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Quantitative determination of cholesterol, sitosterol, and sitostanol in cultured Caco-2 cells by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

In this study, we describe a simple liquid extraction (methanol/choloroform, 1:1, v/v) method for endogenous free cholesterol and administered sterols extracted from cultured Caco-2 cells. To quantify sterol contents in Caco-2 cells, a new HPLC–APCI-MS method was developed. All the sterols were baseline separated using reversed-phase column (C_8, 2.1 mm × 150 mm, 3.5 μm) and isocratic conditions (90%, v/v, methanol–water mixture containing 0.2 mM ammonium acetate). The full scan mass spectra of sterols were measured by an ion trap mass spectrometer equipped with an APCI ion source. The intense fragment ions resulting from the loss of water $[M + H–H_2O]^+$ (*m*/*z* 369, 395, 397 and 399 for cholesterol, stigmasterol, sitosterol, and sitostanol, respectively) were used for determinations. The absolute extraction recovery of sterols from the spiked cell samples were 109.7 ± 26.2, 105.7 ± 5.1, 109.8 ± 5.0 and 99.0 ± 7.0% for cholesterol, stigmasterol, sitosterol, and sitostanol, respectively. Furthermore, no significant matrix effect was observed for the sterols in the cell samples. The sample assay was based on the internal standard method using stigmasterol as an internal standard. The method was linear over the concentration ranges of 0.45–9.0 μM (cholesterol) and 0.225–7.2 μM (sitosterol and sitostanol). The within- and between-day precision was less than 7% and accuracy ranged from 93.51 to 101.77%. The lowest limit of quantitation (LLOQ) was 0.225 μM for sitosterol and sitostanol, and 0.45 μM for cholesterol. The accuracy range was 95–106% and precision was lower than 9% for all LLOQ values.

Keywords: Cholesterol; Phytosterols; HPLC–APCI-MS; Caco-2 cells; Cellular uptake

1. Introduction

A major health concern is high cholesterol levels, which lead to various diseases. Dietary phytosterols and stanols (saturated phytosterols) from plants prevent intestinal absorption of cholesterol. These compounds are structurally related to cholesterol (Fig. 1.) However, phytosterols have comparatively poor intestinal absorption [1,2] and limit cholesterol absorption through competitive inhibition resulting in a reduction of serum cholesterol [3,4]. Most cholesterol and phytosterol absorption studies are performed in vivo with human or animals; however, studies using cultured cell lines (in vitro) are also common [5–8].

Cholesterol and phytosterols in human serum and phytosterols in plants are typically analyzed by gas chromatography (GC) and radioisotope methods [9–12]. In vitro cellular uptake of sterols have been traditionally analyzed by enzyme reactions, radiolabeled isotopes, GC methods, UV–HPLC methods, and thin-layer chromatography (TLC) [5–8,13,14]. UV–HPLC and TLC methods lack either sensitivity or selectivity, whereas enzymatic and radioisotope methods can lack a suitable internal standard as these compounds are not always commercially available. GC methods are sensitive and

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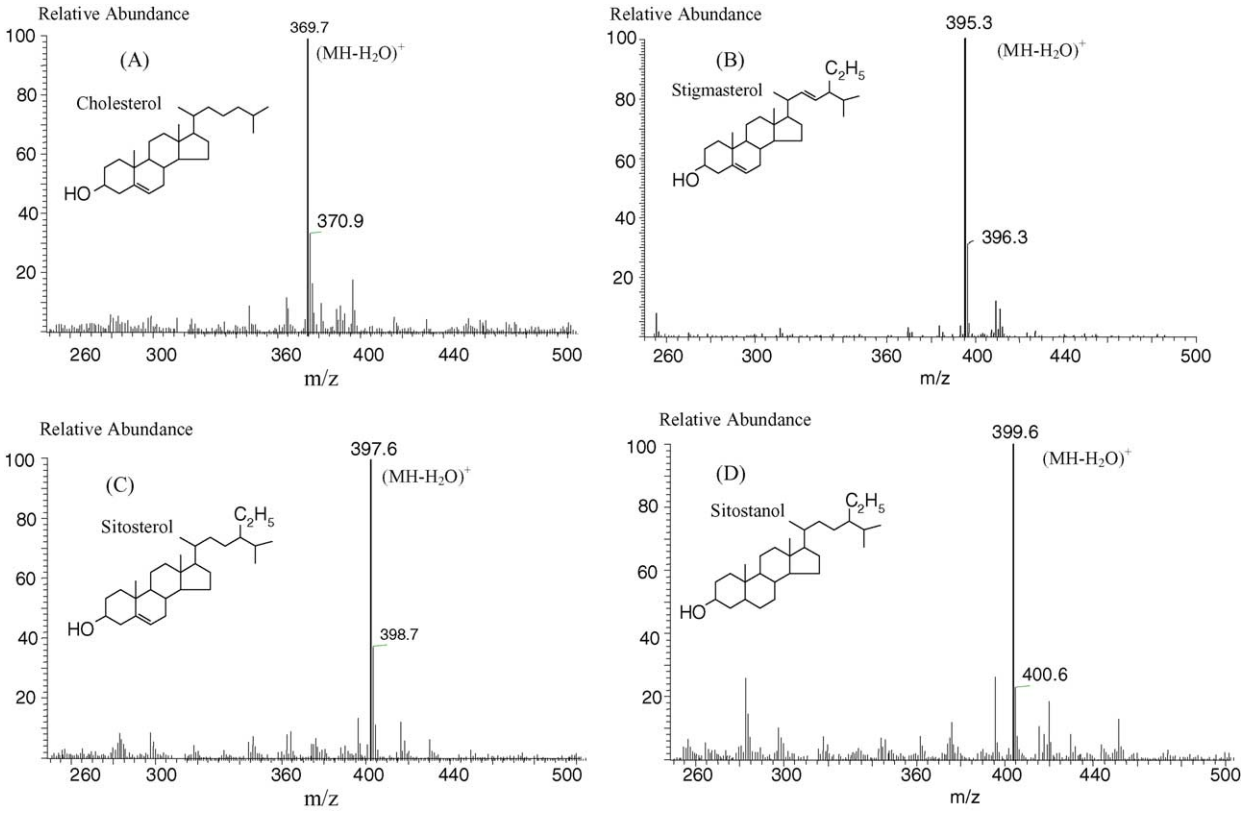

Fig. 1. HPLC–APCI-MS spectrum of standard cholesterol and phytosterols. Mass spectrum of cholesterol (A) ion $(MH–H_2O)^+$ at 369 *m/z*, spectrum of stigmasterol (B) ion at 395 *m/z*, sitosterol (C) ion at 397 *m/z* and sitostanol (D) ion at 399 *m/z* detected as a loss of water $(MH–H_2O)^+$.

selective, however, they require laborious sample derivatization steps before analysis and are suitable only for a limited number of compounds.

Recently, atmospheric pressure chemical ionization (APCI) with liquid chromatography mass spectrometry (LC–MS) was found to be suitable for the sterol analyses in different samples and matrices. LC–APCI-MS was used to identify sterols in soybean oil [15], to characterize phytosterols in spelt [16], to determine ergosterol levels in bulrush [17], and to measure cholesterol oxides in the different food supplies [18]. Phillips et al. [11] reported a precisely validated GC method for the sterols and stanols, but this study was limited to human serum samples.

Because sterols are highly lipophilic with few polar functional groups, they are difficult to ionize through conventional electrospray methods [16,21]. APCI ionization is usable but is not the most sensitive method. Electron impact (EI) ionization in particular and the recently developed atmospheric pressure photoionization (APPI) are effective alternatives for lipophilic compounds [19–21]. However, APCI ionization is most widely used for the sterols analysis, APCI instruments are commonly available, and APCI ionization can be easily coupled with HPLC system.

Extraction recovery of sterols from cultured cells can be varied depending on different properties of solvents [13]. For HPLC–MS studies in cultured cells (such as Caco-2) the matrix effect and the presence of endogenous cholesterol have to be investigated because they might cause potential ion suppression or enhancement of signal intensities. Therefore, an accurate, precise, selective, and sensitive analytical method is required for sterol analysis in cultured cells.

In the present work, we describe a fast and effective extraction method for endogenous free cholesterol and administered sterols extracted from cultured Caco-2 cells. Sterol contents in Caco-2 cells were quantified by a new HPLC–APCI-MS method. This is the first HPLC–APCI-MS method for the quantitation of cholestrol, sitosterol, and sitostanol from cultured cells.

2. Experimental

2.1. Materials and chemicals

All sterol standards were from Sigma (St. Louis, MO, USA). Cholesterol (5-cholesten-3β-ol, purity 99%), sitosterol (β-sitosterol, 24β-ethylcholesterol, purity 95%) and sitostanol (stigmastanol, 24α-ethyl-5α-cholestan-3β-ol, purity 96%) were used as the reference compounds. Stigmasterol (3-β-hydroxy-24-ethyl-5, 22-cholestadiene, purity 95%) was used as an internal standard (I.S.). Methanol and chloroform were obtained from Rathburn (Walkerburn, UK)

and ammonium acetate from Riedel-de Haen (Seelze, Germany) and all the chemicals were of analytical grade.

2.2. Cell culture and cellular uptake study

Caco-2 cells (a human colon adenocarcinoma cell line (HTB 37)) were purchased from ATCC (Manassas, VA, USA). The cells were grown in 75 cm^2 cell culture flasks (Nunc, Denmark) in Dulbecco's Modified Eagle's Medium (DMEM BE12-604F) (BioWhittaker, Belgium) supplemented with 10% heat inactivated foetal bovine serum (HyClone, Belgium), 2 mM L-glutamine, 1× non-essential amino acids, 100 IU/ml penicillin and with 100 μg/ml streptomycin (BioWhittaker, Belgium) in 7% CO_2 atmosphere at 37 °C. Cells were subcultured twice a week and passages 46–49 were used for experiments.

Sodium taurocholate (Fluka Chemie, Switzerland) and oleic acid (Sigma, St. Louis, MO, USA) were used as micelle forming substances. They were dissolved in ethanol as well as cholesterol, sitosterol, and sitostanol (Sigma, St. Louis, MO, USA) to form stock solutions. Substances were mixed in a glass tube, thereafter; ethanol was evaporated under nitrogen flow at 37 °C. The residual, forming a thin film in a glass tube, was dissolved in DMEM and sonicated in a bath sonicator for 15 min at 37 °C. Final concentrations of micelles were 2.5 mM sodium taurocholate, 125 μM oleic acid and 100 μM cholesterol, or 100 μM sitosterol or 100 μM sitostanol. Micelles were protected from light and kept in at 4 °C over night.

Caco-2 cells were seeded to polycarbonate membrane (Transwell® 3401, 12 mm diameter, 0.4 μm pore size) (Corning Incorporated, NY, USA) at a density of 90 000 cells/insert and were cultivated for 22–27 days. Culture media were changed every 2–3 days and on the day before experiment. The integrity of Caco-2 monolayer was controlled by measuring the transport of ^{3}H-mannitol. In uptake experiments, micelles (containing cholesterol, sitosterol, or sitostanol) were administered to cells for 3 h (in 7% CO_2 atmosphere at 37 °C). Cells were washed once with cold 1× PBS, then, cells were lysed by addition of 0.1% Triton X-100 solution for 30 min. Finally, cells were carefully scraped from membranes, suspended by pipeting, and removed to microcentrifuge tubes. All samples were stored at −20 °C until prepared, extracted, and analyzed. Sterol and protein contents of samples were measured by HPLC–APCI-MS and by Bradford methods, respectively.

2.3. Liquid chromatography

The HPLC system consisted of the Ultimate pump and Famos autosampler (LC Packings, Netherlands) with a 20 μl injection volume. The sterol samples were separated under isocratic conditions with a methanol–water (90:10, v/v) mixture containing 0.2 mM ammonium acetate. The chromatographic separation was performed using an Xterra MS C_8 reversed-phase column (2.1 mm × 150 mm, 3.5 μm, Waters, Milford, MA) with a flow rate of 180 μl/min. For the optimization of chromatography different flow rates were used and another Xterra column (1.0 mm × 50 mm, 3.5 μm) with a flow rate of 80 μl/min was used to compare for sensitivity, separation, and speed.

2.4. Mass spectrometry

A Finnigan LCQ quadrupole ion trap mass spectrometer (San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source was used for the mass analysis. The ionization was performed in the positive mode and full scan mass spectra from *m*/*z* 200 to 700 were measured. The optimized operating parameters of the APCI–MS interface were as follows: vaporisation temperature 400 °C; source temperature 150 °C; discharge current 5 μA; sheath gas flow 80 arbitrary (instrument) unit; aux gas flow 0 arbitrary unit; tube lens offset 10 V and ion collection time was 200 ms and three scans were summed. The flow from the HPLC was diverted to waste for the first 5 min preventing the impurities entering the mass spectrometer. Data were processed using the Finnigan LCQ software package version 1.2.

2.5. Validation of the method

2.5.1. Standard solutions and calibration

Individual primary stock solutions (2 mM) of cholesterol, sitosterol, sitostanol and stigmasterol (I.S.) were prepared separately in absolute ethanol and stored in the dark at 4 °C, at which they were found to be stable for at least 3 months. I.S. working solutions were prepared by diluting the primary I.S. stock solution to the final concentration of 200 μM (for samples) and 25 μM (for standards and quality control samples) in methanol. Similarly, appropriate amounts of each primary stock solution (except of I.S.) were combined and diluted with methanol to give 200 μM working solutions (A) for calibration and quality control (QC) standards. Furthermore, all the four primary stock solutions were mixed and prepared to final concentrations of 200 μM (B; for recovery samples) and 20 μM (C; for matrix effect samples). All the working solutions were stored at −20 °C until used.

A working solution (A) was further diluted with methanol and solutions were used for calibration and QC standards. Calibration (0.225, 0.45, 0.9, 2.25, 4.5, 7.2 and 9.0 μM) and QC (1.125 and 3.6 μM) standards were prepared daily through serially dilution and each contained an equal amount of I.S. (2.5 μM). The calibration and QC standards were analyzed (immediately after the preparation) repetitively between samples within each analytical sequence. The calibration curve was constructed by plotting the chromatographic peak area ratios (standard area/I.S. area) versus the concentration in the standard using linear regression and the coefficients of correlation (r^2) was calculated. The lowest limit of quantitation (LLOQ) for each sterol was calculated based on the FDA Guidance for Industry, Bioanalytical Method Val-

idation [22]. Briefly, the analyte response at LLOQ should be five times level of the baseline noise, and the analyte response at LLOQ should be determined with precision <20% and accuracy of 80–120%.

2.5.2. Precision and accuracy

Within-day accuracy and precision of the assay were determined by repetitive measurements ($n = 6$) of QC standards on two concentration levels. Precision was calculated as the relative standard deviation (R.S.D., %) and accuracy was expressed as the mean percent [(mean measured concentration)/(expected concentration) × 100]. Between-day accuracy and precision was evaluated by performing repeated measurements of the same QC standards on three different days and calculated in the same manner as the within-day values.

2.5.3. Extraction recovery and matrix effect

Cultured cells contain endogenous cholesterol; pure blank cells are not available. Therefore, the extraction recoveries of four sterols were determined using the standard addition method (a known amount of sterols was added to a cell sample) and based on liquid extraction without phase separation. Briefly, a cell sample (400 μl, contains endogenous cholesterol) was sonicated for 15 min and 50 μl of methanol and 3550 μl of methanol/chloroform (1:1, v/v) solvent were added to a cell suspension. A spiked cell sample was prepared in the same manner, except 50 μl of methanol was replaced with equal volume of working solution B (200 μM of each sterol). After 15 min vortex-mixing, cell and spiked cell samples were centrifuged at 4000 rpm for 5 min. Clarified supernatants (200 μl) were collected and evaporated to dryness by vacuum centrifugation. Residues were reconstituted in 200 μl of methanol. Finally, all samples were sonicated and vortex-mixed prior to analysis. The absolute recoveries of sterols were calculated in five replicates ($n = 5$) by comparing the peak areas of the samples (cell sample subtracted from spiked cell sample) to those of unextracted (matrix-free) standards at equivalent concentration (2.5 μM) (Eq. (1)). For evaluation of extraction procedure accuracy and precision the calculation was based on calibration curve plotted against internal standard. Contents of spiked sterols ($n = 5$) were calculated and compared to known added (nominal) contents for accuracy determination. Precision values were calculated from spiked sterol contents.

$$\text{Recovery}\,(\%) = \frac{\text{area of spiked cell sample} - \text{area of cell sample}}{\text{area of matrix free standard}} \times 100\% \quad (1)$$

To investigate matrix effect, standard samples were compared to spiked cell samples ($n = 5$) at equivalent concentrations (2.0 μM). Briefly, 90 μl of pre-extracted and reconstituted cell sample (as stated earlier) was spiked with 10 μl of working solution C (20 μM of each sterol). Percent ion suppression for each sterol was calculated as $100 \times (A_s - A_p)/A_s$, where A_s and A_p were the mean peak area of the sterol standard and the spiked sample, respectively.

2.6. Sample preparation and extraction process

After cellular uptake studies, micelles were collected and analyzed. Micelles (100 μl), 50 μl of I.S. solution (200 μM) and methanol (3850 μl) were vigorously mixed for 10 min by vortex. Sterol contents of micelles were analyzed along with other samples using HPLC–APCI-MS. Lysed and scraped cell samples (360 μl) were sonicated for 15 min, then 45 μl of I.S. solution (200 μM) and 3195 μl of methanol/chloroform (1:1, v/v) solvent were added to the cell suspension. After 15 min vortex-mixing, the extracted samples were processed as described in Section 2.5.3.

3. Results and discussion

3.1. Liquid chromatography

The high lipophilicity of the sterols can make sample processing and chromatography difficult. Potential contamination problems in the column or autoinjector were minimized by utilizing high organic solvent content both in the autoinjector and in the mobile phase. After standard or matrix samples, pure methanol samples did not present any traces of carryover. Although normal-phase chromatography is more often used for the separation of sterols [19], reversed-phase column was chosen for the analysis. Fig. 2 shows the chromatographic separation of sterols with two different reversed phase LC-columns. Separation of sterols was achieved in 3.5 min using a short column (Xterra, 1 mm × 50 mm) with isocratic elution (Fig. 2A) and increased sensitivity was observed with the flow rate of 80 μl/min, but impurities and sterols eluted together. In HPLC–MS analysis, baseline separation of peaks is not always necessary due to high specificity and selectivity of the detection method. However, effective chromatographic separation will decrease interference caused by isobaric (same molecular weight) compounds in the sample when the full scan mass mode is used. We found that longer reversed-phase column with MS detection was appropriate for the sterol analysis (Xterra, 2.1 mm × 150 mm, 3.5 μm, isocratic flow rate 180 μl/min). As seen in Fig. 2B, sterols were successfully separated within 15 min and uniform peak shapes were obtained. An interfering compound, which might originate from impurities or from an isotope of sitosterol, was successfully separated from sitostanol (Fig. 2B, ion channel 399 m/z).

To optimize peak intensity and retention times of sterols, four different flow rates were assessed. The flow rate of 80 μl/min offered the highest peak intensity but the retention time was over 30 min. Peak intensities using flow rates of 160 and 180 μl/min were rather equal, however, separation was 2 min faster when the higher flow rate was used giving

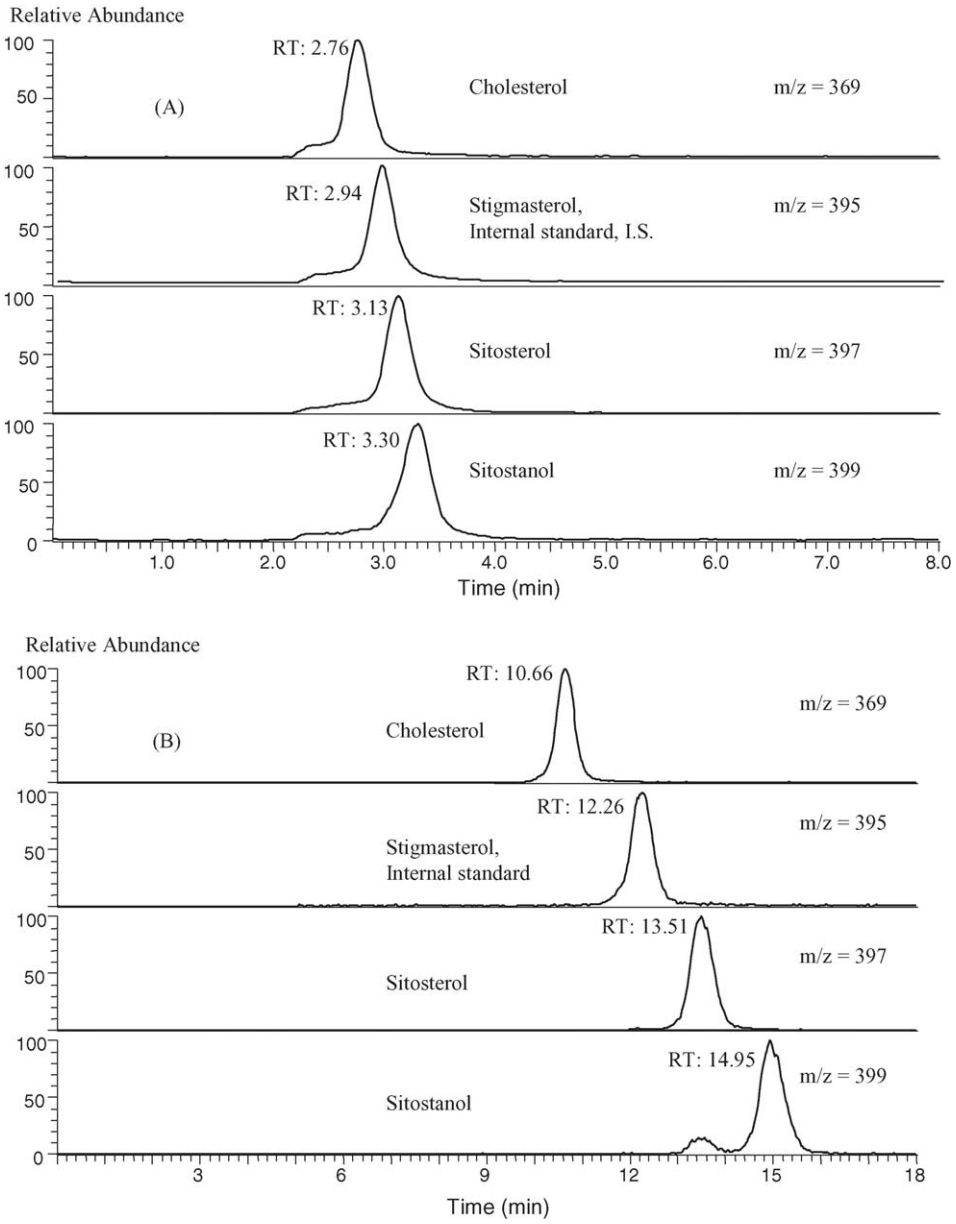

Fig. 2. Ion chromatograms of a standard mixture of sterols by HPLC–APCI-MS. Separation performed in (A) reversed phase Xterra column (1 mm × 50 mm), eluent flow rate 80 μl/min; (B) reversed phase Xterra column (2.1 mm × 150 mm), eluent flow rate 180 μl/min. Concentrations in (A) and (B) 1.0 and 3.6 μM, respectively.

the final retention time around 15 min. Flow rates over the 180 μl/min produced weak peak intensities.

3.2. Mass spectrometry

The positive full scan mass spectra of sterols are shown in Fig. 1. In the mass spectra the protonated sterol molecules $[M + H]^+$ induced weak signal intensities, and sterols were not observed as ammonium adducts. Intense fragment ions (*m*/*z* 369, 395, 397 and 399 for cholesterol, stigmasterol, sitosterol, and sitostanol, respectively) were observed, indicating the loss of water $[M + H–H_2O]^+$. This seems to be characteristic for sterols as dehydration was observed in earlier reports with cholesterol oxides [18], phytosterols [15,16], and ergosterol [17]. The operating parameters of the APCI-MS were manually optimized to maximize the detection of fragments $[M + H–H_2O]^+$ and the analysis of subsequent samples was based on these fragments. Detection using full scan mass mode was adequate for the sterol analysis, since the molecular weights were different and the chromatographic separation was efficient. MS/MS detection is known to be specific and selective. However, we tested MS/MS spectra of sterols and we observed that the product ion spectrums of the $[M + H–H_2O]^+$ ions were quite similar. Additionally, signal-

to-noise ratios of sterols in the MS/MS mode were lower than using the full MS method.

3.3. Validation of the method

3.3.1. Calibration and limit of quantitation

General recommendations for bioanalytical method validation (e.g. FDA-guidelines, ref. [22]) specify the quantitative determination of drug and metabolites in biological matrices such as blood, serum, plasma, or urine. There are no recommendations of method validation for cultured cells. Typically, bioanalytical validation parameters (calibration, LLOQ, precision, and accuracy) should be determined in the same biological matrix as the samples. The quantitation of sterols in cultured cells is difficult, since cells contain high level of endogenous cholesterol. When blank matrix is not available for the preparation of validation standards, the method of choice is using the standard solutions instead of spiked matrix samples [23–25].

In this study, method selectivity was evaluated during method development. First, the method was tested to be selective for sterol analyses in the presence of biological matrix. Second, interference from the solvent used for standard preparation was evaluated. Each sterol was tested individually to ensure that there was no detectable interference at specific ion channels. As stated in Section 3.3.3, cell matrix did not cause any interference for the signal intensities or for the extraction recoveries. Extraction efficiencies in spiked cell sample were excellent with a good precision and accuracy. Furthermore, no significant interfering peaks from the matrix and from the standard solvent were found at the MS retention times and in the ion channels of the analytes. After these confirmations, validation parameters of the analytical method were determined by using standard solutions instead of spiked matrix samples. Recently, similar semi-validation approach has been applied for determinations of endogenous retinoid and retinol levels in plasma and a variety of tissues [23–25].

The calibration curves of cholesterol, sitosterol, and sitostanol were linear over concentration ranges with correlation coefficients (r^2) greater than 0.994 (Table 1). The equations for the curves were calculated using six calibration points with three sets of replicate standards ($n = 3$) per curve. To compensate for analyte losses during sample preparation, the sample assay was based on the internal standard method, which was calculated from the peak area ratios (unknown/I.S. area) versus the calibration curve. Stigmasterol was chosen for the internal standard (I.S.), since it was commercially available and was observed to behave in the same manner as other sterols during sample extraction, chromatographic elution, and mass spectrometric detection. The linearity of the I.S. was tested using concentration range 0.2–5.0 μM and it showed linear response with 0.998 correlation coefficient.

Table 1
Calibration range, linearity (r^2) and LLOQ of the LC–APCI-MS method

Compound	Calibration range (μM)	Linearity (r^2)[a]	LLOQ ($n = 3$)		
			μM	R.S.D. (%)	Accuracy (%)
Cholesterol	0.45–9.0	0.9965	0.45	2.02	105.63
Sitosterol	0.225–7.2	0.9992	0.225	8.38	96.74
Sitostanol	0.225–7.2	0.9944	0.225	4.20	95.41

[a] Correlation coefficient using six calibration points ($n = 3$).

The LLOQ was 0.225 μM for sitosterol and sitostanol, and 0.45 μM for cholesterol (Table 1). The accuracy range was 95–106% and the R.S.D. precision was lower than 9% ($n = 3$) for all LLOQ values, which surpass the minimal FDA criteria. The signal-to-noise ratios obtained at the LLOQ were at least 10:1 and true sample concentrations were well above the LLOQ. This method is about six-fold more sensitive for sitosterol compared to a corresponding HPLC–UV method (sitosterol in the oil samples) [15] and similar to the LLOQ of 0.14 μM for sitosterol recorded from GC analysis of serum samples [10]. Furthermore, Phillips et al. [11] utilized also GC method for phytosterols in the human serum and reported the LLOQ of 1.0 and 0.5 μM for sitosterol and sitostanol, respectively. However, these previous results were performed by using spiked matrix samples.

3.3.2. Precision and accuracy

Within-day precision of the method was evaluated by performing six repetitive analyses of QC standards (1.125 and 3.60 μM), which gave excellent R.S.D. values between 1.27 and 4.36%. The accuracy range was 93–102% (Table 2). The between-day accuracy of the method ranged from 93 to 101% and the R.S.D. precisions were lower than 7%. The within-day precision of stigmasterol (I.S.) was 6.5% R.S.D. ($n = 38$).

3.3.3. Extraction recovery and matrix effect

Extraction recovery and matrix effect were performed by using spiked matrix samples, i.e. real spiked cell samples. A straightforward liquid extraction (methanol/chloroform, 1:1, v/v) method was used for each sterol. The mean absolute recovery of sterols from the spiked cell samples ($n = 5$) were 109.7 ± 26.2, 105.7 ± 5.1, 109.8 ± 5.0 and 99.0 ± 7.0% for cholesterol, stigmasterol (I.S.), sitosterol, and sitostanol, respectively. The extraction procedure accuracy was 90.9–103.7% and precision was lower than 16.2% for added cholesterol, sitosterol, and sitostanol. A previous report measuring radiolabeled cholesterol recovery from primary monocytes using chloroform/methanol (2:1, v/v) resulted in 93 ± 4.0% recovery initially and <50% recovery after evaporation and resuspension [13]. Thus, the extraction recoveries of our method were excellent, and the time of sample preparation was minimized. Matrix associated ion suppression of the response of the sterols was relatively minor with APCI-MS. Values for the spiked cell samples ($n = 5$) ranged from −0.1 to +6.5%. Overall, above-mentioned validation parameters indicate the accuracy, repeatability, linearity, and sensitivity of the analytical method and usefulness of the extraction procedure.

Table 2
Within- and between-day precision and accuracy of LC–APCI-MS method

Compound[a]	Within-day variation ($n = 6$)				Between-day variation (3 days, $n = 9$)			
	QC[b] (μM)	Mean[c] (μM)	R.S.D. (%)	Accuracy (%)	QC[b] (μM)	Mean[c] (μM)	R.S.D. (%)	Accuracy (%)
Cholesterol	1.125	1.145	4.11	101.77	1.125	1.052	6.92	93.51
	3.60	3.416	1.27	94.89	3.60	3.437	2.87	95.47
Sitosterol	1.125	1.122	3.24	99.75	1.125	1.120	3.62	99.56
	3.60	3.488	1.80	96.88	3.60	3.507	2.10	97.42
Sitostanol	1.125	1.133	3.81	100.73	1.125	1.128	3.33	100.27
	3.60	3.381	4.36	93.91	3.60	3.433	3.62	95.36

[a] Compounds are in chromatographical order.
[b] QC: quality control standard (nominal concentration).
[c] Mean observed concentration.

3.4. Sterols assay and cellular uptake of sterols

The extraction procedure and analytical method were utilized to quantify endogenous cholesterol and cellular uptake of cholesterol and phytosterols in Caco-2 cells. In uptake experiments, an individual sterol (cholesterol or sitosterol or sitostanol) was administered to a cell monolayer. After sample preparation sterol content was measured. Fig. 3 shows that all the sterols were successfully extracted from cell samples and identified chromatographically along with the internal standard. Further, no significant interfering peaks from the matrix were found in the ion channels of the sterols or inter-

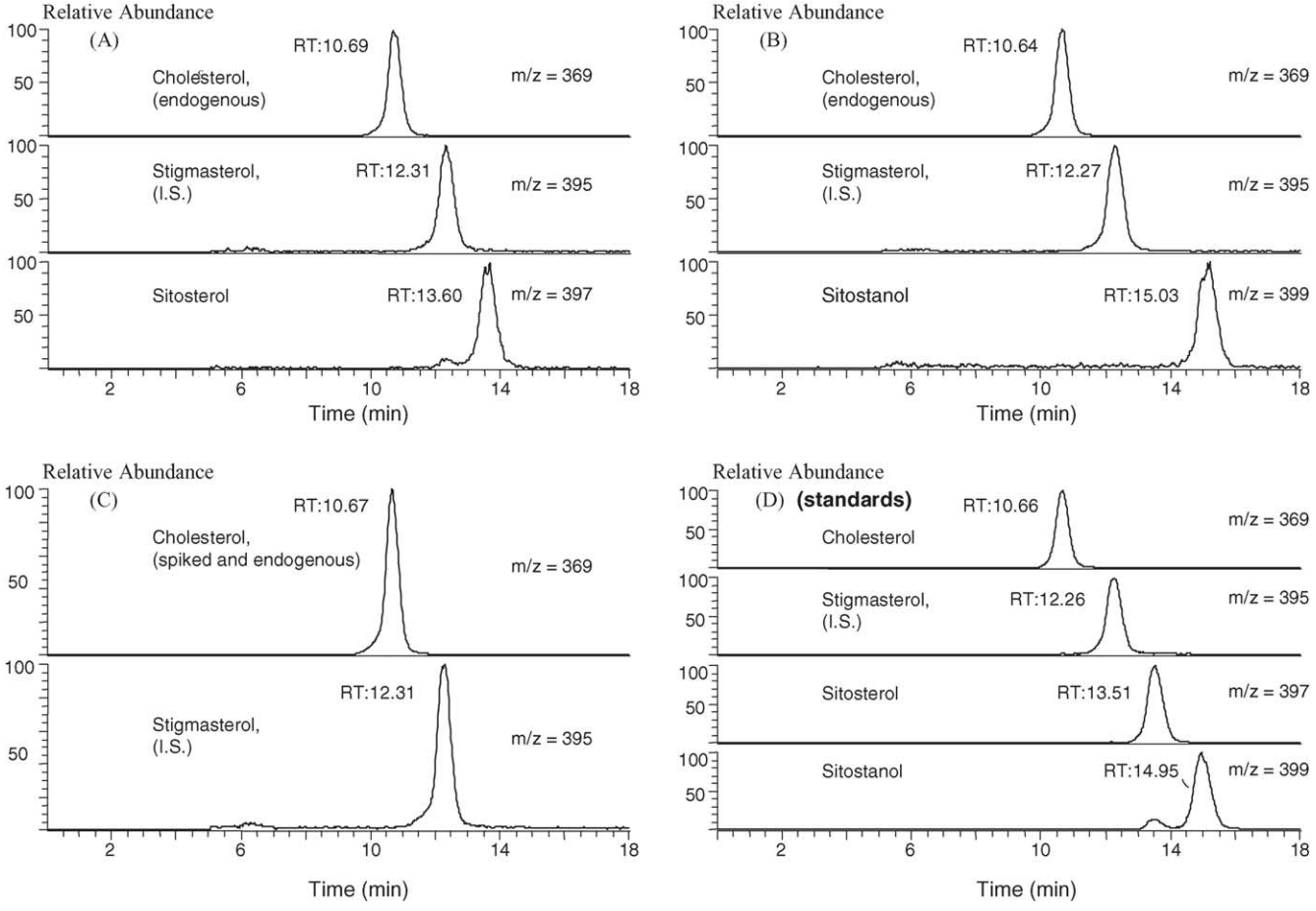

Fig. 3. Ion chromatograms of sterols extracted from cell samples (A–C) and chromatograms of standard sterols (D). Concentrations in a cell sample: (A) cholesterol (6.8 μM), stigmasterol (2.5 μM) and absorbed sitosterol (0.56 μM) after sitosterol administration. Correspondingly, in (B) absorbed sitostanol (0.62 μM); (C) absorbed and endogenous cholesterol (8.3 μM); and (D) each sterol standard (3.6 μM). Conditions as stated in Fig. 1B.

Table 3
Cellular uptake of sterols and amount of endogenous cholesterol in Caco-2 cells

Micelles composition[a] (μg)	Cellular sterol content ($n = 3$)					
	Cholesterol		Sitosterol		Sitostanol	
	Mean ± S.D. (μg)	Mean ± S.D. (μg sterol/mg protein)	Mean ± S.D. (μg)	Mean ± S.D. (μg sterol/mg protein)	Mean ± S.D. (μg)	Mean ± S.D. (μg sterol/mg protein)
Cholesterol 19.335	11.216 ± 0.96[b]	32.38 ± 0.97[b]				
Sitosterol 20.735	9.671 ± 0.95[c]	31.66 ± 6.39[c]	0.799 ± 0.03[d]	2.60 ± 0.18[d]		
Sitostanol 20.835	10.454 ± 1.50[c]	29.95 ± 4.63[c]			0.884 ± 0.13[d]	2.52 ± 0.35[d]

[a] Micelles were administered to the cell monolayer, after 3 h sterols were extracted as described in Section 2.
[b] Endogenous and absorbed cholesterol.
[c] Endogenous cholesterol.
[d] Absorbed sterol.

nal standard. Because of a lack of interfering peaks, cellular uptake could potentially be measured if all the sterols were administered simultaneously.

Table 3 illustrates initial amount of endogenous cholesterol and cellular uptake of sterols in cultured Caco-2 cells. The percentage amount of absorbed sitosterol and sitostanol was 3.85 and 4.25%, respectively. These values correlate with earlier reports of intestinal absorption of phytosterols where less than 5% was absorbed [7,26]. It is believed that cholesterol has high intestinal absorption compared to phytosterols, which we did not observe. The combined endogenous and absorbed cholesterol value was slightly greater than the average endogenous amount. Our findings of endogenous cholesterol content are on the same order with previous studies of Caco-2 cells in which enzyme and TLC [6] or GC [14] methods were used.

Previous estimates of intracellular cholesterol in human macrophages have varied by 5–10 times. The main source of discrepancy may be the limitation of enzymatic methods. The potential incomplete lipid extraction or incomplete solubility of extracted lipids have been reported and discussed [5,13,27]. The method described above could alleviate these issues and provide an accurate value of cholesterol levels.

4. Conclusion

In the present paper we report a rapid and effective procedure for the extraction of sterols and a new HPLC–APCI-MS method for the quantitation of sterols in Caco-2 cells. This new analytical method proved to be suitable for the sensitive quantitation of sterols in a complex cell matrix, even when the endogenous cholesterol was present. This study demonstrates that the liquid chromatography method coupled to MS can be applied in the direct analysis of lipophilic compounds, which are typically processed and derivatized prior to gas chromatographic analysis. The methods described here allow studying of cholesterol levels in cultured cells, so differences in various cell lines can be monitored more precisely. Moreover, these methods are suitable for further investigation of cellular uptake of sterols and they can be used to study the effect of phytosterols on the cellular uptake of cholesterol.

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References

[1] M. Igel, U. Giesa, D. Lütjohann, K. Bergmann, J. Lipid Res. 44 (2003) 533.
[2] D. Lütjohann, I. Björkhem, U. Beil, K. Bergmann, J. Lipid Res. 36 (1995) 1763.
[3] L. Normén, P. Dutta, Å. Lia, H. Andersson, Am. J. Clin. Nutr. 71 (2000) 908.
[4] M. Nissinen, H. Gylling, M. Vuoristo, T. Miettinen, Am. J. Physiol. Gastrointest. Liver Physiol. 282 (2002) G1009.
[5] J. Contreras, M. Castro, C. Bocos, E. Herrera, M. Lasunción, J. Lipid Res. 33 (1992) 931.
[6] L. Cai, E. Eckhardt, W. Shi, Z. Zhao, M. Nasser, W. Villiers, D. Westhuyzen, J. Lipid Res. 45 (2004) 253.
[7] C. Fernández, Y. Suárez, A. Ferruelo, D. Gómez-Coronado, M. Lasunción, Biochem. J. 366 (2002) 109.
[8] F. Field, E. Born, S. Mathur, J. Lipid Res. 38 (1997) 348.
[9] P. Dutta, L. Normén, J. Chromatogr. A 816 (1998) 177.
[10] R. Dyer, C. Hetherington, K. Alberti, M. Laker, J. Chromatogr. B 663 (1995) 1.
[11] K. Phillips, D. Ruggio, J. Bailey, J. Chromatogr. B 732 (1999) 17.
[12] N. Matthan, A. Lichtenstein, Atherosclerosis 174 (2004) 197.
[13] P. Cullen, K. Tegelkamp, M. Fobker, F. Kannenberg, G. Assmann, Anal. Biochem. 251 (1997) 39.
[14] H. Chen, E. Born, S. Mathur, F. Field, J. Lipid Res. 34 (1993) 2159.
[15] M. Careri, L. Elviri, A. Mangia, J. Chromatogr. A 935 (2001) 249.
[16] R. Rozenberg, N. Ruibal-Mendieta, G. Petitjean, P. Cani, D. Delacroix, N. Delzenne, M. Meurens, J. Quetin-Leclercq, J.-L. Habib-Jiwan, J. Cereal Sci. 38 (2003) 189.
[17] J. Headley, K. Peru, B. Verna, R. Robarts, J. Chromatogr. A 958 (2002) 149.
[18] E. Razzazi-Fazeli, S. Kleineisen, W. Luf, J. Chromatogr. A 896 (2000) 321.
[19] L. Heimark, P. Shipkova, J. Greene, H. Munyyer, T. Yarosh-Tomaine, B. DiDomenico, R. Hare, B. Pramanik, J. Mass Spectrom. 37 (2002) 265.

[20] R. Kock, B. Delvoux, H. Greiling, Clin. Chem. 43 (1997) 1896.
[21] E. Trösken, E. Straube, W. Lutz, W. Völkel, J. Am. Soc. Mass Spectrom. 15 (2004) 1216.
[22] Food and Drug Administration, Guidance for Industry, Bioanalytical method validation, 2001, http://www.fda.gov/cder/guidance/index.htm.
[23] C. Schmidt, A. Brouwer, H. Nau, Anal. Biochem. 315 (2003) 36.
[24] R. van Breemen, D. Nikolic, X. Xu, Y. Xiong, M. van Lieshout, C. West, A. Schilling, J. Chromatogr. A 794 (1998) 245.
[25] Y. Wang, W. Chang, G. Prins, R. van Breemen, J. Mass Spectrom. 36 (2001) 882.
[26] M. Moghadasian, Life Sci. 67 (2000) 605.
[27] P. Cullen, M. Fobker, K. Tegelkamp, K. Meyer, F. Kannenberg, A. Cignarella, A. Benninghoven, G. Assmann, J. Lipid Res. 38 (1997) 401.