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Derivatization-independent cholesterol analysis in crude lipid extracts by liquid chromatography/mass spectrometry: Applications to a rabbit model for atherosclerosis

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ABSTRACT

Direct measurement of various sterols in crude lipid extracts in a single experiment from limited biological samples is challenging. Current mass spectrometry (MS) based approaches usually require chemical derivatization before subjecting to MS analysis. Here, we present a derivatization-independent method for analyzing various sterols, including cholesterol and its congeners, using liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. Based on the specific tandem mass spectrometry pattern of cholesterol, multiple reaction monitoring (MRM) transitions were used to quantify free cholesterol and its fatty acyl esters. Several cholesterol oxidation products could also be measured using the upfront liquid chromatography separation and specific MRM transitions. The method was validated alongside established enzymatic assays in measuring total cholesterol. As a proof of concept, we analyzed plasma sterols in rabbits administrated with a high cholesterol diet (HCD) which is a classical atherosclerotic model. Free cholesterol, cholesterol esters, 7-hydroxycholesterol, and 7-ketocholesterol were elevated in plasma of rabbits on HCD. This method could also serve as an excellent tool for quantitative analysis of other sterols such as ergosterol and sitosterol in other organisms beside mammalian. In *Saccharomyces cerevisiae*, our results indicated dramatic increases of the ratio of ergosterol esters to free ergosterol in both *yeh2*Δ and *tgl1*Δ cells, which are consistent with the function of the respective enzymes.

1. Introduction

Cholesterol (cholest-5-en-3β-ol) is an essential component of blood plasma and mammalian cell membranes [1]. Cholesteryl esters are the major transport and storage forms of cholesterol in mammalian cells and lipoprotein particles. Aberrant levels of cholesterol and cholesteryl esters are associated with many human diseases, such as coronary heart disease, stroke, Smith-Lemli-Opitz syndrome, Antley-Bixler syndrome, and Niemann-Pick type C disease [2–6]. A total plasma cholesterol level above 180 mg/dL and a low-density lipoprotein cholesterol above 100 mg/dL are considered atherogenic when accompanied by other risk factors [7]. Aberrant levels of 7-dehydrocholesterol, which is a cholesterol precursor in serum, are shown to be associated with Smith-Lemli-Opitz syndrome [8]. Oxysterols can be formed from cholesterol via enzymatic oxidation or autoxidation [9–12]. Levels of cholest-4-en-3-one, an oxysterol identified in brain [13,14], are increased by almost 2-fold in brain tissue from patients with Alzheimer's Disease [14]. 7β-Hydroxycholesterol and 7-ketocholesterol are formed as a result of cholesterol oxidation and serve as biomarkers for oxidative stress in patients with atherosclerosis and other diseases [15–17].

Analysis of sterol levels in biological samples including serum, cells, and tissues is thus important for clinical studies. Laboratory measurement of serum sterols is currently done using several methods based on enzymatic assays, gas chromatography coupled with mass spectrometry, and recently liquid chromatography coupled with mass spectrometry (LC–MS) [18–27]. Use of LC–MS circumvents the need for sample derivatization and

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renders the overall analysis simpler and faster [18–26]. Liquid chromatography-atmosphere chemical ionization (LC-APCI) based selected ion monitoring of dehydrated protonated sterol molecules has been used to analyze cholesterol [28,18,29,30]. However, selected ion monitoring is confounded with noise from the matrix, complicating analysis of minor sterol species like trace amounts of oxysterols in a crude lipid extract in particular. Recently, MS/MS approaches, which are based on the loss of a water molecule, were used to quantify sterols [22]. However, loss of water is not a specific event, as any compound containing a hydroxyl group could theoretically lose one water molecule during APCI. To increase sensitivity and specificity, a few derivatization approaches were recently developed to enhance the LC–MS/MS measurement of sterols [19,21,23,31]. Cholesterol and cholesterol esters have been reported to be analyzed simultaneously in a single LC–MS run after acetyl chloride derivatization [23]. Derivatisation of oxysterols into picolinyl esters facilitates the sensitive measurement of key regulatory oxysterols and sterols in human serum [20,21]. However, to our knowledge, none of the existing MS methods detect free cholesterol, cholesterol esters, 7-dehydrocholesterol, and cholesterol oxidation products in a single experimental setup mainly due to concentration and solubility issues. While the solubility issues could be resolved using chloroform-based mobile phases, the concentration issue requires the method to have a wide dynamic linearity range. In this study, we used chloroform-based mobile phase in LC separation to improve the solubility of various sterols and optimized multiple reaction monitoring (MRM) transitions to have a wide dynamic linearity range for direct analysis of sterols in crude lipid extracts from biological samples via a one-step LC–MS method.

2. Experimental

2.1. Chemicals

Squalene, cholesterol, 7-ketocholesterol, 7β-hydroxycholesterol, 7-dehydrocholesterol, 24-hydroxycholesterol, 27-hydroxycholesterol, 4-cholestene 3-one, and 5,6-epoxycholesterol were from Sigma–Aldrich (St. Louis, MO). Cholesteryl butyrate, cholesteryl pelargonate, cholesteryl myristate, cholesteryl palmitate, and cholesteryl stearate were obtained from Steraloids Inc. (Newport, RI). Cholesterol-26,26,26,27,27,27-d6, cholesterol-26,26,26,27,27,27-d6-stearate, cholesterol-2,2,3,4,4,6-d6-stearate, 7-ketocholesterol-25,26,26,26,27,27,27-d7, and 7β-hydroxycholesterol-25,26,26,26,27,27,27-d7 were from CDN Isotopes Inc. (Quebec, Canada). Zymosterol, zymosterol-2,2,3,4,4-d5, 14-demethyl-lanosterol-d6, 24-hydroxycholesterol-25,26,26,26,27,27,27-d7 were from Medical Isotopes Inc. (Pelham, NH). Deferasirox (ICL670A, Exjade) was supplied by Novartis Institutes for BioMedical Research, Basel, Switzerland.

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

2.2. Animal experiments

New Zealand white rabbits were obtained from the Laboratory Animal Centre (Singapore), weighing an average of 2–2.5 kg. These animals were fed a normal diet for 2 weeks for acclimatization to the environment, and then were divided into two groups: Group 1 ($n = 5$) was fed a normal diet (GFS, Glen Forrest Stockfeeders, Western Australia) as control, group 2 ($n = 5$) was fed 1% (w/w) cholesterol-containing food (GFS + 1% cholesterol; high cholesterol diet (HCD)) [33]. Rabbits were sacrificed after 8 weeks of Exjade treatment (8-week animals) by i.v. injection of Hypnorm. This study was approved by the NUS local Animal Care and Use Committee (IAUAC No. 709/04A).

2.3. Human plasma sample collection

Blood samples from 10 volunteers were collected in EDTA tubes at National University Hospital, Singapore, under an institutional review board–approved clinical protocol (NUS-IRB No. 04-115) to study lipidomics of membrane signaling as a tool for clinical prognostics.

2.4. Yeast strains and media

The strains used for this study were purchased from EUROpean Saccharoymces Cerevisiae ARchive for Functional analysis (EUROSCARF) library. BY4741 is a wild type strain, while Δ*yeh2* and Δ*tgl1* are two deletion mutants for genes encoding steryl ester hydrolase. Strains were grown on either YPD medium (1% yeast extract, 2% Bacto-peptone, and 2% glucose) or synthetic complete (SC) medium prepared as previously described [34]. The cells from each strain ($n = 4$) were grown to an O.D of 0.8–0.9 and harvested at 3500 rpm for 2 min. The cells were washed in 1 mL of Milli-Q water and transferred to a 2 mL eppendorf tube and pellets were stored at −80 °C.

2.5. Plasma lipid extraction

Ice-cold solvent mixture (900 μL of chloroform:methanol, 1:2 (v/v), +0.01% butylated hydroxytoluene) was added to 100 μL of plasma sample, vortexed, and incubated on ice in a vacuum chamber in a dark room for 1 h with agitation. After incubation, 0.3 mL of chloroform was added to the homogenate, followed by 0.35 mL of ice-cold water. The homogenate was then vortexed for 30 s and centrifuged for 2 min at 9000 rpm. The bottom organic phase was carefully transferred to an empty tube, and 0.5 mL of ice-cold chloroform was added for the second extraction. The two organic extracts were then combined and dried under nitrogen.

2.6. Yeast lipid extraction

Lipid extraction was carried out as described previously with slight modification [35]. Briefly, 150 μL of acid washed glass beads and 900 μL of chlorofom:methanol (1:2) was added into cell pellet, and the suspension was vortexed vigorously for 10 min. The tubes were transferred into a vacuum container and incubated overnight at 4 °C with agitation at 1100 rpm. 300 μL of chloroform and 300 μL of H_2O were added and the mixture was vortexed for 30 s, then incubated for 2 min on ice. The phases were separated at 9000 rpm for 2 min, and lower organic phase was collected. 500 μL of chloroform was added for the second extraction. The two organic extracts were pooled and dried using a Speed-Vac (Thermo Savant, Milford, USA). The dried lipid film is stored in −80 °C freezer and reconstituted in chloroform:methanol (1:1) before LC/MS analysis.

2.7. Total cholesterol measurement using an enzymatic assay

Cholesterol concentrations in human plasma were determined using an enzymatic-colorimetric procedure (kit #352-100; Sigma Chemical Co., St. Louis, MO).

2.8. LC/APCI/MS and LC/APCI/MS/MS

Sterols were analyzed using an Agilent HPLC 1100 system (Agilent) coupled with an Applied Biosystems 3200 QTrap mass spectrometer (Applied Biosystems, Foster City, CA). In brief, separation of sterols was carried out using an Agilent Zorbax Eclipse

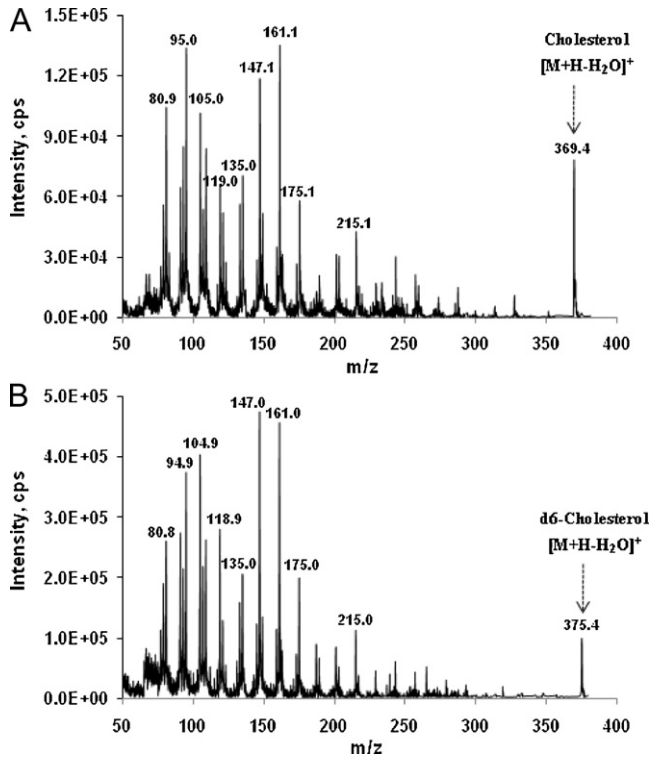

Fig. 1. Tandem mass spectra (MS/MS) of free cholesterol (A) and 26,26,26,27,27,27-d6 cholesterol (B).

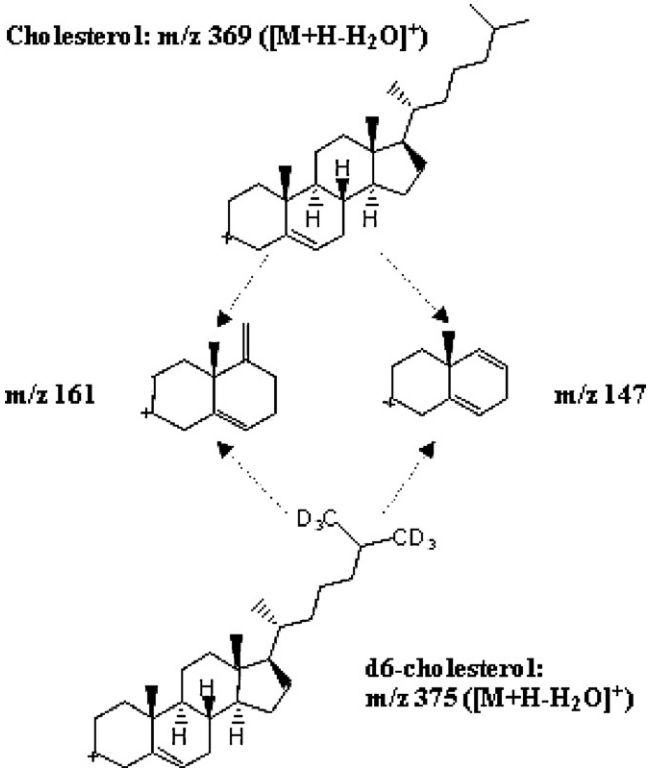

Fig. 2. MS/MS of free cholesterol generates major daughter ion species at *m*/*z* 161 and 147 for both free and deuterated cholesterols.

XDB-C18 column (i.d. 4.6 mm × 150 mm). HPLC conditions were as follows: (1) chloroform:methanol 1:1 (v/v) as the mobile phase at a flow rate of 0.5 mL/min; (2) column temperature: 30 °C; (3) injection volume: 10 μL. The LC–MS instrument was operated in the positive atmospheric pressure chemical ionization (APCI) mode with a vaporizer temperature of 500 °C, corona current of 3 μA, and capillary temperature of 250 °C. Declustering and entrance potential were set at 30 and 10 V, respectively. The fragment ions resulting from the loss of water at *m*/*z* 369 and 375 for cholesterol and d6-cholesterol, respectively, were monitored as parent ions. MS/MS spectra of cholesterol at *m*/*z* 369.4 and cholesterol-26,26,26,27,27,27-d6 at *m*/*z* 375 were obtained, and parameters for the respective specific MRM transitions were optimized (Fig. 1). The collision energy ranged from 40 to 45 V. MRM transitions of 369.3 → 161.0 and 375.3 → 161.0 were found to be the optimal transitions for analysis of cholesterol and cholesterol-26,26,26,27,27,27-d6-stearate, respectively (Fig. 2). Thus, d6-cholesterol (ester) is an ideal internal standard for rapid analysis of cholesterol in biological samples. MS/MS of other sterols was carried out as described above (Supplementary Fig. 1), and the respective MRM transitions were then established for sterol analysis in this study (Fig. 3).

2.9. Method validation

Method reproducibility was investigated using a crude lipid extract spiked with deuterated standards. This preparation was run six times ($n = 6$) under the selected MRM transitions to obtain the MRM ratios of endogenous sterols to deuterated standard.

The linear range of free cholesterol (ester) and oxysterols was obtained using selected deuterated internal standards. 50 ng, 100 ng, 1 μg, 10 μg, 50 μg, and 100 μg per milliliter of cholesterol was spiked with deuterated cholesterol-26,26,26,27,27,27-d6 as the internal standard, and measured using the LC-MRM approach. The LC-MRM results were subjected to linear regression analysis, and the squared correlation coefficient (r^2) was calculated. Similar validation was carried out for 24-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and cholesteryl stearate.

Accuracy and precision were assessed using two different concentrations of endogenous sterols into crude lipid extracts con-

Table 1
Precision, accuracy, LOD and LOQ data obtained from LC-MRM analysis.

Sterol	LOD (ng/mL)	LOQ (ng/mL)	Amount added (μg/mL)	Calculated concentration (μg/mL)	Accuracy (%) ($n = 3$)	Precision (RSD[a], %)
Cholesterol	15.1	49.8	10	9.3	93	1.1
			2	1.9	96	4.5
Cholesterol ester	24.3	80.3	10	9.4	94	6.3
			2	2.0	102	5.5
7-Ketocholesterol	6.5	21.5	0.2	0.21	104	0.3
			0.05	0.05	110	5.3
7β-Hydroxycholesterol	5.3	17.5	0.2	0.19	96	4.5
			0.05	0.05	102	7.4
24-Hydroxycholesterol	4.9	16.3	0.2	0.17	87	4.4
			0.05	0.05	107	6.9

[a] Relative standard deviation.

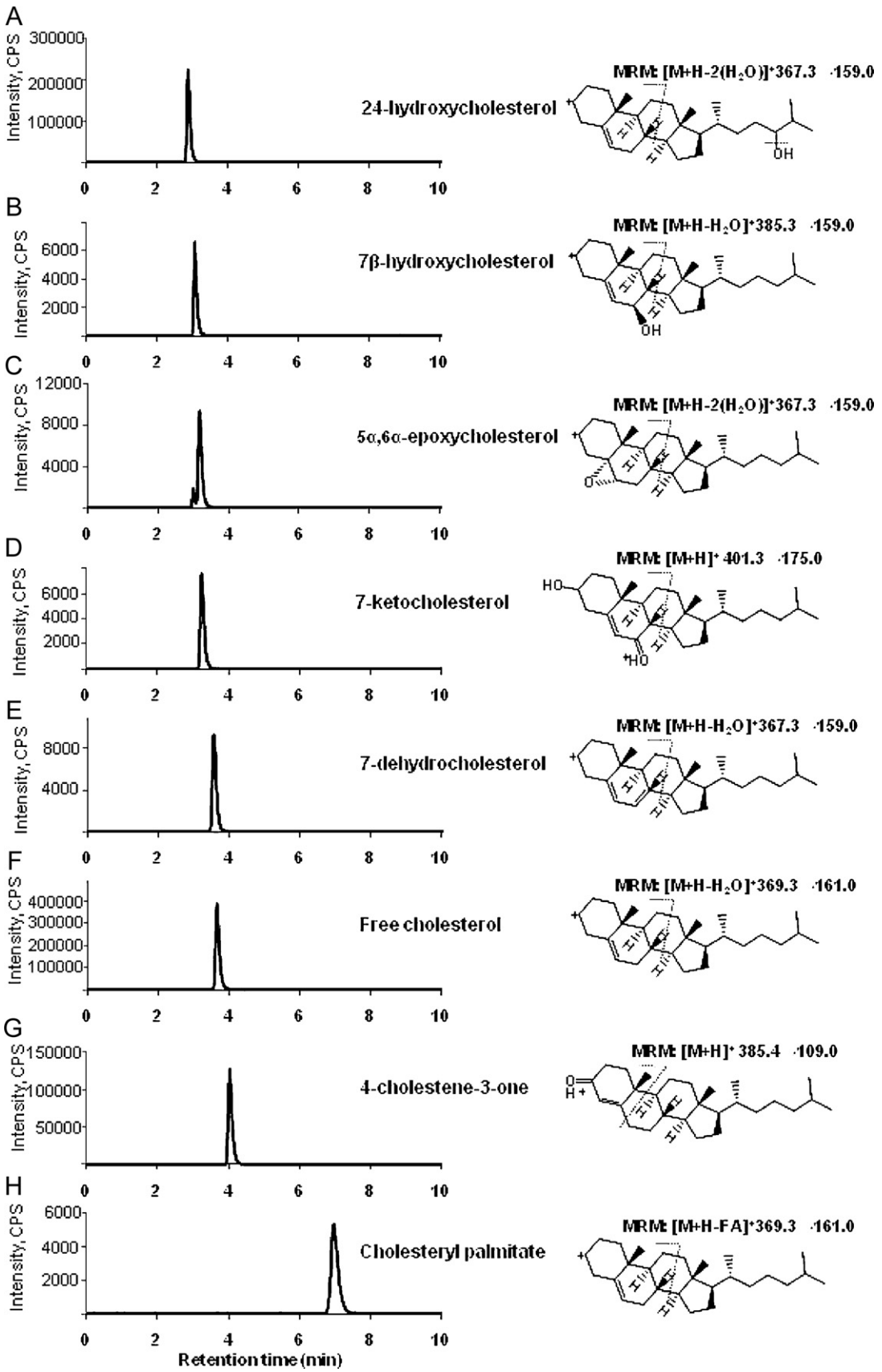

Fig. 3. LC-MRM analyses of sterols. (A) 24-Hydroxycholesterol; (B) 7β-hydroxycholesterol; (C) 5α,6α-epoxycholesterol; (D) 7-ketocholesterol; (E) 7-dehydrocholesterol; (F) free cholesterol; (G) 4-cholestene-3-one; (H) cholesteryl palmitate.

taining corresponding deuterated sterol standards. The amount of each added sterol is listed in Table 1. Accuracy and precision were calculated as the percentage and relative standard deviation, respectively, of the ratio of the mean calculated concentration and true value of the known added amounts of corresponding sterol.

The limit of detection (LOD) was defined as the lowest amount of sterol that could be reliably identified with ion signal-to-noise ratio (determined by peak height) ≥3:1 with a satisfactory chromatography. The limit of quantification (LOQ) was defined as the lowest concentration that met LOD criteria with a deviation target <20% in six replicates.

2.10. LC-MRM analysis of plasma sterols from HCD-fed rabbits

Lipid extract containing deuterated standards from 100 μL of plasma sample was resuspended in 100 μL of mobile phase. To improve accuracy, different amounts of internal standards were spiked into control and HCD-fed rabbits. We found that both the levels of cholesterol and cholesteryl esters in HCD-fed rabbits were very high, and further dilution was performed using the same mobile phase to check system errors. LC-MRM quantification was carried out as described above.

2.11. LC-MRM analysis of yeast ergosterol and its esters

To analyze ergosterol and its esters in crude lipid extracts from *Saccharomyces cerevisiae* wild type and mutant cells, free cholesterol and cholesteryl stearate were used as corresponding internal standards, respectively. MRM transition of 379.4/179 was the optimal transition for quantification of both free ergosterol and ergosterol esters.

3. Results

3.1. LC–MS/MS analysis of sterols

Cholesterol is a relatively neutral molecule and does not ionize effectively with electrospray ionization. However APCI allows for effective ionization of cholesterol into dehydrated protonated molecules at *m/z* 369.4 [32]. MS/MS spectra of cholesterol and cholesterol-26,26,26,27,27,27-d6 show similar fragment ions. Thus, synthetic and pure d6-cholesterol can serve as internal standards for quantification of endogenous cholesterol (Fig. 1A and B). The pathways of major fragment ions at *m/z* 161 and *m/z* 147 for both cholesterol and cholesterol-26,26,26,27,27,27-d6 are shown (Fig. 2).

Based on the above information and product ion analysis of other synthetic sterol standards (Supplementary Fig. 1), individual MRM transitions for the cholesterol oxidation products, 7-dehydrocholesterol and 4-cholesten-3-one, were set up as a single LC-MRM method. In combination with the upfront column separation, these MRM transitions enabled quantitative analysis of selected sterols. 24-Hydroxycholesterol, with retention time at 2.8 min, could be selectively followed by an MRM transition of 367.4 → 159. Likewise, we also observed 7β-hydroxycholesterol, which eluted at 3.06 min with an MRM transition of 385.4 → 159, and 5,6-epoxycholesterol, which eluted at 3.13 min with a sensitive MRM transition of 367.4 → 159, etc. (Fig. 3A–H). Cholesteryl esters, eluted with much longer retention times were monitored with the same MRM transition as free cholesterol (Fig. 3F and H). Using isocratic elution conditions, the retention time of cholesteryl ester increased with the increase in the length of the fatty acyl chains (Supplementary Fig. 2). Although it is still a challenge to quantify individual cholesteryl ester using HPLC/APCI/MS, the fact that cholesterol equivalent of individual cholesteryl ester (C14, C16, and C18) has approximately the same response factor to MS (data not shown), facilitated rapid quantification of total cholesterol esters in a crude lipid extract.

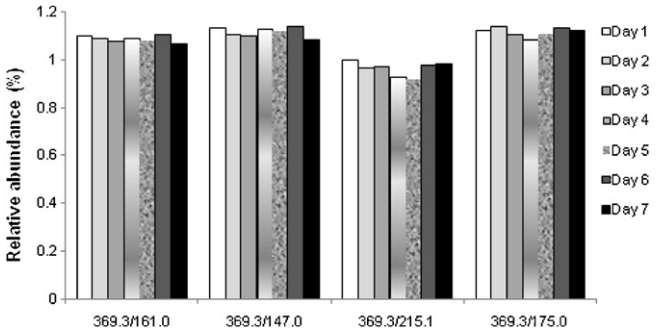

Fig. 4. Inter-day repeatability for cholesterol analysis using different MRM transitions. The relative standard deviation ($n = 7$) for 369.3/161, 369.3/147, 369.3/175 and 369.3/215.1 are 1.21%, 1.48%, 1.24% and 2.38%, respectively.

Using d6-cholesterol as an internal standard, the relative standard deviation for the ratio of the signal from cholesterol (MRM transition at *m/z* 369.3 → 161) versus that of the standard (375.3 → 161) was 0.48% ($n = 6$), which indicated high reproducibility of this mode of analysis. In addition, simple linear regression based standard curve showed linearity with r^2 equaling 0.98 for levels of cholesterol ranged from 0.05 to 100 μg/mL (Fig. 5A). The linearity range of the APCI/MS/MS detector for free cholesterol was estimated to be at least three orders of magnitude. Similar results were obtained for cholesterol esters and selected oxysterols (Fig. 4B and C). The accuracies of the known amounts of sterols ranged from 87% to 104% (Table 1). The LOD for cholesterol was approximately 15 ng/mL, and the LOQ was estimated to be 50 ng/mL. Cholesterol-26,26,26,27,27,27-d6-stearate (MRM 375.3 → 161) was a more appropriate internal standard for quantification of total cholesteryl esters than cholesterol-2,2,3,4,4,6-d6-stearate. When presented as cholesterol equivalents, the approximate response factors for C14, C16, and C18 cholesteryl esters facilitated rapid analysis of total cholesterol. When cholesterol-2,2,3,4,4,6-d6-stearate was used as an internal standard for quantification of cholesteryl esters, an MRM transition of 375.3 → 167 was used but with a different response factor (approximately one-third of 369.4 → 161) (Fig. 5B). Using other prominent fragment ions at *m/z* 161, 147, 175 and 215 as daughter ions for MRM transition was investigated, and the results demonstrated excellent inter-day reproducibility with relative standard deviations of 0.99%, 1.48, 1.24, 2.38, respectively (Fig. 4). While MRM transitions of 369.4 to 161, 147 and 175 gave a dynamic linearity range of approximate three orders of magnitude, MRM of 369.4 → 215.1 showed narrowed linearity range for cholesterol analysis (Supplementary Fig. 3A–D). The two MRM transitions using daughter ions at C are most intensive fragment ions including A + B rings of cholesterol, gave approximate results for cholesterol analysis, in terms of reproducibility and dynamic linearity range. Similar result was obtained when both ions at *m/z* 161 and 147 are used (Supplementary Fig. 3F).

24-Hydroxycholesterol (MRM 367.3 → 159) using 24-hydroxycholesterol-25,26,26,26,27,27,27-d7 (MRM 374.3 → 159) as the internal standard, i.e., with a linearity range of three orders of magnitude, the LOD was lower than 5 ng/mL, and the LOQ was about 15 ng/mL (Fig. 5C). 7-Ketocholesterol (MRM 401.3 → 159) and 7β-hydroxycholesterol (MRM 385.3 → 159) also showed similar results using 7-ketocholesterol-25,26,26,26,27,27,27-d7 (MRM 408.3 → 159) and 7β-hydroxycholesterol-25,26,26,26,27,27,27-d7 (MRM 392.3 → 159) as internal standards, respectively (data not shown).

In order to validate our approach, we compared the total concentration of cholesterol in human plasma using commer-

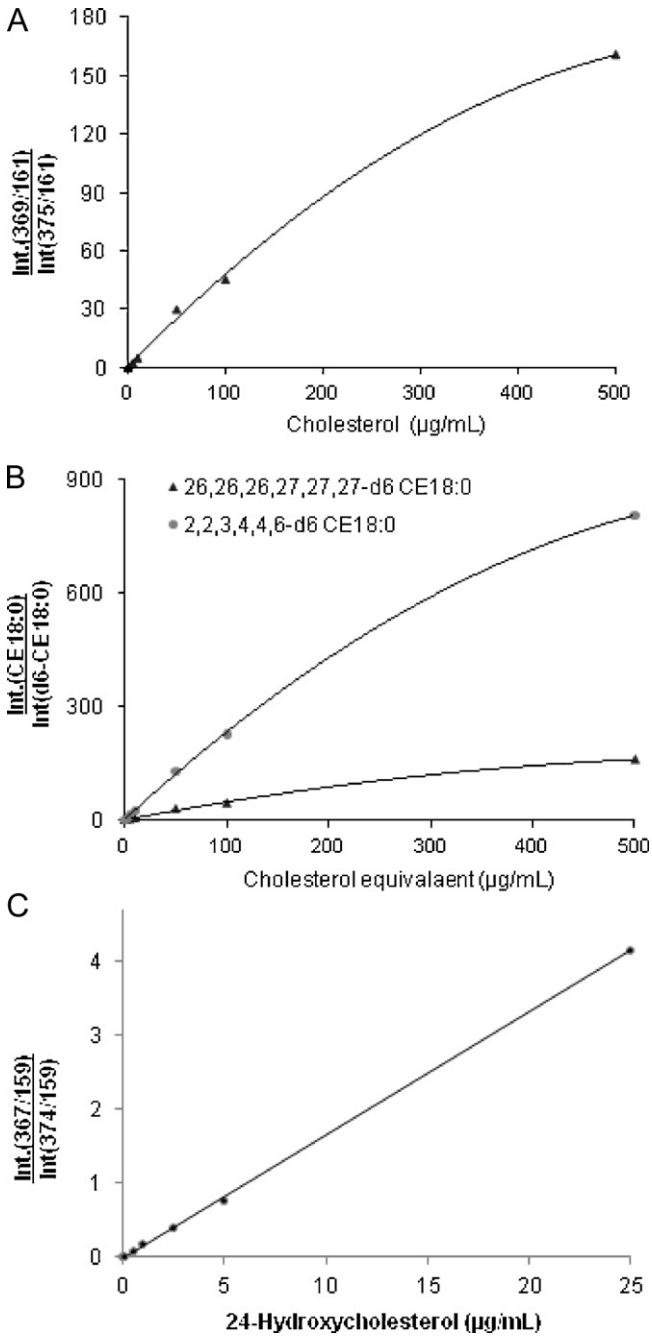

Fig. 5. Validation of MRM quantification of sterols. (A) Investigation of the linear range for detection of cholesterol using 26,26,26,27,27,27-d6 cholesterol as the standard (r^2 = 0.98 for levels of cholesterol ranged from 0.05 to 100 μg/mL). (B) Investigation of the linear range for detection of cholesterol (regression ranged from 0.05 to 100 μg/mL) using 26,26,26,27,27,27-d6 cholesterol CE18:0 (r^2 = 0.99) and 2,2,3,4,4,6-d6 CE18:0 (r^2 = 0.98) as internal standards, respectively. (C) Investigation of the linear range for detection of 24-hydroxycholesterol using 25,26,26,26,27,27,27-d7 24-hydroxycholesterol as an internal standard (r^2 = 0.98).

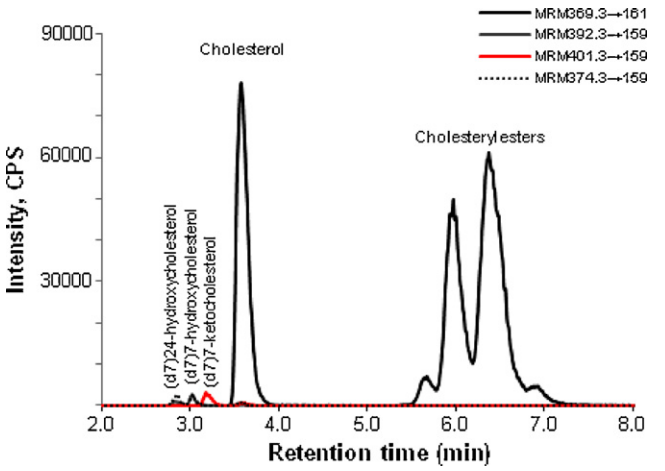

Fig. 6. A representative HPLC-MRM chromatogram of sterols in crude lipid extracts from rabbits fed a normal diet. MRM 369.3 → 161 for free cholesterol (retention time 3.6 min) and cholesteryl esters (5.5–8 min). Other oxysterols eluted between 2.6 and 3.4 min.

cially available enzymatic assay kits. Our LC–MS method yielded a total cholesterol concentration of 4.0 mM or 1.55 ± 0.11 mg/mL (0.59 ± 0.05 mg/mL for free cholesterol and 0.96 ± 0.06 mg/mL cholesterol equivalent for cholesterol esters), which was in good agreement with the results (1.59 ± 0.12 mg/mL) obtained from the commercial kit (Table 2).

3.2. Analysis of plasma sterols in HCD-fed rabbits

This simple MRM-based LC–MS method was used to analyze sterol levels in plasma samples from rabbits fed an HCD, which is known to induce atherosclerosis [33]. Crude lipid extracts from plasma samples were subjected to LC-MRM analysis without additional sample processing. The major sterols (free cholesterol and cholesteryl esters) showed a good separation from the oxysterols (Fig. 6).

Free cholesterol levels and cholesteryl ester levels in the plasma of rabbits fed a normal diet for 10 weeks were found to be 0.13 and 0.06 mg/mL, respectively (Fig. 7A and B). These values significantly increased to 1.41 and 1.57 mg/mL in HCD-fed rabbits. The method also allowed analysis of minor sterols, such as oxidized cholesterols, in the same experimental run. The level of 7-hydroxycholesterol and 7-ketocholesterol, the main oxysterols in control rabbit plasma, was 0.12 and 0.08 μg/mL, respectively (Fig. 7C and D). In plasma from HCD-fed rabbits, 7-hydroxycholesterol and 7-ketocholesterol levels were elevated (0.82 and 0.34 μg/mL, respectively) as compared to the control (Fig. 7C and D). Oxysterols, 7-hydroxycholesterol, 7-ketocholesterol and minor species such as 24-hydroxycholesterol (Supplementary Fig. 4), were thus significantly increased in HCD-fed rabbits.

3.3. Direct analysis of ergosterol and its esters in crude lipid extract from *S. cerevisiae tgl1*Δ *and yeh2*Δ *cells*

We further validated our LC-MRM approach using *S. cerevisiae deletion mutants*, *tgl1*Δ and *yeh2*Δ cells which encode the steryl

Table 2
Total cholesterol levels in human plasma using an enzymatic assay kit and our new LC–MS method (n = 10).

Total cholesterol level from the enzymatic assay kit (mg/mL)	LC–MS (mg/mL, cholesterol equivalent)		
	Free cholesterol	Cholesteryl esters	Total cholesterol level
1.59 ± 0.15	0.59 ± 0.07	0.96 ± 0.08	1.55 ± 0.13

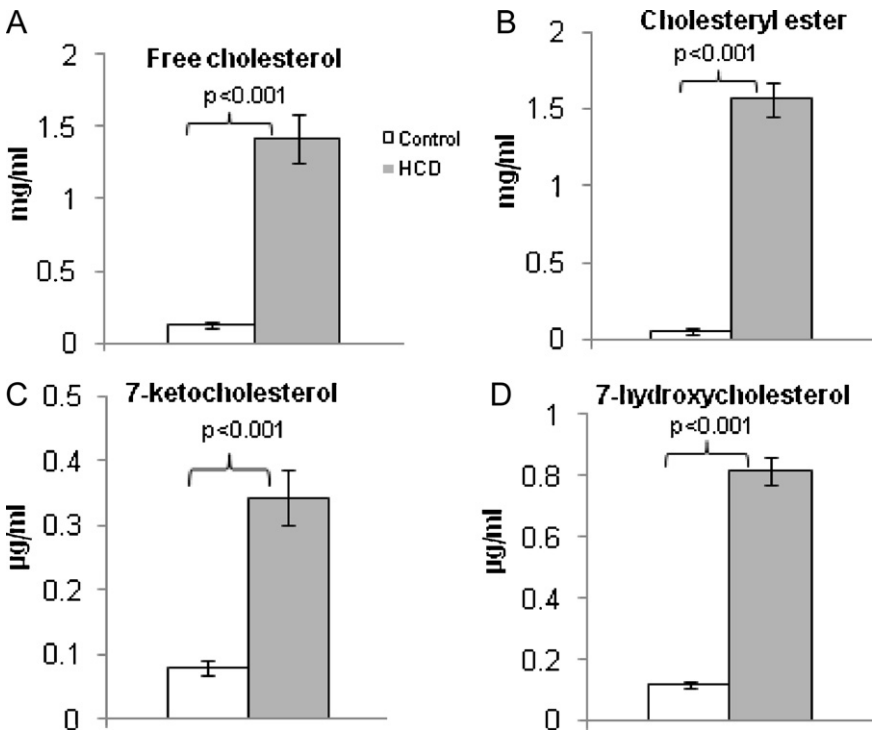

Fig. 7. Comparative analysis of sterols in plasma from the three groups of rabbits: control ($n = 5$), HCD-fed ($n = 5$). (A) Free cholesterol; (B) total cholesteryl esters; (C) 7-ketocholesterol; (D) 7-hydroxycholesterol.

ester hydrolase. These mutants have been previously reported to have an accumulation of sterol esters [36]. Relative content of sterols were obtained using free cholesterol and C18-cholesteryl esters as internal standards. Similar to the fragmentation pattern of cholesterol in tandem mass spectrometry, MRM transition of 379.4 → 179 was set up for their quantification (Fig. 8A). Levels of ergosterol esters are found to be dramatically increased in both mutants consistent with the previously reported findings (Fig. 8B) in *S. cerevisiae* [36].

4. Discussion

The present method forgoes the derivatization step, by using the specific transition of 369.4 → 161 for rapid, sensitive, and selective quantification of total cholesterol levels [32]. In combination with upfront chromatography, this approach is easily extended for quantitative analysis of free cholesterol, total cholesterol esters and some oxysterols. MRM analysis of sterols demonstrated excellent signal-to-noise ratios (Fig. 3), which allows direct analysis of minor sterols in crude lipid extracts without sample pretreatment such as solid-phase extraction or chemical derivatization. The advantages of the current approach include its simplicity, sensitivity and more importantly specificity. To our knowledge this is the first report which directly analyzes different types of sterols including some oxysterols from a crude lipid extract in a single LC–MS run. While other derivatization-based approaches are more sensitive, in particular for minor oxysterol analysis [19,21,23,31], our current approach is more robust for major sterol analysis including sterols and total sterol esters.

Deuterated sterols were selected as internal standards and were crucial for accurate quantitation. For free cholesterol and cholesterol esters, d6-cholesterol and d6-cholesteryl stearate were important internal standards, respectively, as they gave almost identical MS/MS patterns as cholesterol (Fig. 3). Similarly, d7-7-ketocholesterol and d7-7β-hydroxycholesterol are excellent internal standards for the corresponding endogenous molecules.

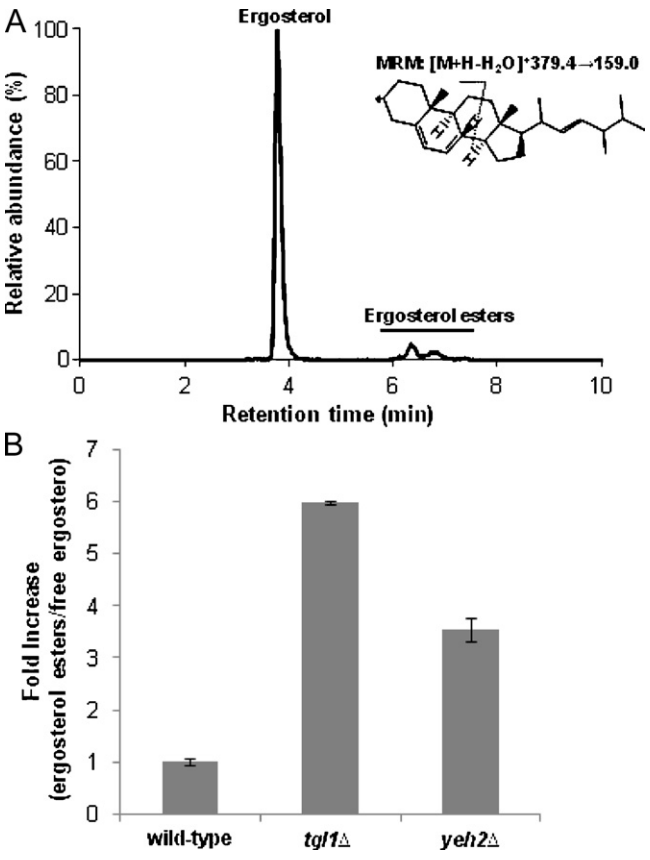

Fig. 8. LC-MRM analysis of ergosterol and ergoesterol esters in crude lipid extracts from yeast *Saccharomyces cerevisiae*. (A) A typical LC-MRM (379.4 → 159.0) elution profile. The insert shows the corresponding fragmentation pattern for selected MRM transition at 379.4 → 159.0. (B) Increased ergosteryl esters in *yeh2*Δ and *tgl1* Δ cells were presented as increased folds for ratio of ergosteryl esters to free ergosterol. Values are mean ± SE.

However, if corresponding deuterated standards are not available, another deuterated sterol standard(s) with a similar MRM transition and close elution time may be used for preliminary comparison. For instance, we found that 5,6-epoxycholesterol was dramatically increased in HCD-fed rabbits using neighboring 7β-hydroxycholesterol-d7 as the internal standard (data not shown). With the use of an appropriate internal standard, 27-hydroxycholesterol can be measured using its specific MRM transition of 385.4 → 109.1 (Supplementary Fig. 5).

The purpose of this study was not to provide an all-encompassing solution for analysis of all sterols but to introduce specific MRM transitions for direct analysis of non-derivatized sterols. Improved chromatographic separation makes analysis of additional oxysterol isomers possible. For instance, two oxysterol isomers, 24-hydroxycholesterol and 27-hydroxycholesterol, which cannot be separated using the above-described isocratic LC elution, could be selectively detected using gradient LC separation combined with sensitive MRM transitions (Supplementary Fig. 5). Indeed, 27-hydroxycholesterol could be measured using its specific MRM transition 385.4 → 109.1 under optimized experimental conditions, whereas 24-hydroxycholesterol has a negligible contribution to this specific MRM transition (Supplementary Fig. 5).

Hypercholesterolemia is a major risk factor for atherosclerosis, coronary heart disease and hepatic fibrosis. It is well established that cholesterol and oxidized lipids, including cholesterol oxidation products, accumulate during formation of atherosclerotic lesions [37,38]. We previously reported that rabbits developed atherosclerosis after challenge with an HCD for 8 weeks [33,39] and that the pathology was accompanied by dramatic increases in plasma sterols. Levels of plasma cholesterol or cholesteryl esters in rabbits fed with a normal diet were much lower than those in human plasma (Fig. 7A and B, Table 2). Elevated levels of 7-hydroxycholesterol and 7-ketocholesterol, the main predictors of progression to carotid atherosclerosis, were consistent with levels reported in other studies [15,16].

While the main point of this study was to present a robust method for analysis of major sterols and cholesteryl esters as well as minor levels of some cholesterol oxidation products in a single run, an isocratic LC elution condition was selected to avoid fluctuations in retention times and ionization that may be encountered during gradient LC elution. Of note, some oxysterol isomers may have been coeluted. For instance, both 24- and 27-hydroxycholesterol have the same retention times. To obtain accurate data regarding these isomers, gradient LC elution programs [22,40], in combination with newly selected MRM transitions, will provide more specific and accurate quantification. Separation of 7α- and 7β-hydroxycholesterol is still challenging using liquid chromatography hence the current approach could only be used to analyze the level of total 7-hydroxycholesterol.

The LC–MS approach also demonstrated its applications in various studies related to aberrant cholesterol biosynthesis, thus providing an insight look at levels of various intermediates during cholesterol biosynthesis, such as squalene, zymosterol, etc. (data not shown and Supplementary Fig. 6). For instance, quantitation of zymosterol and 14-demethyl-lanosterol could be achieved using d5-zymosterol and d6-demethyl-lanosterol as internal standards (Blanc et al., Plos Biol., in revision). When applied to yeast *S. cerevisiae*, we found dramatically elevated ergosteryl esters in *tgl1*Δ and *yeh2*Δ cells, which is consistent with previous results [41] and description of *TGL1* and *YEH2*, which have been reported to encode the steryl ester hydrolase in *S. cerevisiae* [36].

5. Conclusion

In summary, we have developed a simple (derivatization-independent) and sensitive LC-MRM assay for rapid analysis of free cholesterol (ergosterol, sitosterol) and cholesterol esters (other sterol esters) in a crude lipid extract from different types of biological samples. This method may also serve as a tool to qualitatively or quantitatively monitor sterol intermediates and oxysterol metabolites, such as zymosterol, 7-dehydrocholesterol, 7-ketocholesterol, 7-hydroxycholesterol, 4-cholesten-3-one and side-chain hydroxylcholesterols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.011.

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