Graphical Abstract

Online solid-phase extraction–liquid chromatography–mass spectrometry to determine free sterols in human serum

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Highlights

- The method allows the analysis of 2 cholesterol precursors and 3 phytosterols.
- The organic phase from the Bligh–Dyer extraction was injected in the online system.
- The method avoids the time-consuming evaporation and reconstitution steps.
- A water cross-flow solved the problem of having two reversed phases in the system.
- The method is able to process 25 samples per day with 2 hours of sample handling.

Online solid-phase extraction–liquid chromatography–mass spectrometry to determine free sterols in human serum

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An automated method for analyzing free non-cholesterol sterols in human serum using online solid phase extraction–liquid chromatography–mass spectrometry is proposed herein. The method allows the determination of three phytosterols (sitosterol, stigmasterol and campesterol) and two cholesterol precursors (desmosterol and lanosterol). The analysis of sterols in human serum is critical in the study of cholesterol-related disorders, such as inherited familial hypercholesterolemias. Special effort was made to isolate the analytes from the serum lipoproteins, their natural conveyance through the bloodstream. The sample treatment consisted of a Bligh–Dyer extraction followed by dilution of the extract. This treatment allowed the sample to be injected into the online system and ensured the correct detection of the analytes, while avoiding the matrix effects commonly related to serum samples.

The analytical performance showed linear ranges that covered two orders of magnitude, with correlation coefficients above 0.99. Limits of detection and quantification ranged from 0.2 ng/mL to 13 ng/mL and from 1.0 ng/mL to 43 ng/mL, respectively. Recovery when spiking serum with a half or a tenth of the average concentration reported in human serum ranged from 99% to 111% and from 102% to 120%, respectively. Intra-day precision and inter-day precision were below 20%.

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1. Introduction

Sterols are metabolites related to cholesterol metabolism. The analysis of these compounds in human serum is an essential step in the diagnosis of cholesterol-related disorders, such as inherited familial hypercholesterolemias, cerebrotendinous xanthomatosis and sitosterolemia [1]. Cholesterol and related sterols are regulated in the human body *via* a complex set of mechanisms. Cholesterol can be absorbed through diet at intestinal level [2]. The rate of cholesterol consumption can be inferred from the presence of phytosterols such as sitosterol, stigmasterol and campesterol in human serum, since they undergo the same absorption and secretion processes [3]. Cholesterol is also endogenously produced and regulated in the liver. Some sterols such as desmosterol and lanosterol are intermediate products (precursors) in this reaction mechanism. Since these compounds leak into the bloodstream at a rate proportional to their formation in the cholesterol synthetic pathway, the circulating levels of these endogenous precursors reflect the rate of cholesterol synthesis [4,5].

Sterols have a non-polar structure and are carried through the circulatory system by lipoproteins. Lipoproteins are aggregates of lipids surrounded by a 2-nm amphiphilic layer composed of phospholipids, apoproteins and free cholesterol [6]. Therefore, the analysis of sterols involves the breakage of these aggregates.

The extraction of sterols has been typically performed using the Bligh–Dyer method [7,8], where a mixture of chloroform and methanol solubilizes the lipids from the lipoproteins, allowing the apoproteins to be separated. Sterols have been traditionally analyzed using gas chromatography, *via* a time-consuming derivatization step [4,9–11]. Liquid chromatography (LC) coupled to mass spectrometry (MS) avoids this derivatization step, diminishing the sample processing time [8,12]. However, the sample cleanup prior to LC–MS analysis is still strongly recommended and a solid phase extraction (SPE) step is generally performed [8,13].

An interesting choice is the use of hyphenated techniques that couple SPE to LC–MS using a switching valve, which is common in the analysis of biological samples and enables rapid and reliable sample preparation [14–17]. Several publications dealing with online systems are focused on determining drugs in plasma or urine; but scarce literature has been found related to the determination of endogenous metabolites [18,19]. A previous work of the authors aimed at the reduction of the sample processing time for determining free and bonded sterols in human serum [20,21] and another contribution demonstrated the capacity of coupling a Restricted Access Material (RAM) cartridge to LC–MS for determining bile acids

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in human serum [22]. To the best of our knowledge, there is not such a system to determine sterols in human serum, so the present contribution aims at the routine analysis of sterols in a faster way by using an online system.

2. Materials and methods

2.1. Chemicals and reagents

The sterols under study (Fig. 1) were provided by Sigma-Aldrich (Madrid, Spain): desmosterol (purity 85%, Chemical Abstract Service identification number: 303-04-2), campesterol (65%, 474-62-4), stigmasterol (95%, 83-48-7), lanosterol (97%, 79-63-0), sitosterol (95%, 83-46-5), and cholesterol-26,26,26,27,27,27-D6 (internal standard, IS, 97%, 60816-17-3). The solvents used were methanol (supragradient LC–MS grade, 67-56-1), 2-propanol (LC–MS grade, 67-63-0), and chloroform (HPLC grade, 67-66-3). The solvents were supplied by Sharlab (Barcelona, Spain). Water was obtained from a Milli-Q Plus 186 device from Millipore (Billerica, MS, USA).

Stock solutions of 1000 mg/L in methanol were prepared for each analyte. A stock solution of the IS at 100 mg/L was prepared in 2-propanol. A working solution with all of the sterols in methanol, at a concentration of approximately 10 mg/L, was prepared and stored in the dark at 4 °C.

2.2. Pooled serum

The pool of serum was obtained by mixing individual serum samples from 150–200 informed healthy volunteers. The pool was provided by the Lipids Unit of the Aragon Institute of Health Sciences (Zaragoza, Spain), and stored in the dark at −80 °C prior to the analysis.

2.3. Sample preparation

The sample was prepared using the following method: 300 µL of serum were extracted through a modification of the Bligh–Dyer method [23]. A volume of 1 mL of chloroform–methanol (1:2, v/v) was mixed with 30 µL of the IS solution and then added to the serum. The mixture was then centrifuged at 9000 rpm (7500g) for 10 min in a Microfuge® 18 Centrifuge, Beckman Coulter™ (Brea, CA, USA). The lower organic phase was recovered using a glass Pasteur pipet and transferred to a 2-mL vial. The extract was then accurately weighed, and 1 mL of methanol was added. The resulting extract was filtered using a 0.22-µm Nylon syringe filter. After this sample treatment, the final serum sample extract was ready to be injected into the online system described below.

Fig. 2 illustrates the online system. The mobile phases were delivered using three pumps; a 600E Controler Multisolvent Delivery System (pump 1) with a 717 plus Autosampler (Waters, Milford, MS, USA), a Kontron 322 System auxiliary binary pump (Kontron Instruments, Neufahrn, Germany) (pump 2) and an Alliance® 2795 Separations Module (Waters) (pump 3). The fluidic paths were controlled using a Cheminert C72 × 1690ED 10-port valve controlled by microelectric actuators, purchased from VICI® Valco Instruments (Houston, TX, USA). The analytes were detected using a Quattro micro™ API Mass Spectrometer (Waters). All these instruments were connected to operate simultaneously. This is explained in detail in the Supplementary information section S1.

Table 1 shows the steps of the online method, flow rate and composition of pumps 1 and 3, as well as the valve position program. The online method comprises four steps: loading, washing, eluting and detection.

For loading, 300 µL of the treated serum were injected using the 717 plus Autosampler and transferred to a 20 × 4 mm BioTrap 500C18 cartridge, supplied by ChromTech Ltd. (Cheshire, UK) at a 2.0 mL/min flow of 60% methanol and 40% water. A 2-µm filter was included before the cartridge to prevent clogging. This step lasted 4 min.

The washing step consisted of a 2.0 mL/min flow of water. The elution of large, non-retained molecules was monitored at 220 nm using a 2487 dual λ absorbance detector (Waters). Their elimination was considered to be complete when the UV signal had fallen below 0.05 absorbance units. Then, the valve was automatically switched to the eluting position.
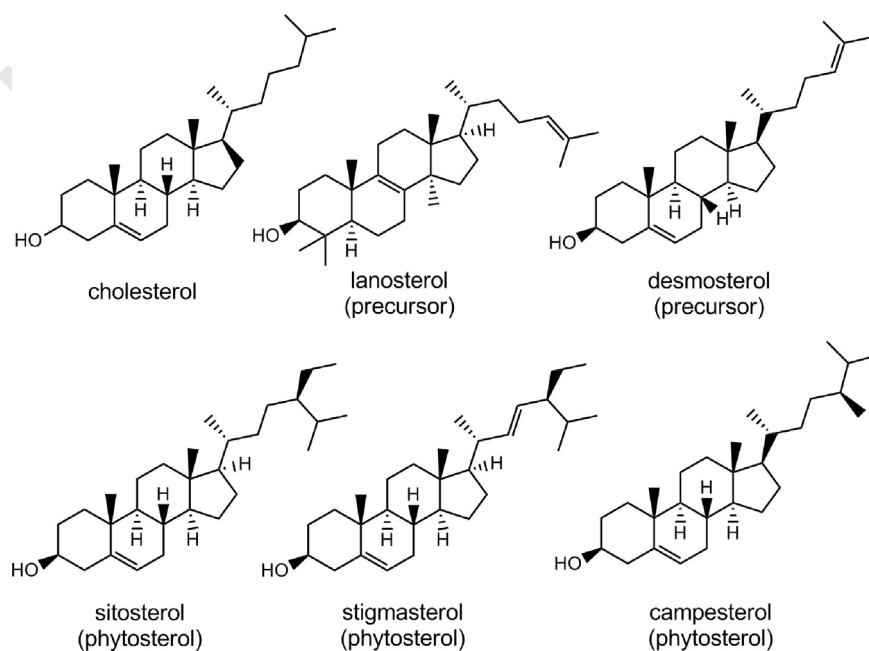

Fig. 1. Chemical structures of the sterols under study and cholesterol.
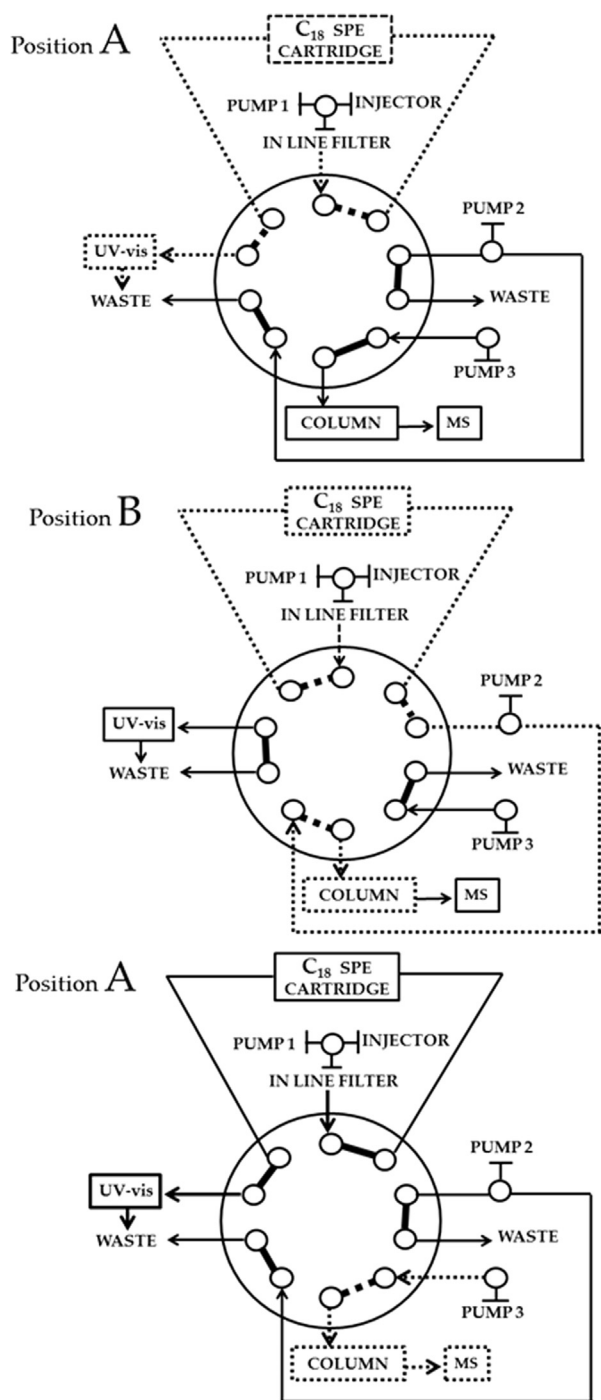
Fig. 2. SPE–LC–MS flowchart scheme. (A) Loading and detection steps. (B) Eluting step.

2.4.3. Elution

From min 9 to 35, the retained compounds were backflushed from the cartridge using 2-propanol at a flow rate of 0.4 mL/min. Before entering the analytical column, a water cross-flow of 0.6 mL/min was added (pump 2), leading to a total flow of 1 mL/min of 60% water and 40% 2-propanol. This flow composition ensured that the sterols were retained at the head of the analytical column.

2.4.4. Detection

The analytes were separated using an XTerra® MS C18 column (4.6 × 100 mm and 3.5 μm particle size, Waters). A guard column of the same stationary phase material and 5 μm particle size was used to protect the analytical column. The column temperature was set at 60 °C. The analytical gradient started at 35 min, using an 85% methanol and 15% water, at a flow rate of 0.7 mL/min. Then, the gradient was increased in 9 min to 100% methanol, where it was held for 5 min. The last compound (sitosterol) was eluted after 13.8 min of chromatography.

The compounds of interest were determined using atmospheric pressure chemical ionization (APCI) detecting in positive mode. The detection parameters were as follows: corona current, 10 μA; source temperature, 130 °C; desolvation temperature, 600 °C and desolvation nitrogen flow, 600 L/h. The optimized cone voltage was + 20 V for all the compounds. The chromatograms were registered in selected ion monitoring (SIM) mode. The protonated molecules (m/z) of the target analytes are listed in Table 2. MassLynx v4.1 software from Waters was used to acquire and process the generated data.

Complementary information dealing with the steps of the online system can be found in the Supplementary information, section S2.

2.5. Evaluation of the method performance

The following analytical parameters were studied: calibration curve, limits of detection (LODs) and quantification (LOQs), linear dynamic range, recovery and reproducibility. The linear dynamic range was determined from the response of nine standard solutions spiked with the IS (30 μL of a 100 mg/L 2-propanol solution). The calibration curve was prepared in a 1:2 (v/v) chloroform: methanol solution, coinciding with the composition of the resulting mixture after the sample treatment. These solutions were injected into the system in triplicate. The LOD and LOQ were calculated as three and ten times the standard deviation of the area of the peaks detected, respectively, when processing blank samples (chloroform–methanol, 1:2 v/v). The determination of the intra-day precision ($n=5$), the inter-day precision ($n=3$) and the recovery was carried out by analyzing serum spiked at two concentration levels: half and a tenth of the average concentration of sterols according to the Human Metabolome Database [24].

Matrix effects were investigated by a post-extraction addition experiment, as explained by Trufelli et al. [25]. Four sets of samples were analyzed: a standard solution with a known concentration of the analytes (set A), serum samples spiked with the same amount of analytes after the sample treatment (set B), serum samples spiked with the same amount of analytes before the sample treatment (set C) and serum samples non-spiked (set D) to subtract the endogenous concentration levels. The response of the analytes was used in the following equations to quantify the matrix effects (Eq. (1)), the extraction recovery (Eq. (2)) and the process efficiency (Eq. (3)).

$$\text{Matrix effects}(\%) = \frac{100 \times (B-D)}{A} \tag{1}$$

$$\text{Extraction recovery}(\%) = \frac{100 \times (C-D)}{(B-D)} \tag{2}$$

$$\text{Process efficiency}(\%) = \frac{100 \times (C-D)}{A} \tag{3}$$

3. Results and discussion

3.1. Sample treatment

The Bligh–Dyer method [26] is commonly used with human serum samples to extract the lipids and remove the protein fraction due to sterols are carried through the bloodstream by lipoproteins [7,8]. For this reason, protein precipitation approach,

Table 1

SPE and LC mobile phase flow rates, composition and 10-port switching valve position program. The shaded cells correspond to flows that do not carry analytes; but condition the path for the following injection.

Step	Time (min)	Valve position	Flow through the SPE cartridge (Pump 1)				Flow through the analytical column (Pump 3)		
			Flow rate (mL/min)	Methanol water (60/40) (%)	Water (%)	2-Propanol (%)	Flow rate (mL/ min)	Methanol (%)	Water (%)
Loading	0	A	2.0	100	0	0	0.3	0	0[a]
Washing	4		2.0	0	100	0	0.3	100	0
Eluting	9	B	0.4	0	0	100	1.0	0	100
Detection[b]	0	A	0.4	0	0	100	0.7	85	15
	9		0.4	100	0	0	0.7	100	0
	14		0.4	100	0	0	0.7	100	0

[a] From min 0 to 4, the composition was 100% 2-propanol (see section S2).

[b] Time of the chromatographic separation.

Table 2

Detection parameters, linear dynamic range and limits of detection and quantification of the analysis of sterols by the proposed online SPE–LC–MS method.

Sterol	CAS number[a]	Retention time (min)	m/z	Linear dynamic range (µg/ mL)	Correlation coefficient	LOD (ng/ mL)		Concentration in pooled serum (µg/mL)
Desmosterol	303-04-2	11.2	367.5	0.017–2.70	0.991	5.0	17	0.83 ± 0.23
Lanosterol	79-63-0	12.9	409.5	0.001–0.527	0.999	0.2	1.0	0.17 ± 0.04
Stigmasterol	83-48-7	13.0	395.5	0.025–5.22	0.994	7.5	25	1.35 ± 0.42
Campesterol	474-62-4	13.1	383.5	0.025–9.27	0.990	7.5	25	1.49 ± 0.26
Sitosterol	83-46-5	13.8	397.5	0.043–7.03	0.999	13	43	1.28 ± 0.07

[a] Chemical Abstract Service identification number.

results in the undesirable precipitation of the lipoproteins and the subsequent loss of the analytes (Fig. S3). Thus, a lipid extraction step is essential to determine sterols even when using an online system.

The composition of the final organic phase is mainly chloroform, which is not miscible with water and is not suitable for direct injection in reversed-phase liquid chromatography. Thus, the extract needs to be dried and reconstituted in a compatible solvent [8]. The aim of the sample treatment was to obtain a final extract suitable to be directly injected into the system. Herein, we took advantage of the miscibility properties of the water–methanol–chloroform tertiary system (as the Bligh–Dyer method does). First, the composition of the initial mobile phase in the online system was adjusted by increasing the proportion of methanol to the maximum that still allows the analytes to be retained in the C18 SPE cartridge, which is 60% methanol and 40% water. Next, the extract was diluted using methanol in such a way that the mixture with the initial composition of the mobile phase gives a single liquid phase. Fig. S4 shows the composition of the extract along the sample treatment in a ternary diagram for methanol–water–chloroform mixtures.

3.2. Online system setup

3.2.1. Loading and washing steps

In the loading step, the analytes were injected and taken to a C18 SPE cartridge. The strong non-polar properties of sterols permitted the use of C18 cartridges to concentrate them, while other less non-polar compounds (even lipids) were eluted to waste.

The solvent used in the washing step was water. This solvent showed satisfactory results removing interferences in a previous work [20].

3.2.2. Elution step and water cross-flow

Preliminary tests showed that 2-propanol was a powerful solvent for recovering the sterols from a C18 SPE cartridge. The elution profile was determined by collecting 0.4-mL fractions from the SPE cartridge in backflush mode every minute for 40 min. The fractions were later analyzed by direct flow-MS (i.e. without using the analytical column) in full-scan mode from 340 to 400 Da. In this way, each fraction appeared as a single peak (Fig. S5). As can be seen, 10 mL (25 fractions × 0.4 mL/fraction) of 2-propanol quantitatively recovered the analytes from the cartridge. Thus, the eluting stage lasted from 9 min to 35 min.

Because the cartridge and the analytical column were C18, the designed system did not have truly orthogonal features. This means that the eluting strength of 2-propanol would prevent the retention of the analytes in the head of the analytical column, compromising the chromatographic step. This problem was overcome by adding water to the 2-propanol eluent after the cartridge. To find out the necessary amount of water required to reduce the eluotropic strength of the flow, the following experiment was carried out. A standard solution of 1 µg/mL of sterols was prepared in 2-propanol. Increasing volumes of water were added to 1 mL of the standard solution and the resulting solutions were manually injected into the LC-MS, using an auxiliary sample loop and the MS divert valve. Fig. 3 shows how the added water reduced the eluotropic strength of the solution, enabling the chromatographic process. From these results, it was concluded that the flow composition entering the column should contain at least 60% (v/v) water.

A flow of 40% 2-propanol and 60% water (v/v) produced high backpressure in the analytical column. Hence, the total flow rate entering the column was cut back to 1.0 mL/min (working backpressure of 200 bar), 0.4 mL/min of 2-propanol were used to backflush the analytes from the cartridge and 0.6 mL/min water cross flow was added to reduce the eluotropic strength of the eluent.

3.3. Detection step

Sterols were detected in SIM mode as $[M+H-H_2O]^+$, which corresponds to the loss of the hydroxy group as a molecule of water (Table 2). This is commonly observed in the chemical ionization of alcohols [27]. Sterols crashed into several daughter

ions when the Multiple Reaction Monitoring (MRM) detection mode was selected (even when using soft collision voltages). The signal/noise values obtained in SIR mode were much higher than those of the MRM mode. In this case, the selectivity provided by the MRM mode was not enough to make up for the loss of sensitivity.

3.4. Cross contamination

The Bligh–Dyer extraction method is intended for removing proteins and simultaneously extracting lipids from blood and other tissues, so it is not sterol-selective. During the development of the method, cross-contamination was detected (blank injections with ghost peaks). Phospholipids and triacylglycerols usually build up in analytical or guard columns, especially when using aqueous mobile phases [28]. This problem was solved by extending the chromatographic gradient for 5 min after the elution of the last compound. Fig. S6 shows the improvement when a column cleaning step with 2-propanol was included. This is consistent with Ismaiel et al., who found that 35% of 2-propanol in the mobile phase was essential to elute lipids from the C18 guard column [28].

3.5. Evaluation of the method performance

One of the major drawbacks in biological analysis is the presence of matrix effects, which affect the recoveries, quantitative performance and the efficiency of the analytical process [15,25,29]. Hence, verifying the absence of matrix effects is advisable. In accordance with Trufelli et al. [25], post-extraction addition experiments were performed to evaluate matrix effects, extraction recovery and process efficiency.
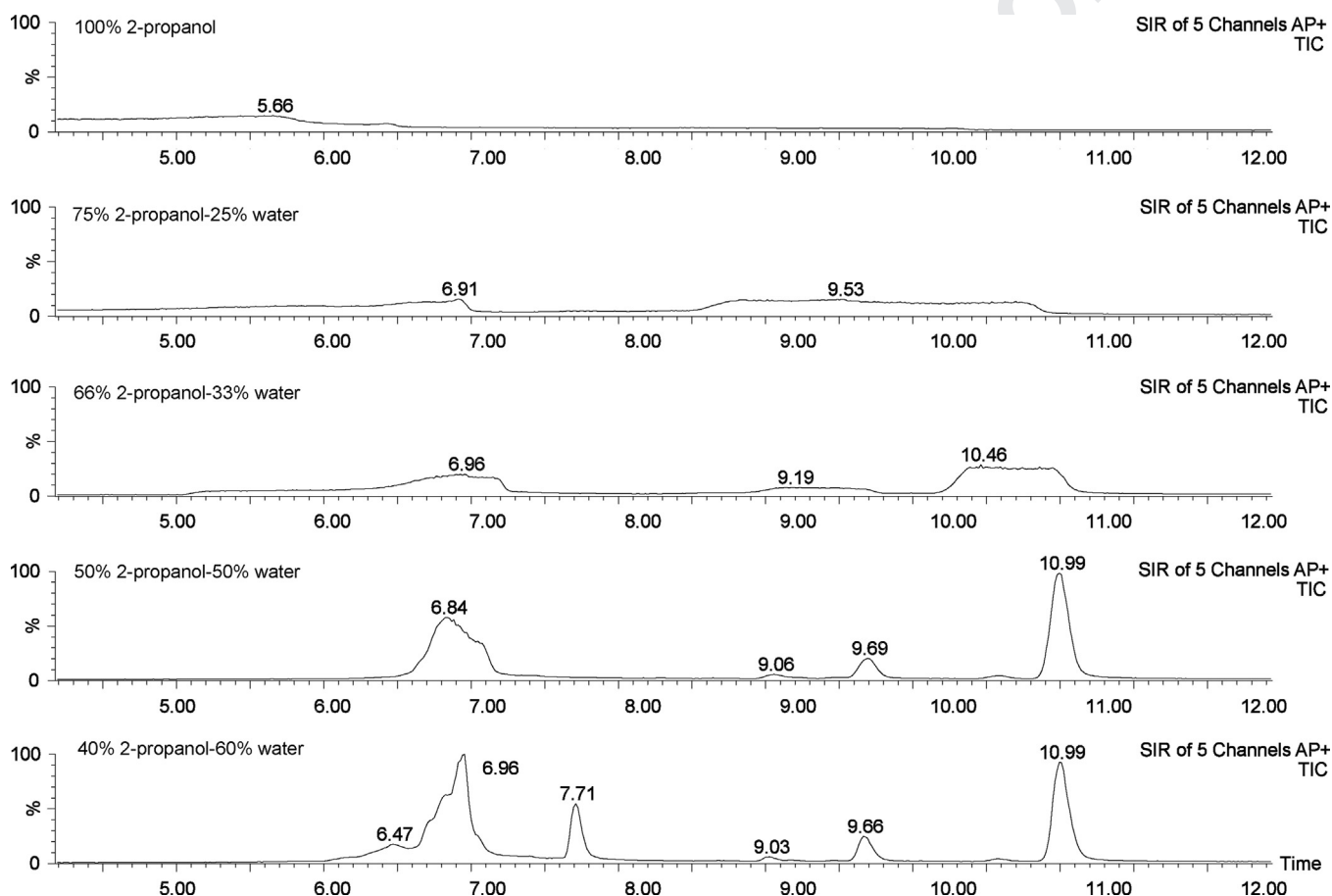

Fig. 3. Study of the eluotropic strength of the flow to be transferred to the LC analytical column. Increasing the ratio 2-propanol/water led to a weaker initial mobile phase. The chromatographic process was possible with at least 1.5 times more of water than 2-propanol.

Table 3
Recovery, intra- and inter-day precision of the analysis of sterols by the proposed online SPE–LC–MS method.

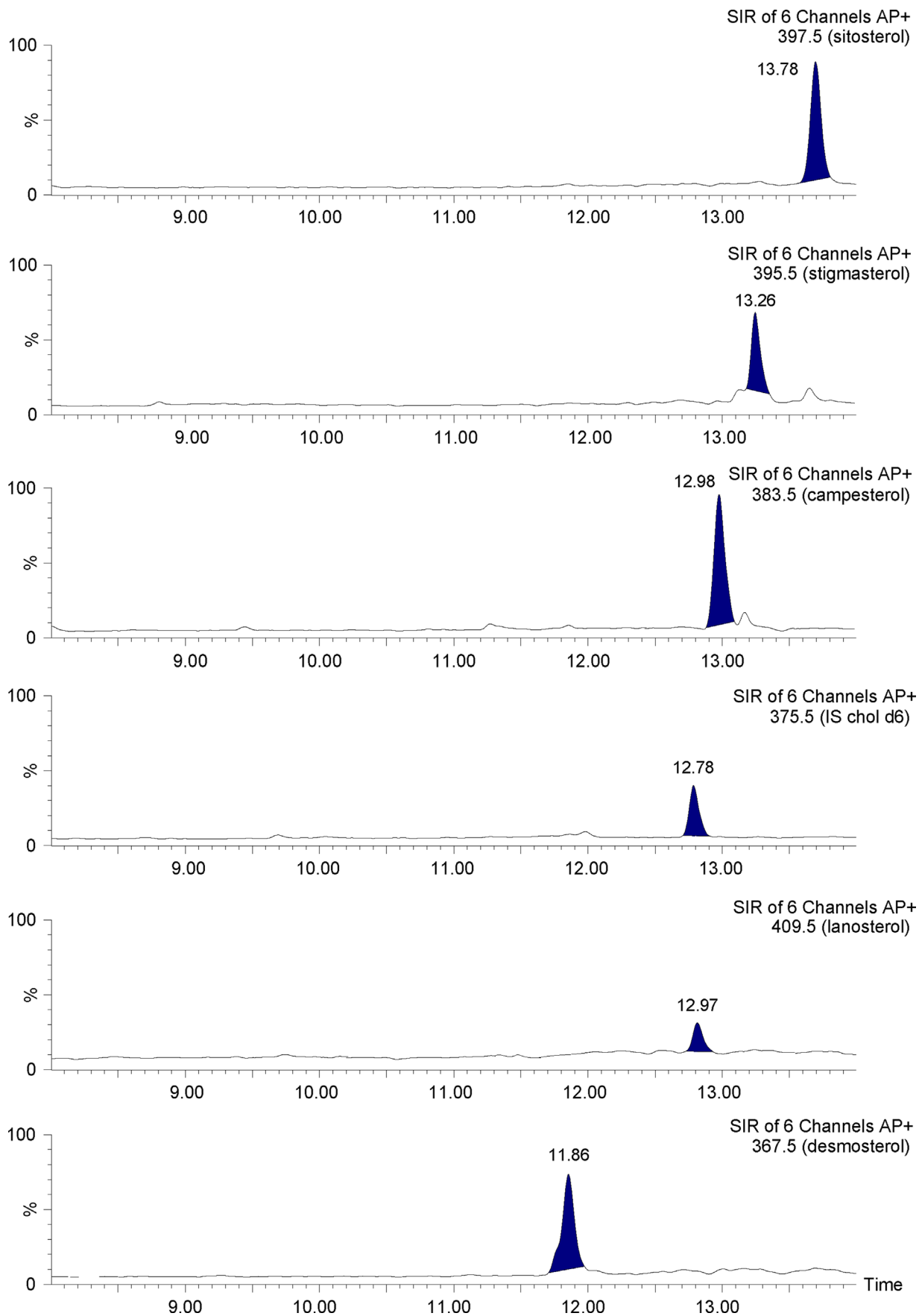| Sterol	Spiked concentration (ng/mL)	Calculated concentration (mean ± std deviation, ng/mL)	Recovery (%)	Intra-day precision (RSD %, $n=5$)	Inter-day precision (RSD %, $n=3$)
Desmosterol	205	210 ± 12	102	6	12
	42	47 ± 5	112	10	18
Lanosterol	38	42 ± 6	111	10	16
	10	12 ± 3	120	15	20
Stigmasterol	398	403 ± 12	101	7	10
	76	80 ± 9	105	11	14
Campesterol	736	730 ± 7	99	5	13
	154	157 ± 9	102	12	19
Sitosterol	440	448 ± 10	102	4	15
	87	92 ± 6	106	11	17

Fig. 4. Chromatograms of the studied sterols obtained after processing pooled serum.

Matrix effects ranged from 89% (stigmasterol) to 106% (lanosterol). This means that the signals observed were slightly affected by other compounds coming from the sample. Thus, there were almost no losses during the sample treatment. Finally, the process efficiency was also determined. The values obtained ranged from 90% (stigmasterol and campesterol) to 99% (desmosterol or lanosterol). These results showed that matrix effects do not hinder the determination of sterols *via* the proposed method. Since the response of the spiked samples was similar to the response of the sterol standard solution, an external calibration could be performed without applying a matrix matched calibration or standard additions.

3.6. Analytical features

The following analytical parameters were determined: linear dynamic range, LODs and LOQs, recovery, intra-day precision and inter-day precision. The results related to the linear dynamic range, limits of detection and quantification are listed in Table 2. Calibration was performed by plotting the ratio of the signal of the analyte and the deuterated IS versus the ratio of \the concentrations. The calibration curves were linear to two orders of magnitude with correlation coefficients that ranged from 0.990 (campesterol) to 0.999 (sitosterol or lanosterol). The LOD ranged from 0.2 ng/mL (lanosterol) to 13 ng/mL (sitosterol), and the LOQ ranged from 1.0 ng/mL (lanosterol) to 43 ng/mL (sitosterol). All of them were low enough to analyze the sterols in human serum.

Recovery and reproducibility (intra-day precision and inter-day precision) were evaluated by spiking the serum samples with standard solutions as it is described in Section 2.5. The results are summarized in Table 3. Recovery of the analytes at two levels of concentration was consistent and reproducible. The values obtained ranged from 99% to 111% when spiking with half of the average concentration in serum, and ranged from 102% to 120% when spiking with one tenth of the average concentration in serum. The results of intra-day precision ($n=5$) and inter-day precision ($n=3$) range from 4 to 20%. These results showed that the method meets the standards required for the study of hypercholesterolemia diseases.

3.7. Analysis of pooled serum

The chromatograms of the analytes in pooled serum are showed in Fig. 4. The concentration of sterols in the pool of serum was also determined (Table 2). It can be seen that the concentrations obtained were in agreement with the values found in literature [2,7]. As said before, serum levels of cholesterol precursors are related to liver cholesterol synthesis and they were found in much less concentration than cholesterol in human serum. The concentration of desmosterol and lanosterol resulted in 0.83 ± 0.23 μg/mL and 0.17 ± 0.04 μg/mL (Table 2), similar to the concentration values reported by McDonald et al. (0.71 μg/mL and 0.20 μg/mL, respectively) [7]. The content of phytosterols is typically higher than the content of cholesterol precursors, and it is more sample-dependant. The concentration of stigmasterol (1.35 ± 0.42 μg/mL), campesterol (1.49 ± 0.26 μg/mL) and sitosterol (1.28 ± 0.07 μg/mL) (Table 2) fall within the ranges reported in the Human Metabolome Database [24]. Due to the association between non-cholesterol sterols with cholesterol absorption, synthesis and metabolism, the determination of these compounds is a useful tool to explore their behavior in the human body.

4. Conclusions

An online SPE–LC–MS system to determine non-cholesterol sterols in human serum has been developed and described herein. The method allows the determination of two cholesterol precursors,

desmosterol and lanosterol, and three phytosterols, stigmasterol, campesterol and sitosterol.

This protocol substantially reduces the sample treatment, avoiding the time-consuming evaporation and reconstitution steps. The lipoproteins were extracted using the well-established Bligh–Dyer method, and the losses associated to a protein precipitation step with methanol or acetonitrile were eluded. This was achieved thanks to the careful selection of both the final extract and the initial mobile phase solvent compositions.

Two LC instruments have been coupled to operate simultaneously by using event analog signals. As a consequence, no additional tasks have to be executed by the analyst to run the online system. The use of the C18 cartridge combined with C18 LC was not a problem thanks to a water cross-flow added between both stationary phases. This can be performed by using a 10-port switching valve instead of an ordinary 6-port valve. Matrix effects were thoroughly studied and reduced to an extent that external standard calibration could be used.

All this work led to a sample treatment time reduction of 75%; that is to say, using this protocol, 25 samples can be handled in only 2 h. In previous works, the authors needed a working day (8 h) to handle these samples, using the alternative Bligh–Dyer, evaporation, reconstitution and SPE clean-up protocol based on the work of McDonald et al. [8].

The significant reduction in the analysis time would help in the research of cholesterol related diseases, which demands the processing of high number of samples to infer reliable conclusions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.10.029.

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