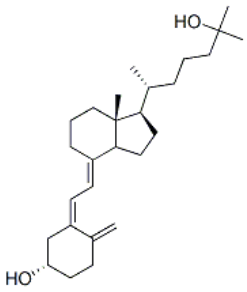
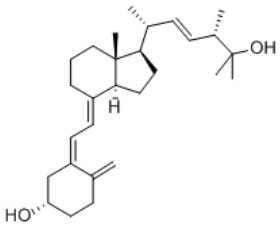
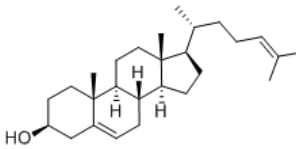
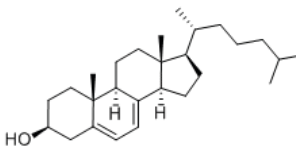
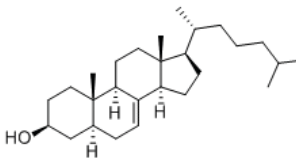
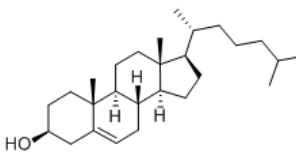
The analysis of cholesterol and related compounds, such as cholestenol, β-sitosterol, desmosterol, and lathosterol, can be done by GC and LC. HPLC has become more widely used in the analysis of sterols, and is often coupled to triple quadrupole mass spectrometers for quantitative analysis to enhance sensitivity and selectivity. Similarly, improvements in ionization and ion transfer into the mass spectrometer have enhanced the ability to measure low-level metabolites in biological matrices.

Detection of sterols using LC can be by MS, UV, or evaporative light scattering detection (ELSD). The choice of detector is influenced by the sensitivity needed in the separation, with LC/MS/MS providing very high sensitivity. We developed a method using MS with an Agilent 6460A triple quadrupole LC/MS and atmospheric pressure chemical ionization (APCI) in positive ion mode for the sterols in Table 1. The nonpolar nature of sterol compounds makes APCI the best choice of ionization source, since it provides good sensitivity for the compounds in positive mode without derivatization.

As many sterols are positional isomers, chromatographic resolution remains crucial for the analysis because the MS cannot differentiate between the isobaric compounds. Lathosterol and cholesterol are the most challenging compounds in this separation for two reasons: they are difficult to resolve chromatographically, and they are isobaric. In a plasma sample, cholesterol is present at a much higher concentration than lathosterol, which further increases the difficulty of the analysis.

We used InfinityLab Poroshell 120 columns in this method, which deliver higher efficiencies and throughput at reduced backpressure compared to totally porous particles. The 1.9 µm InfinityLab Poroshell 120 column delivers the highest efficiency. The 2.7 µm InfinityLab Poroshell 120 column offers backpressure below 600 bar, and superior robustness with a 2 µm frit that is less likely to plug with dirty samples such as plasma.

Table 1. Analytes in this study.

No.	Compound	Molecular Weight (g/mol)	CAS No.	Structure
1	25-Hydrovitamin D3 (Calcifediol)	400.64	63283-36-3	
2	25-Hydrovitamin D2	412.65	21343-40-8	
3	Desmosterol	384.64	313-04-2	
4	7-Dehydrocholesterol (Provitamin D3)	384.64	434-16-2	
5	Lathosterol	386.65	80-99-9	
6	Cholesterol	386.65	57-88-5	

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher. HPLC grade acetonitrile and methanol were bought from J. T. Baker (Center Valley, PA, USA.). Water was purified using an ELGA PURELAB Chorus system (High Wycombe, UK). The standards were from Sigma-Aldrich (St. Louis, MO, USA).

Equipment and materials

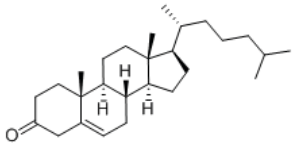
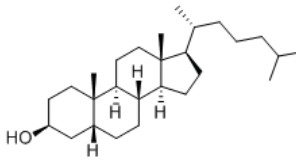
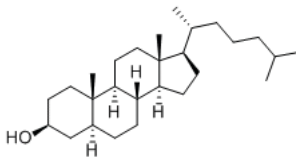
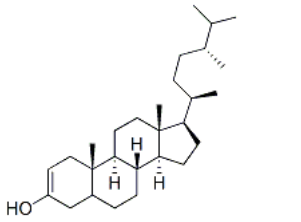
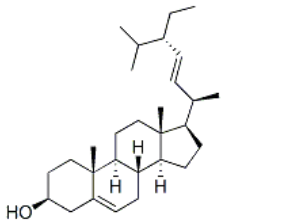
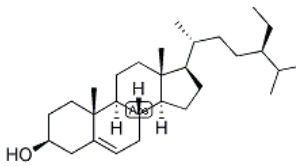
- Column inlet: Agilent InfinityLab Quick Connect LC fitting (p/n 50675965)
- Column outlet: Agilent InfinityLab Quick Turn LC fitting (p/n 5067-5966)
- Agilent Captiva Econofilter, PTFE membrane, 13 mm diameter, 0.2 µm pore size (p/n 5190-5265)
- Agilent vial, screw top, amber, write-on spot, certified, 2 mL (p/n 5182-0716)
- Agilent bonded screw cap, bonded blue, PTFE/red silicone septa (p/n 5190-7024)
- Agilent InfinityLab solvent bottle, amber, 1,000 mL (p/n 9301-6526)
- Agilent InfinityLab Stay Safe cap, GL45, 3-port, 1-vent valve (p/n 5043-1219)
- Eppendorf pipettes and repeater
- Sonicator (VWR, Radnor, PA, USA)

Instrumentation

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6460 triple quadrupole LC/MS (G6460A)

Software

- Agilent MassHunter LC/MS data acquisition software, version B.08.00
- Agilent MassHunter qualitative analysis software, version B.07.00

No.	Compound	Molecular Weight (g/mol)	CAS No.	Structure
7	5-Cholesten-3-one	384.64	601-54-7	
8	Coprostanol	388.67	360-68-9	
9	Cholestanol	388.67	80-97-7	
10	Campesterol	400.68	474-62-4	
11	Stigmasterol	412.69	83-48-7	
12	Sitosterol	414.71	64997-52-0	

HPLC Conditions	
Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302) Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 100 mm, 2.7 µm (p/n 685975-302) Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 1.9 µm (p/n 695675-302)
Mobile Phase A	Water
Mobile Phase B	Methanol
Gradient	0 to 10 minutes: 92 to 96% B, 10 to 12 minutes: 96% B, 12 to 16 minutes: 96 to 100% B, Stop time: 20 minutes, Post time: 2 minutes
Flow Rate	0.60 mL/min
Column Temperature	15 °C
Injection Volume	20 µL

MS Conditions	
Ion Mode	APCI, Positive
Drying Gas Temperature	325 °C
Vaporizer	350 °C
Drying Gas Flow	4 L/min
Nebulizer Pressure	30 psi
Capillary Voltage (+)	2,000 V
MRM Condition	ΔEMV, 500

Table 2. APCI acquisition parameters and transitions.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell	Fragmentor Voltage	Collision Energy	Cell Accelerator Voltage	Polarity
Provitamin D3	367.3	159.2	18	130	13	4	Positive
Provitamin D3	367.3	145.1	18	130	17	4	Positive
Desmosterol	367.3	95	18	100	22	4	Positive
Desmosterol	367.3	161.1	18	100	17	4	Positive
Cholesterol	369.4	161.2	18	166	10	4	Positive
Cholesterol	369.4	95.2	18	166	38	4	Positive
Lathosterol	369.4	95.1	18	112	29	4	Positive
Lathosterol	369.4	81.1	18	112	40	4	Positive
Coprostanol	371.4	221.2	18	140	21	4	Positive
Coprostanol	371.4	95.1	18	140	21	4	Positive
Cholestanol	371.4	149	18	150	15	4	Positive
Cholestanol	371.4	95	18	150	30	4	Positive
Calcifediol	383.3	211.2	18	144	25	4	Positive
Calcifediol	383.3	107.1	18	144	25	4	Positive
Campesterol	383.4	161.2	18	142	16	4	Positive
Campesterol	383.4	95	18	142	30	4	Positive
5-Cholesten-3-one	385.4	109.1	18	128	40	4	Positive
5-Cholesten-3-one	385.4	97	18	128	21	4	Positive
25-Hydroxyvitamin D2	395.3	377.4	18	166	9	4	Positive
25-Hydroxyvitamin D2	395.3	269.2	18	166	9	4	Positive
Stigmasterol	395.4	83.1	18	148	17	4	Positive
Stigmasterol	395.4	81.1	18	148	37	4	Positive
Sitosterol	397.4	161	18	125	18	4	Positive
Sitosterol	397.4	135.2	18	125	12	4	Positive

Results and discussion

In total, 12 sterols were separated on InfinityLab Poroshell 120 EC-C18 and SB-C18 columns using the LC-MS/MS. The separation was done using a gradient mobile phase with water/methanol at a reduced temperature of 15 °C for better resolution. Previously, experiments with acetonitrile mobile phase could easily separate critical pairs, but severely suppressed the APCI signal. The upper and mid chromatograms in Figure 1 showed different selectivity between the EC-C18 and the SB-C18 chemistry. The challenging isobaric compounds lathosterol (peak 5) and cholesterol (peak 6) are resolved on the InfinityLab Poroshell 120 EC-C18, but not on the InfinityLab Poroshell 120 SB-C18 column. The bottom chromatogram shows the results on a 1.9 µm InfinityLab Poroshell 120 EC-C18 column, which provided superior resolution for some isomers such as lathosterol and cholesterol.

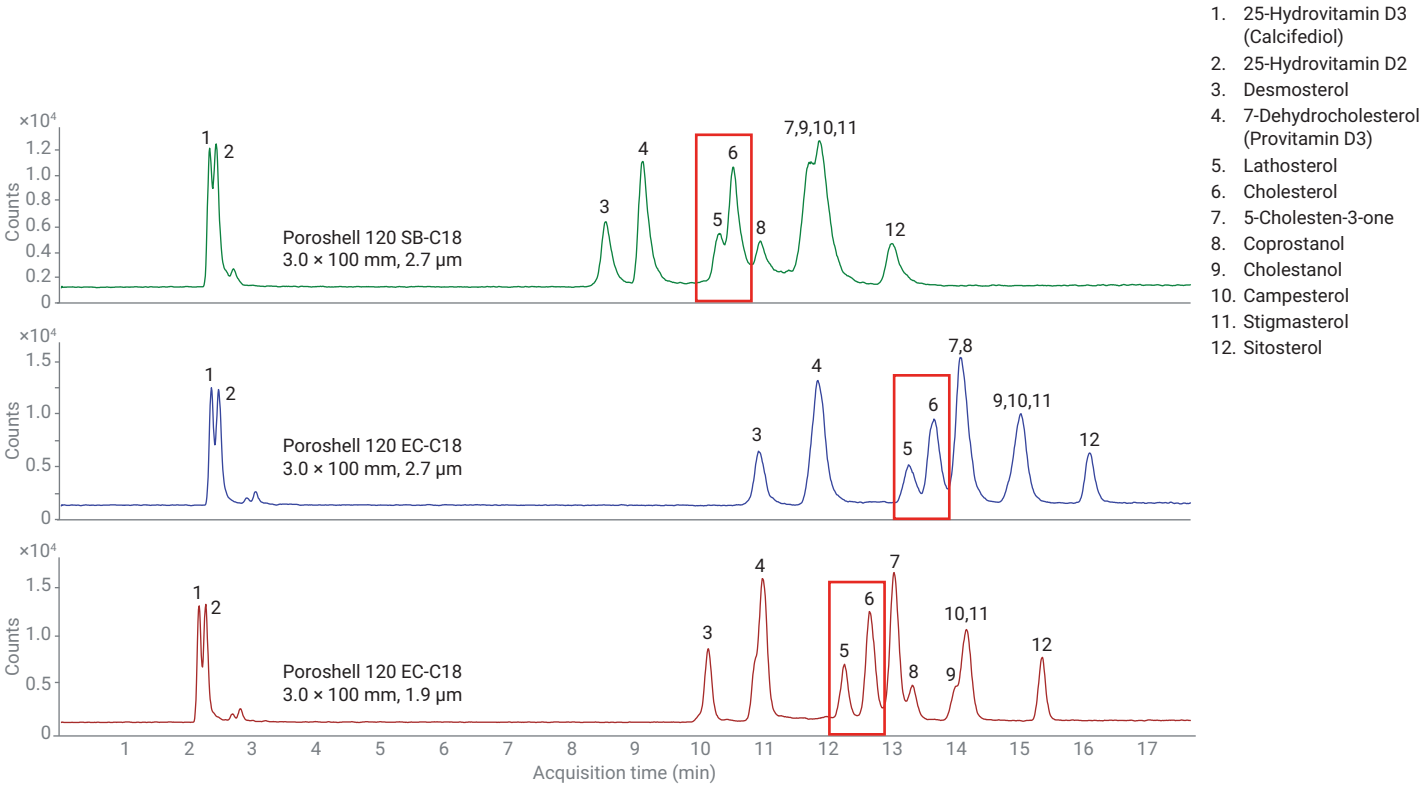


Figure 1. TIC chromatograms of 12 sterols on InfinityLab Poroshell 120 SB-C18 and EC-C18 phases.

To achieve higher analytical sensitivity, the APCI parameters, including capillary voltage, gas flow, and nebulizer, were optimized. The optimized method applied an InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 1.9 µm column. MRM chromatograms of 12 sterols in Figure 2 show complete separation at the 10 ppb level.

We found that the capillary voltage was crucial for the signal. A higher capillary voltage, such as 4,000 V, could severely suppress the APCI signal and lead to an unstable signal. The optimized capillary voltage in this Application Note was 2,000 V. Good reproducibility was shown by overlaying 10 injections of 100 ppb standards (Figure 3).

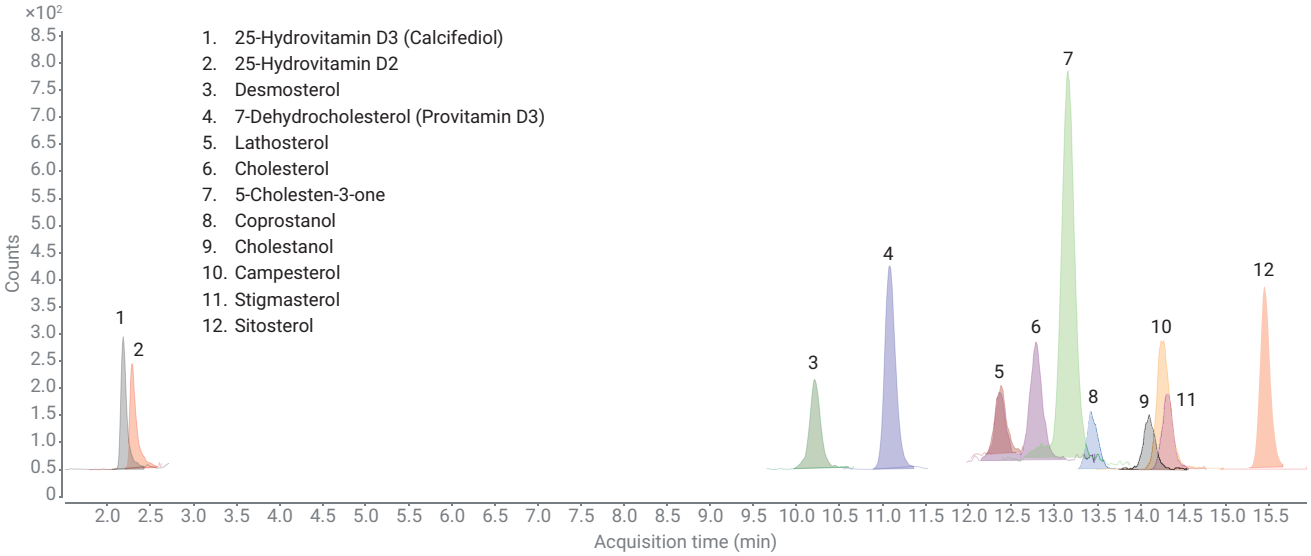


Figure 2. MRM chromatograms of 12 sterols on an InfinityLab Poroshell EC-C18, 3.0 × 100 mm, 1.9 µm column at the 10 ppb level.

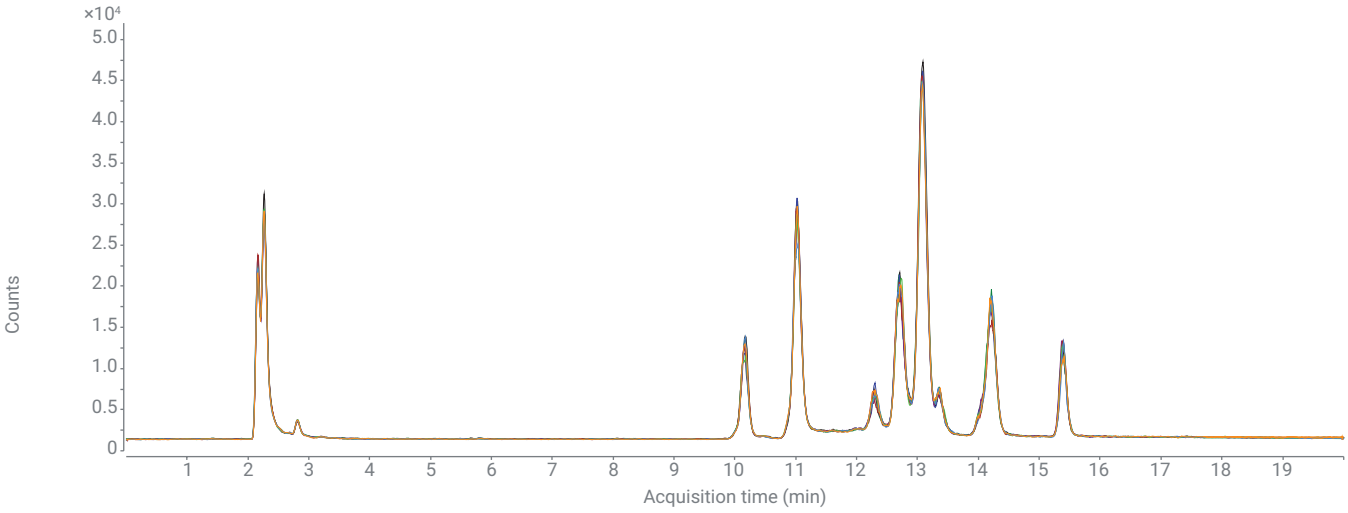


Figure 3. Overlay of 10 injections of 100 ppb standards on an InfinityLab Poroshell 120 EC-C18, 3 × 100 mm, 1.9 µm column.

In a real plasma sample, cholesterol is present at a much higher concentration than other sterols. The ratio of cholesterol to lathosterol is approximately 2,000:1 in a plasma sample, and it is very difficult for lathosterol to separate from a high concentration of cholesterol. Figure 4 shows a chromatogram of the plasma sample before and after being spiked with 12 sterols at 10 ppb.

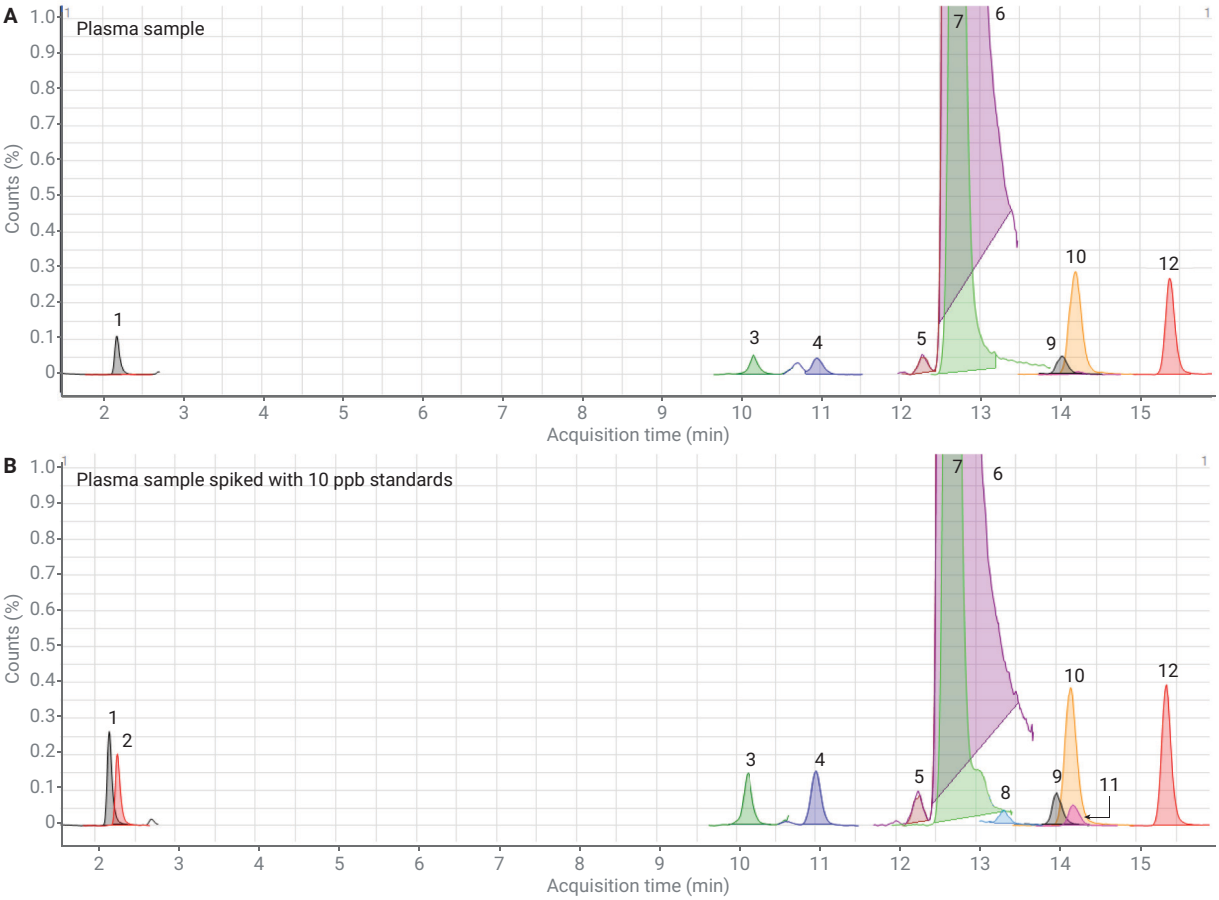


Figure 4. MRM chromatograms of blank plasma sample and 10 ppb standards spiked sample separated on an InfinityLab Poroshell 120 EC-C18, 3 × 100 mm, 1.9 µm column.

Conclusions

The separation of cholesterol, some of its metabolites, and other phytosterols was most effectively performed with an InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 1.9 µm column with APCI detection in positive ion mode on a 6460A triple quadrupole LC/MS. This column achieved baseline separation between the critical pair of cholesterol and lathosterol, even at a ratio of 2,000:1. This was critical, as the two compounds have the same molecular weight, and resolution was needed to effectively quantitate these two analytes.

Reference

1. McDonald, J. G.; *et al.* A comprehensive method for extraction and quantitative analysis of sterols and secosteroids from human plasma. *J. Lipid Res*. **2012**, *53(7)*, 1399–1409.

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