APPENDIX 1

Summary of the procedure used for the measurement of mass concentration of "total" sterols (esterified + non-esterified) in serum by UHPLC-APCI-MS/MS

1. Reagents

- β-Sitostanol (Sigma-Aldrich-Merck; Ref: S462330-250MG)
- β-Sitostanol-D7 (Toronto Research Chemicals; Ref: S495002)
- β-Sitosterol (Toronto Research Chemicals; Ref: S497050)
- β-Sitosterol-D7 (Toronto Research Chemicals; Ref: S497052)
- 2,6-Di-tert-butyl-4-methylphenol (BHT) (Sigma-Aldrich-Merck; Ref: B1378-100G)
- 2-Propanol (isopropanol) LC-MS (Sigma-Aldrich-Merck; Ref: 1.02781.1000)
- Absolute ethanol (Merck; Ref: 1.07017)
- Acetonitrile UHPLC-MS (Sigma-Aldrich; Ref: 34967-1L)
- Brasicasterol (Toronto Research Chemicals; Ref: B676850)
- Campesterol (Toronto Research Chemicals; Ref: C155360)
- Campesterol-D3 (Toronto Research Chemicals; Ref: C155362)
- Desmosterol (Toronto Research Chemicals; Ref: D296860)
- Desmosterol-D6 (Toronto Research Chemicals; Ref: D296862)
- Dihydrolanosterol (Toronto Research Chemicals; Ref: D449855)
- Ergosterol (Toronto Research Chemicals; Ref: E599240)
- Hexane HPLC (Sigma-Aldrich; Ref: 34859-1L)
- Lanosterol (Toronto Research Chemicals; Ref: L174580)
- Methanol LC-MS (Sigma-Aldrich; Ref: 14262-1L)

- Potassium hydroxide (Sigma-Aldrich-Merck; Ref: 000000001050125000)
- Stigmasterol (Toronto Research Chemicals; Ref: S686750)
- Stigmasterol-D3 (Toronto Research Chemicals; Ref: S686753)
- Water UHPLC-MS (Sigma-Aldrich; Ref: 14263-1L)

2. Materials and equipment

- Analytical balance ADA-120/L (Adam Equipment)
- Centrifuge Biofuge® 13 (Heraeus Holding GmbH)
- Repetitive dispenser Nichimate® Stepper (Nichiryo Co Ltd)
- Nitrogen evaporator/concentrator MD200-2 (Xian Toption Instrument Co., Ltd.)
- Volumetric flasks of 10 mL, 50 mL and 100 mL BLAUBRAND® (BRAND GMBH + CO KG)
- Adjustable volume mechanical pipette 100-1000 µL Acura® 825 (Socorex)
- Adjustable volume mechanical pipette 20-100 µL Nichipet® EX II (Nichiryo Co Ltd)
- Adjustable volume mechanical pipette 2-10 µL Acura® 825 (Socorex)
- Sonicator Branson® 3510 MTH Ultrasonic (Branson)
- Beakers of 50 mL and 100 mL BLAUBRAND® (BRAND GMBH + CO KG)

3. Preparation of calibration and control materials

1. Two primary solutions of 1000 mg/L are prepared for each of the sterols in 2-propanol.

2. From the different primary solutions, two secondary solutions of 100 mg/L in 2-propanol containing all sterols are prepared. One of the solutions will be used to prepare the calibration materials and another one for the control materials.

3. From the secondary solutions, 9 calibration materials (0.10, 0.10, 0.25, 0.50, 1.00, 5.00, 10.0, 30.0 and 50.0 mg/L) and 3 control materials (0.30, 20.0 and 40.0 mg/L) are prepared in 2-propanol.

All solutions and materials are stored at -80ºC.

4. Preparation of working solution of internal standards

Relationship between internal standard and sterol:

Sterol	Internal standard
Brassicasterol	Colesterol-D_6
Campesterol	Campesterol-D_3
Desmosterol	Desmosterol-D_6
Ergosterol	Colesterol-D_6
Lanosterol	Dihydrolanosterol
β-Sitostanol	β-Sitostanol-D_7
β-Sitosterol	β-Sitosterol-D_7
Stigmasterol	Stigmasterol-D_3

Preparation of working solution of internal standard:

1. Primary solutions of 500 mg/L are prepared for each internal standard. Solutions are aliquoted in Eppendorf tubes and stored at -80ºC.

2. At the time of the analysis, and from the different primary solutions, a working solution of 20 mg/L in 2-propanol is prepared, which contains all the internal standards.

5. Preparation and treatment of serum samples, calibration materials (CAL) and control materials (CTRL)

The preparation and treatment consists of an alkaline hydrolysis, followed by a liquid-liquid extraction, an evaporation of the extract with nitrogen and a subsequent reconstitution with methanol:

250 µL serum or CAL or CTRL

+

750 µL KOH 0,7 M in EtOH/H_2O (96/4 *v/v*)

+

50 µL working sol. IS (20 mg/L in 2-propanol)

+

10 µL BHT 1000 mg/L in MeOH

↓ **Alcaline hydrolisis: 68°C, 30 min in sonicator**

500 µL H_2O

+

1.5 mL Hexane

↓ **1) Vortex 5 s**
2) Centrifuge 2500 *g* 20 min

Collect supernatant (1200 µL) in glass tube

↓ **Evaporate with N_2 until dryness (approx. 10 min)**

Reconstitute with 600 µL MeOH and inject onto UHPLC-APCI-MS/MS

6. Chromatographic and mass spectrometry equipment

An ACQUITY®-UPLC® measuring system coupled to a triple quadrupole mass spectrometer ACQUITY®-TQD®, both from Waters SA Chromatography, is used.

Chromatographic conditions:

- Column: Acquity UPLC® BEH™ 2.1 x 100 mm; 1.7 µm (Waters)
- Pre-column: Acquity® UPLC® BEH™ C_{18} VanGuard Pre-column (5 mm x 2.1 mm; 130 Å, 1.7 µm)
- Filter: 0.2 µm ACQUITY UPLC® Col. In-Line Filter Kit (Waters)
- Column temperature: 30°C
- Sampling temperature: 15°C
- Injection volume: 10 µL
- Mobile phase A: water
- Mobile phase B: methanol
- Flow: 0.5 mL/min
- Elution: gradient

Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Type of elution in gradient
0.0	0.5	15	85	-
0.2	0.5	15	85	linear
0.5	0.5	0	100	linear
3.3	0.5	0	100	non-linear
4.0	0.5	15	85	non-linear

- Total time of chromatography: 5.5 min

Mass spectrometry conditions:

- Ionisation: Atmospheric pressure chemical ionisation (APCI)
- Triple quadrupole mode: Multiple reaction monitoring (MRM)
- Nebulizer gas: nitrogen
- Collision gas: argon

- Intensity of corona: 10 µA
- Temperature of ionisation source: 130ºC
- Temperature of desolvatisation: 600ºC
- Flow of desolvatisation gas: 600 L/h
- Flow of nitrogen in cone: 60 L/h
- Flow of collision gas: 0.20 mL/min
- Dwell time: 0.04 s
- Other parameters:

Sterol	***m/z*** **Ion precursor**	***m/z*** **Ion product**	**Cone potential (V)**	**Collision energy (eV)**
Brassicasterol	381.5	161.3	30	30
Campesterol	383.5	161.3	30	20
Campesterol-D_3	386.5	164.3	30	20
Colesterol-D_6	375.5	167.5	30	20
Desmosterol	367.5	161.3	30	20
Desmosterol-D6	373.5	167.3	30	20
Ergosterol	379.5	161.3	30	20
Lanosterol	409.5	149.3	30	25
Dihydrolanosterol	411.5	205.5	30	25
β-Sitostanol	399.5	81.4	30	30
β-Sitostanol-D_7	406.5	81.4	30	30
β-Sitosterol	397.5	161.3	30	20
β-Sitosterol-D_7	404.5	168.3	30	20
Stigmasterol	395.5	161.3	30	20
Stigmasterol-D_3	398.5	164.3	30	20