# Direct analysis of sterols from dried plasma/blood spots by an atmospheric pressure thermal desorption chemical ionization mass spectrometry (APTDCI-MS) method for a rapid screening of Smith–Lemli–Opitz syndrome†

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Here is proposed a rapid and sensitive method involving atmospheric pressure thermal desorption chemical ionization mass spectrometry (APTDCI-MS) for specific laboratory screening of the Smith–Lemli–Opitz syndrome (SLOS), an inherited defect of cholesterol biosynthesis. Biochemical findings in the blood of SLOS patients are low cholesterol (Chol), high 7- and 8-dehydrocholesterol (DHCs) levels and high DHCs/Chol ratios. The APTDCI proposed method is able to ionize sterols for qualitative and quantitative analysis directly from dried plasma/blood spots. Critical APTDCI parameters – desolvation gas flow and temperature – were optimized analyzing Chol, 7-DHC and cholesteryl stearate standards spotted onto a glass slide acquiring the full scan spectra in positive ion mode. Chol levels in dried plasma spots of unaffected controls ($n = 23$) obtained by the proposed method were compared with those of the enzymatic method ($y = 0.9166x + 0.3811$; $r = 0.8831$) while Chol and DHCs of SLOS patients ($n = 9$) were compared with the gas chromatography flame ionization detection (GC-FID) method ($y = 0.8214x + 0.7388$; $r = 0.8288$). The APTDCI-MS method is also able to differentiate normal from SLOS samples directly analyzing whole blood and washed red cells spotted on paper. In conclusion, the intrinsic analytical high-throughput of APTDCI-MS method for sterol analysis could be useful to screen SLO syndrome.

## Introduction

Smith–Lemli–Opitz Syndrome (SLOS; OMIM 270400) is an autosomal recessive multiple malformation syndrome with a very broad phenotypic spectrum. Infants affected by the severe form typically die in the perinatal period while the milder variant causes distinct behavioural and learning problems, growth failure and intellectual disability.[1–3] The discovery of the deficiency of sterol delta7-reductase as a causative factor of SLOS made this syndrome the first true metabolic syndrome of multiple congenital malformations.[4] Several mutations in the human gene sterol delta7-reductase (*DHCR7*) are implicated in the syndrome causing the deficiency of the enzyme activity (DHCR7; EC 1.3.1.21) which catalyzes the last step of cholesterol biosynthesis,[5–8] resulting in low plasma cholesterol and increased 7- and 8-dehydrocholesterol (7-DHC, 8-DHC) levels. The worldwide incidence of SLOS is variable and difficult to estimate accurately: it ranges from 1/20 000 to 1/70 000 and is more common in Northern European individuals.[9] Analysis by GC-MS of cholesterol and DHCs levels in blood, tissues or cells is the reference method for the diagnosis of SLOS;[4] also the prenatal diagnosis can be made by measuring the sterols in amniotic fluid or chorionic villus cells.[10] Analysis of sterols by GC/MS also allows the identification of the rare SLOS-like syndromes such as lathosterolosis and desmosterolosis.[11,12] On the other hand, GC-MS is time-consuming because of the long time of both sample clean up and chromatographic analysis, so several attempts to use other MS techniques have been done. To date, a fast screening method for the diagnosis of these syndromes is lacking.

Recently, different ambient desorption ionization (ADI) techniques have been introduced to analyze many compounds by direct sampling/ionization of the analytes from raw samples providing a stimulating concept of MS analysis without sample preparation which could lead to the development of high-throughput analysis.[13] ADI techniques are mainly based on two fundamental principles described by Cooks *et al.* and by Cody *et al.* named desorption electrospray ionization (DESI)[14,15] and direct analysis in real time (DART),[16] respectively. ADI has been used to perform analysis in different fields for biological,[17–20] forensic[21,22] and pharmaceutical applications.[23–25] Nevertheless, the quantitative performance of ADI techniques has not yet been sufficiently investigated, even if Ifa *et al.* demonstrate that DESI-MS systems can provide good quantitative response when an internal standard is used.[26] Moreover, compounds with a lower proton affinity, such as sterols, are difficult to ionize with the ambient ionization and only few ADI methods have been reported for the analysis of these compounds.

Recently, Wu *et al.* successfully developed a reactive-DESI method for the analysis of free cholesterol in human serum,[27]

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while McEwen and Gutteridge developed a technique named atmospheric solids analysis probe (ASAP)[28] which has been employed for the desorption/ionization of a variety of sterols from liquids, solids and biological tissues. In this study a technique based on atmospheric pressure thermal desorption chemical ionization (APTDCI), previously described by us,[29] has been employed to analyze sterols. In both ASAP and APTDCI methods the heated nitrogen desolvation gas, from either an ESI or APCI probe, has been employed as vaporization source. The ionization of the thermally induced vapors occurs by corona discharge under standard APCI conditions. In the APTDCI technique the solid sample is positioned on a sample holder near the ion source region and analyzed in its native form, while in ASAP the sample must be introduced into a capillary melting point tube before the analysis. Moreover, APTDCI does not need source modification because the sample holder can be easily inserted into the ionization chamber.

In this work the sterols are desorbed and ionized directly from dried plasma/blood spotted on paper for neonatal screening using APTDCI and quantitatively analyzed by MS. The developed APTDCI-MS method is able to differentiate unaffected subjects from SLOS patients. The dried spot samples offer significant advantages such as simple sample collection, less invasive nature and no risks associated with the use and disposal of needles and syringes.[30] Besides, dried sample spots, adequately stored, can be long-term archived.[31,32]

## Experimental

### Materials and reagents

The analytical solvents of HPLC grade, methanol, n-hexane, acetone, ethanol, 2-propanol and chloroform, were obtained from J.T.Baker (Deventer, Netherlands). NaCl 0.9% was from Bieffe Medital (Grosotto, Sondrio, Italy). KOH was purchased from Merck (Merck KGaA, Darmstadt, Germany). 5α-Cholestane, cholesterol (Chol), 7-dehydrocholesterol (7-DHC), lathosterol, sitosterol and cholesteryl stearate (C18:0 CE) standards were purchased from Sigma Aldrich Ltd. (St. Louis, MO, USA). The labeled internal standard (IS), cholesterol-26,26,26,27,27,27-$d_6$ ($d_6$-Chol; 97–98%), was purchased from C/D/N Isotopes Inc. (Quebec, Canada).

Chol, 7-DHC, sitosterol, C18:0 CE and $d_6$-Chol standards were prepared as stock solutions of 1 g/L in ethanol–2-propanol (7:3) and stored at −20 °C until the analysis by APTDCI-MS. A working solution for MS tuning containing not labeled standards (Chol, 7-DHC and C18:0 CE) was prepared in chloroform.

For gas chromatography/flame ionization detector (GC/FID) analysis, 5α-cholestane (IS), Chol, 7-DHC, lathosterol and sitosterol standards were dissolved in a chloroform–methanol mixture (2:1, v/v) to a final concentration of 1 g/L and stored at −20 °C.

### Samples and patients

Plasma samples ($n = 23$) from apparently healthy subjects and with cholesterol ranging from 100 to 300 mg/dL were anonymously selected from those routinely analyzed in the Clinical Biochemistry Laboratory of Foggia Hospital. Samples from SLOS patients, plasma ($n = 9$) and whole blood ($n = 2$), were selected from those collected at the diagnostic Laboratory of Department of Biochemistry and Medical Biotechnologies of Naples. A few drops of whole blood were directly spotted onto paper, then plasma and blood red cells were obtained following standard separation procedures; after centrifugation the plasma was recovered and used for the preparation of the spots. In order to remove the excess of plasma, blood red cells were washed three times with NaCl solution (0.9%) and spotted onto paper. All spots were dried and stored protected from light at 4 °C until the analysis by APTDCI-MS for the qualitative evaluation. All plasma samples were stored at −20 °C.

For quantitative analysis, $d_6$-Chol was used as internal standard. The calibration curves were prepared following two different procedures to add the internal standard to the samples. Calibrators containing Chol and 7-DHC standards were prepared at the following levels: 0, 0.90, 2.3, 3.2 and 5.2 mM for Chol; 0, 0.21, 0.42, 0.83 and 1.66 mM for 7-DHC. These calibrators were divided into two groups (A and B) and 5 μL of internal standard (1.02 mM) were added only to group A. Both groups were dried using a sample concentrator dry-block (DB-3D, Techne, Cambridge, UK), then resuspended by adding 50 μL of plasma and mixed vigorously. 25 μL from each calibration point of group A and 25 μL from each calibration point of group B were spotted onto paper separately and allowed to dry. The blank was prepared by spotting NaCl 0.9% containing the same amount of internal standard for the group A and without internal standard for the group B. Disks of 6 mm (∅) punched from dried plasma spots of group A were directly analyzed by APTDCI-MS, while 5 μL of internal standard (1.02 mM) were added to the disks punched from dried plasma spots of group B before analysis by APTDCI-MS. Each point of both calibration curves was prepared in triplicate. The dried plasma spots from group B were employed for the quantitative study.

### Analytical procedure

**APTDCI-MS.** A triple quadrupole mass spectrometer (Micromass QuattroMicro, Waters-USA) was equipped with a home-made APTDCI device previously described.[29] The geometry of the system was evaluated using standard solutions spotted onto glass slides (Chol 52 pmol, 7-DHC 52 pmol and C18:0 CE 31 pmol). The sprayer–surface distance and MS inlet–sample distance were set at about 14 mm and 1 mm, respectively. The collection angle between the MS inlet and sample was of 20° and the incidence angle between the sprayer and sample was of 82°. The spectra were acquired in positive ion mode scanning a mass range from 350 to 450 *m*/*z*, the scan time and interscan delay were of 1 s and 0.1 s, respectively. The cone voltage, the source temperature, the desolvation gas flow, the desolvation gas temperature and the nebulizer gas pressure were set at 20 V, 150 °C, 450 L/h, 500 °C and 80 psi, respectively. During the APTDCI process, the analytes were desorbed by heated nitrogen gas flow and the ionization was induced by a corona discharge (2 μA). No solvent was sprayed during the APTDCI experiments.

**GC/FID analysis.** A gas chromatograph (HP-5890; Agilent Laboratories, CA, USA) equipped with a SAC-5 capillary

 

column (30 m length, 0.25 mm I.D., 0.25 μm film thickness; Supelco, Germany) was used to separate the sterols using nitrogen as a carrier gas with a column flow rate of 25 cm/s and a total flow rate of 20 mL/min. The head column gas pressure was 19.5 psi. The total run time was 25 min using a constant oven temperature of 280 °C and a constant injector and detector temperature of 300 °C. The retention times ($t_R$) of chromatographic peaks obtained from plasma samples were compared with those obtained from standard compounds. The relative retention time ($Rt_R$) of each peak, compared to 5α-cholestane, was 1.55 for cholesterol, 1.58 for 8-DHC, 1.67 for 7-DHC, 1.72 for lathosterol and 2.23 for sitosterol. Sterol concentrations were obtained by interpolating the peak area ratios (analyte/IS) on the calibration curves. A plasma pool with a cholesterol concentration of 142 mg/dL was spiked with 7-DHC, lathosterol and sitosterol standards at a final concentration of 50 mg/dL, respectively, and used for the calibration curves, the solution was diluted with NaCl (0.9%) and spotted onto paper to prepare different calibration points. The analysis of total sterols from calibrators and patient samples was performed using sample spots of 6 mm (∅) and 40 μg of internal standard (5α-cholestane); then 3 mL of 1 N KOH in 90% ethanol were added and the mixture was incubated for 60 min at 80 °C. After the hydrolysis, the samples were mixed with 3 mL of distilled water and extracted three times with 3 mL of hexane. The upper organic phases were pooled and evaporated under a gentle stream of nitrogen at 40 °C. The dry residue was dissolved with 100 μL of dichloromethane; 1 μL of the solution was injected into the GC/FID.

8-DHC concentrations of SLOS patients were calculated using the calibration curve of 7-DHC because the 8-DHC standard is not commercially available.[32]

**Enzymatic method.** Total cholesterol of plasma samples from healthy subjects was analyzed by the enzymatic method based on cholesterol esterase/cholesterol oxidase (ILAB-600; Instrumentation Laboratory, MA, USA).

**Statistical analysis.** The Pearson correlation coefficient and simple regression analysis were used to correlate the results of the proposed method with the enzymatic and the GC/FID methods.

## Results and discussion

The aim of this work is to demonstrate that the APTDCI-MS method offers a simple tool to screen quickly the Smith–Lemli–Opitz syndrome, analyzing directly a dried sample spot without sample preparation. The diagnostic metabolites of SLOS – Chol, 7- and 8-DHC – are difficult to ionize using DESI and here it is demonstrated that the desorption/ionization mechanism of the APTDCI technique is suitable for analysis of the sterols. In Fig. 1 the procedure employed to perform the analysis on dried samples by APTDCI device inserted in the ionization chamber is depicted step by step.

In Fig. 2 are shown the APTDCI-MS spectra obtained from standard solutions of Chol, 7-DHC and C18:0 CE spotted onto the glass slide. During the analysis of Chol the thermal desorption mechanism promotes the formation of cholestadiene, then it is suddenly protonated using corona discharge (Fig. 2a). This

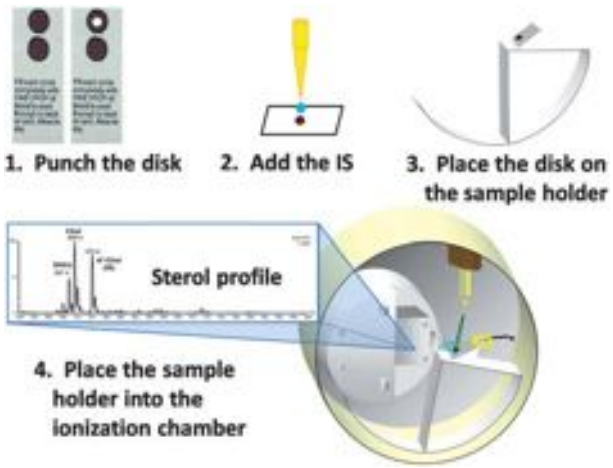


**Fig. 1** APTDCI-MS analysis procedure steps.

mechanism also causes the desorption/ionization of other sterols and of cholesteryl esters. As shown, the APTDCI analysis of both Chol and C18:0 CE produces the same ion at *m*/*z* 369 resulting from the loss of water (Fig. 2a) or from the loss of the fatty acid (Fig. 2c), while the 7-DHC produces the ion at *m*/*z* 367 (Fig. 2b). The monitoring of the cholestadiene ion, produced from both cholesterol and cholesteryl ester, allows the determination of total cholesterol without any pre-analytical step to convert cholesteryl esters into free cholesterol. Also, other free sterols and their esters can be detected as one signal corresponding to the mass of sterol less water or acyl chain (data not shown).

The influence of the temperature on the signal intensity of Chol and 7-DHC was also investigated by maintaining the desolvation gas flow constant at 450 L/h and varying the desolvation gas temperature from 100 to 600 °C. The signal intensity for both Chol and 7-DHC increased with the temperature, achieving the optimum at 500 °C (Fig. 3). APTDCI is similar to the desorption atmospheric pressure chemical ionization technique (DAPCI),[33] but in DAPCI a solvent is sprayed through the capillary nebulizer gas to facilitate the desorption/ionization of compounds. In Fig. 3 is depicted the ionization efficiency of APTDCI compared to DAPCI analysis performed spraying a mixture of water–methanol (1:1) at the flow rate of 10 μL/min and using the same APTDCI parameters set up. The solvent employed in the DAPCI technique does not improve the desorption/ionization response.

### Qualitative analysis

The qualitative response of diagnostic sterols during the APTDCI-MS process was investigated using three different complex matrices – plasma, washed red cells and whole blood – spotted onto paper. As represented in Fig. 4, looking at the full scan spectra, it is possible to distinguish between normal and SLOS. In fact, the SLOS spectra display a reduction of the cholesterol ion intensity (*m*/*z* 369) and an increase of the DHCs ion intensity (*m*/*z* 367). The ion at *m*/*z* 367 is well resolved from that of cholesterol and it represents the sum of 7-DHC and 8-DHC.

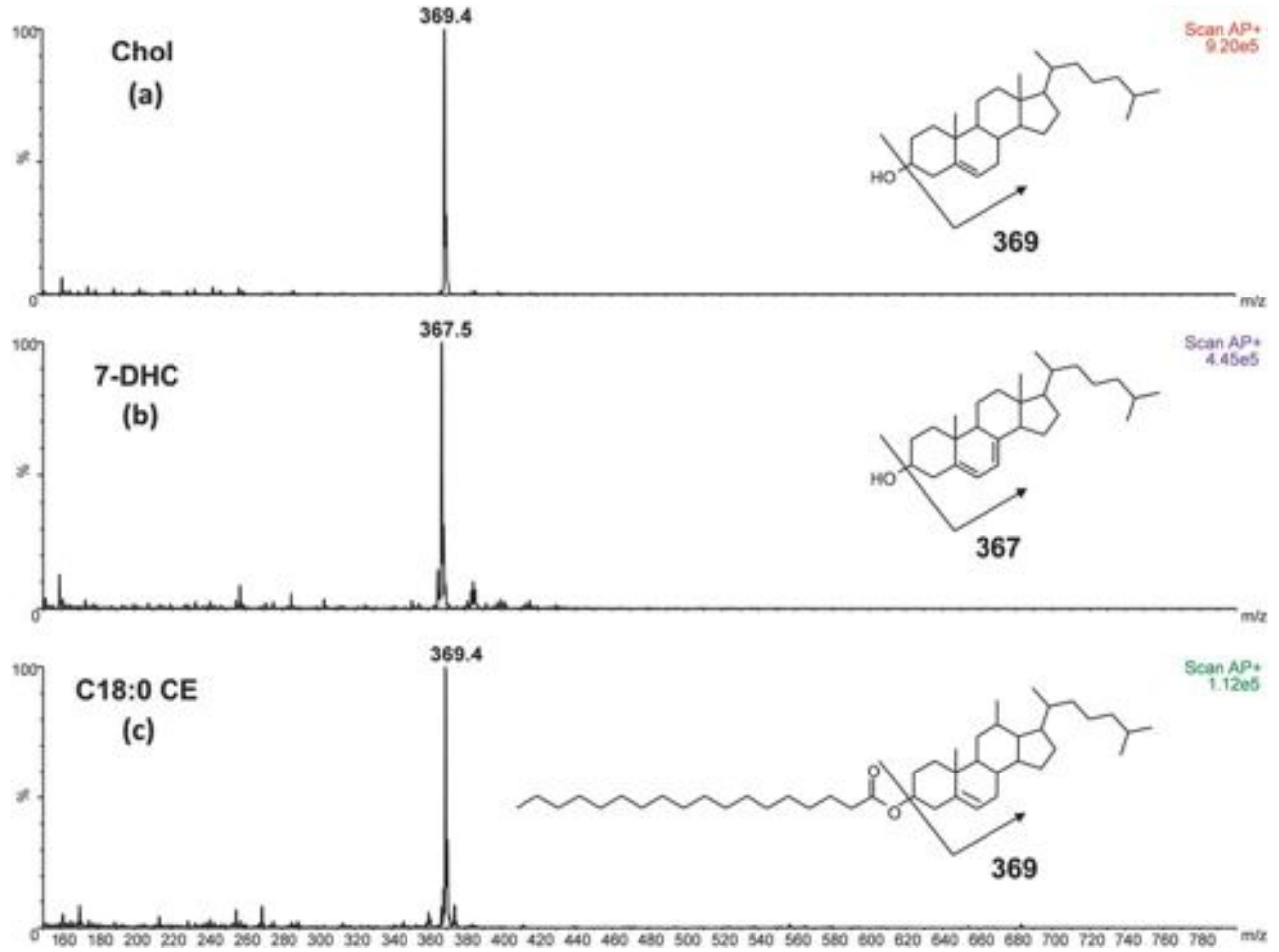


**Fig. 2** APTDCI-MS spectra obtained spotting 2 μL of standard solutions of cholesterol (a), 7-dehydrocholesterol (b) and cholesteryl stearate (c) on glass slides.

The DHCs ion intensity in SLOS samples is about 20% compared to the Chol ion intensity. Instead, in control samples the ion intensity at *m*/*z* 367 is less than 2% compared to Chol. Measuring this signal, which represents the sum of both isomers, the diagnosis of SLOS is not missed, even if caution should be given for blood samples stored on paper for long periods.[32] In addition, lower cholesterol and higher DHCs lead to an increase of the DHCs/Chol ratio which could improve the diagnostic

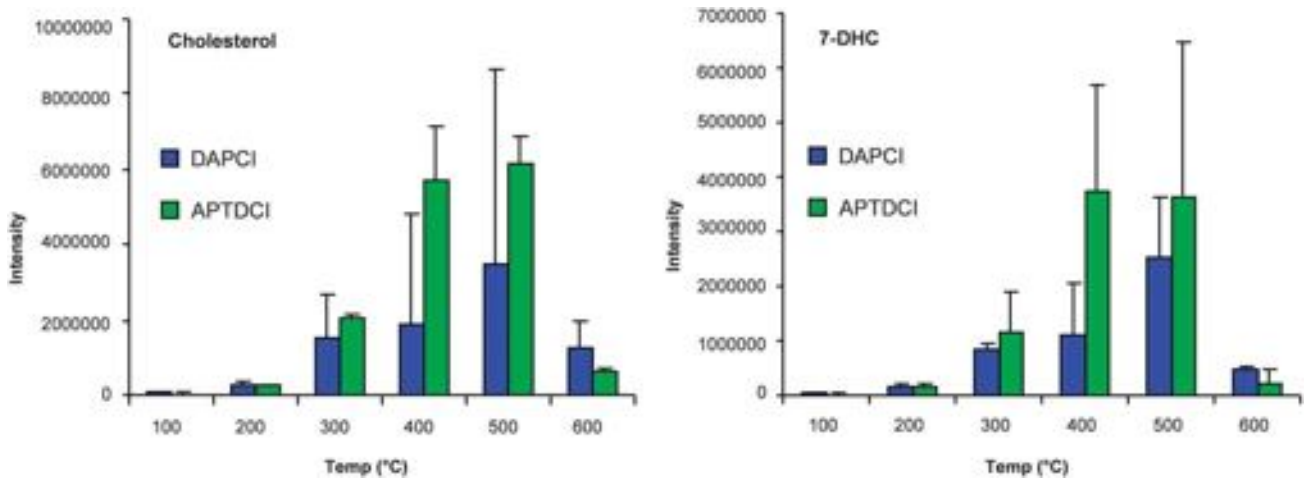


**Fig. 3** Influence of desolvation gas temperature on the signal intensity of cholesterol and 7-dehydrocholesterol during APTDCI-MS and DAPCI-MS experiments (desolvation gas flow is constant at 450 L/h).

 

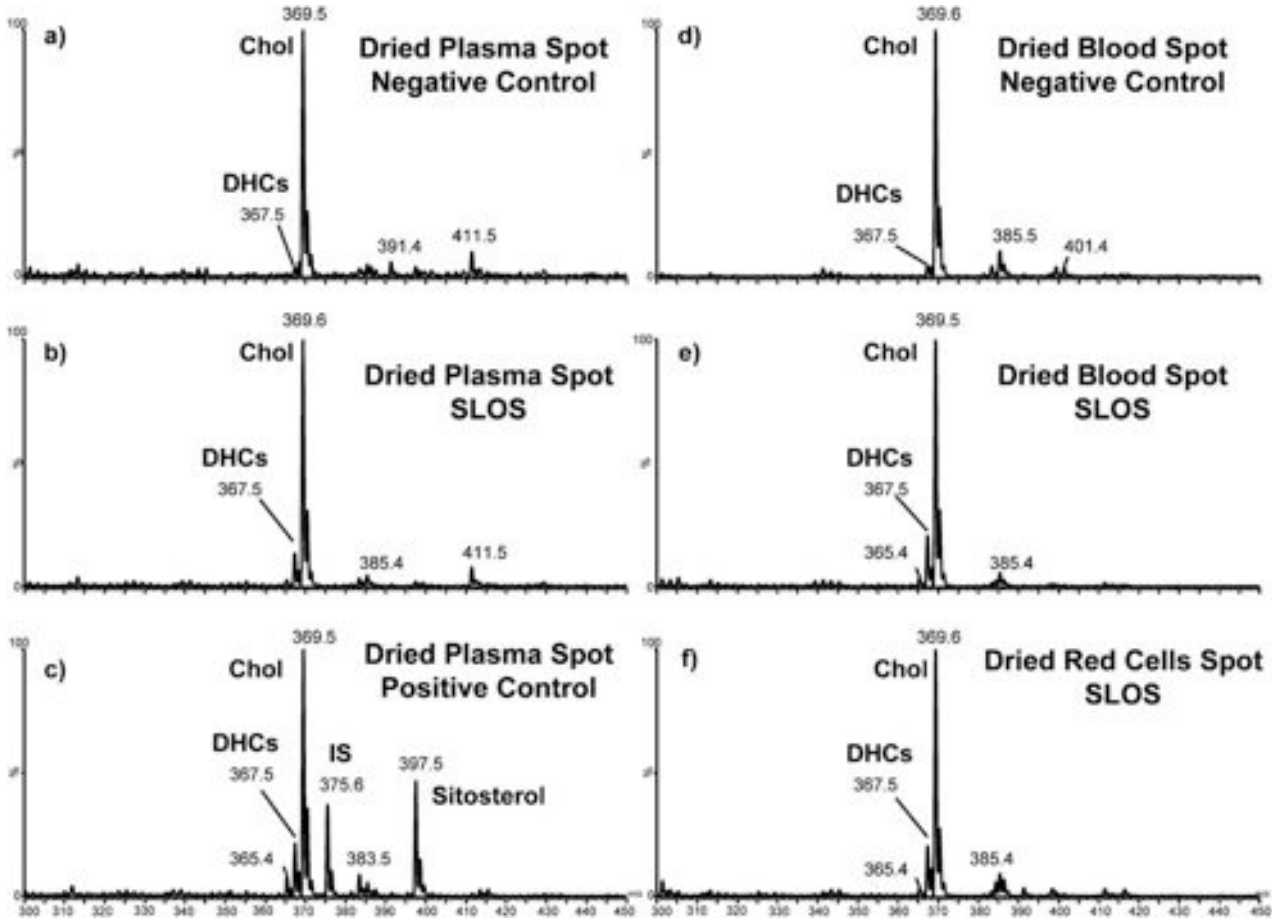


**Fig. 4** APTDCI-MS spectra obtained from dried plasma spots, dried blood spots and dried red cells spots of negative and positive controls and SLOS samples.

sensitivity compared to the measurement of DHCs concentration. Plasma concentrations of cholesterol isobars, such as lathosterol, are very low (less than 0.3% of total cholesterol) and normally do not influence the analysis of Chol significantly.[34] In Fig. 4c is also depicted the full scan spectrum obtained by the APTDCI-MS analysis of a dried plasma spot from a positive control spiked with 7-DHC, internal standard and sitosterol. Sitosterol is also measurable by this method as the ion at *m*/*z* 397 after the loss of water; this compound has been confirmed by tandem MS analysis (data not shown).

A specificity study was conducted to confirm the identity of Chol and DHCs; they were analyzed by a collision induced dissociation study (the mass range was from *m*/*z* 50 to 400; the collision energy was 25 eV); the fragmentation spectra are shown in Fig. 5. The product ion spectra of *m*/*z* 369 and 367 obtained from standards (panels **a** and **c**) compared to the product ion spectra of Chol and of DHCs from a dried spot of a SLOS patient (panels **b** and **d**) are well overlapped.

In Table 1 the DHCs/Chol ratio of two SLOS patients by APTDCI-MS, calculated using the ion intensity, are compared with those obtained by GC/FID. The results of the patient #1 provide similar ratios, while the APTDCI-MS ratios of patient #2 are lower than those of the GC/FID method – this comparison should be improved by analyzing a consistent number of patients to obtain more significant results. The CV% obtained from 3 separate experiments did not exceed 10% for both APTDCI-MS and GC/FID methods.

## Quantitative analysis

**Internal standard.** The use of the internal standard is critical for the quantitative analysis by ambient desorption ionization as reported previously.[27,35,36] In this study the possibility of adding the internal standard to the dried plasma spot has been evaluated by preparing two calibration curves using dried plasma spots containing internal standard (group A) and dried plasma spots without internal standard (group B). The disks obtained from dried plasma spots containing the internal standard (group A) were directly analyzed by APTDCI-MS, while the disks obtained from dried plasma spots without the internal standard (group B) were spiked with 5 μL of internal standard before the analysis. The comparison of both calibration curves, for the range of cholesterol concentrations employed in this study, shows a good linearity providing the following equations: $y = 2.4176x - 0.9749$ and $r = 0.9695$ (group A), $y = 0.6587x + 0.1110$ and $r = 0.9944$ (group B). These data demonstrate that the internal standard can be directly spotted onto the dried spot; in fact, this procedure shows a better calibration response compared to the addition of the internal standard in solution before the preparation of the spots. The internal standard is essential for the quantitative

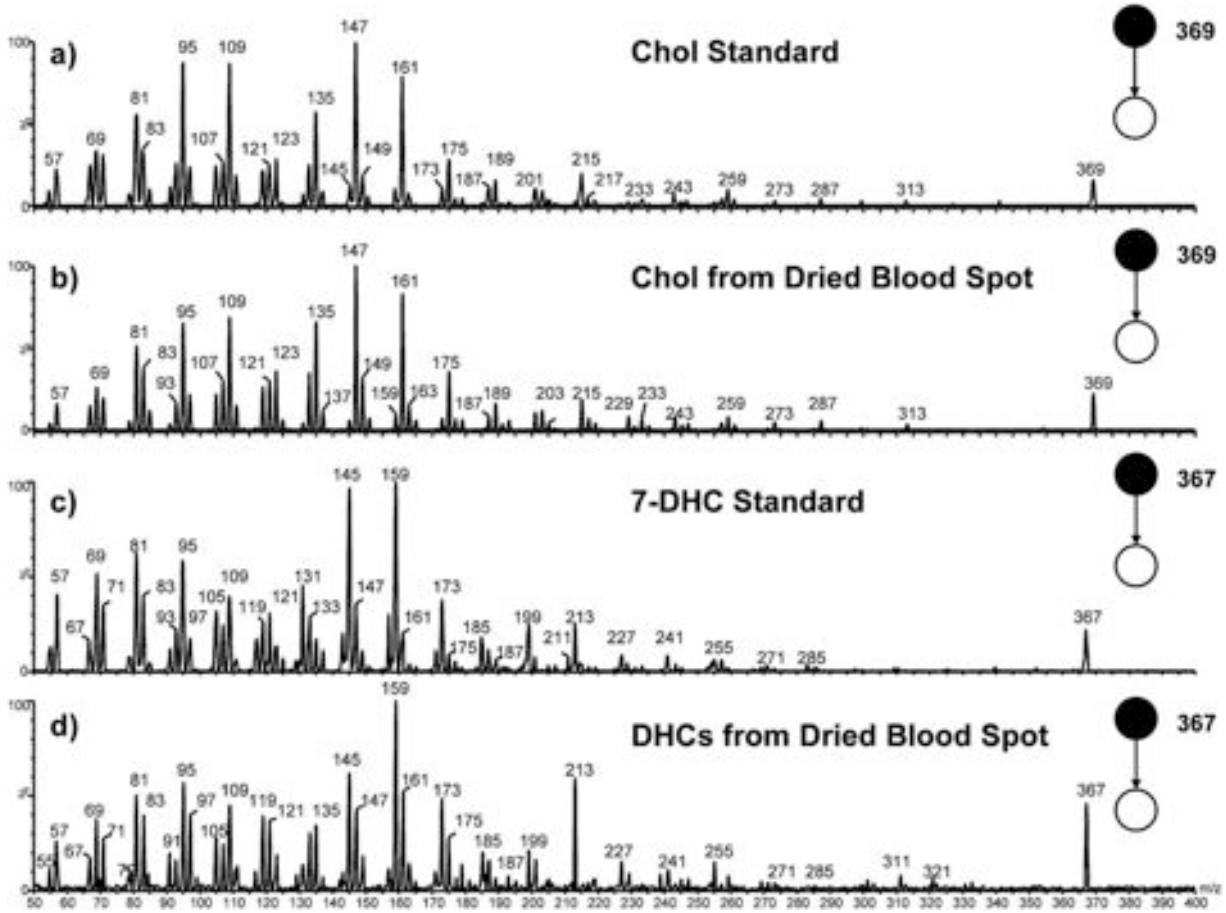


**Fig. 5** APTDCI-MS/MS product ion spectra obtained from standard solutions of cholesterol (a) and 7-DHC (c) and from cholesterol (b) and DHCs (d) of a SLOS dried blood spot.

**Table 1** Comparison of DHCs/Chol ratio obtained from dried blood spots, dried red cells spots and dried plasma spots of two SLOS samples analyzed by APTDCI-MS and GC/FID methods

| | | DHCs/Chol[a] | | | |
|---|---|---|---|---|---|
| | Matrix | APTDCI-MS | CV (%) | GC/FID | CV (%) |
| SLOS sample (#1) | Dried blood spot | 0.22 | 10 | 0.28 | 10 |
| | Dried red cells spot | 0.19 | 8 | 0.27 | 4 |
| | Dried plasma spot | 0.20 | 7 | 0.26 | 10 |
| SLOS sample (#2) | Dried blood spot | 0.31 | 2 | 0.63 | 10 |
| | Dried red cells spot | 0.36 | 10 | 0.72 | 9 |
| | Dried plasma spot | 0.53 | 2 | 0.81 | 5 |

[a] The ratio DHCs/Chol was calculated using the signal intensity ($n = 3$) of the ions obtained from the spectra.

analysis and the possibility of adding it directly to dried spots enables APTDCI-MS as a screening method to analyze dried plasma/blood spots collected at different times and places. The collection of dried blood spots on filter paper is less invasive for the patient and has significant advantages due to the samples' longer lifespan with the reduced need for refrigeration; it is simple to perform, requires minimal training, and the samples are easy to ship. In addition there are economic benefits; in fact, the transport and the laboratory handling of dried spots are considerably cheaper than samples collected by venipuncture.[30]

**Comparison between methods.** The concentrations of Chol and DHCs in dried plasma spot of patients and healthy subjects were calculated using the calibration curves obtained by analyzing calibrators of group B (Chol: $y = 0.6587x + 0.1110$, $r = 0.9944$; 7-DHC: $y = 0.1801x + 0.0895$; $r = 0.9969$).

Cholesterol plasma levels in healthy subjects ($n = 23$) measured by APTDCI-MS were compared with those obtained by the enzymatic method. As depicted in Fig. 6a the comparison between the two methods shows a significant linear correlation ($r = 0.8831$; $y = 0.9166x + 0.3811$) even if the value of the intercept, due to a high baseline noise of the full scan spectra, influences positively the concentrations obtained by APTDCI-MS. Moreover, Chol and DHCs levels obtained by APTDCI-MS from SLOS samples ($n = 9$) were compared with those analyzed by GC/FID (Fig. 6b): although the number of data pairs are few, the correlation is also significant ($r = 0.8288$; $y = 0.8214x + 0.7388$) and the value of intercept is positive too.

The ranges of the DHCs/Chol ratio of both APTDCI-MS and GC/FID methods are shown in Table 2; they were calculated from concentrations (mM) of 32 dried plasma spots (normal $n = 23$; SLOS $n = 9$). The DHCs/Chol ratio by APTDCI-MS ranged from 0.01 to 0.17 for normal samples and from 0.26 to

 

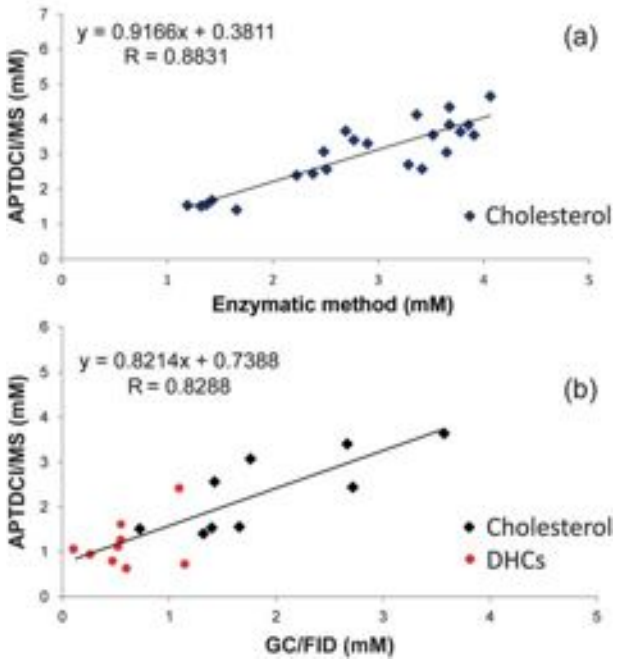


**Fig. 6** Comparison between methods: (a) comparison of cholesterol concentrations from normal plasma samples obtained by APTDCI-MS and by the enzymatic method; (b) comparison of cholesterol and DHCs concentrations from SLOS plasma samples obtained by APTDCI-MS and by GC/FID.

**Table 2** Comparison of DHCs/Chol ratio obtained from 32 dried plasma spots (23 normal and 9 SLOS) analyzed by APTDCI-MS and GC/FID methods

| | DHCs/Chol[a] | |
|---|---|---|
| | Range (APTDCI/MS) | Range (GC/FID) |
| Normal ($n = 23$) | 0.01–0.17 | 0–0.002 |
| SLOS ($n = 9$) | 0.26–1.59 | 0.07–0.71 |

[a] Data referred to a dried plasma spot. The ratio DHCs/Chol was calculated using the concentrations (mM) obtained from calibration curve.

1.59 for SLOS samples (Table 2). These ranges are not overlapped and allow us to distinguish affected patients from normal subjects. Even if the proposed method is able to differentiate normal from SLOS samples, it is evident that the DHCs/Chol ratio obtained by APTDCI-MS is influenced by the baseline noise and the dynamic range of the MS method is narrower than by GC-FID. In this method all APTDCI-MS spectra were acquired using the full scan mode. In particular, the results in normal samples show that the high background influences the concentrations of DHCs more than those of cholesterol; then, the noise observed by APTDCI-MS increases the DHCs/Chol ratios of normal samples which are significantly higher than those of the GC/FID method (Table 2).

Further experiments using a higher number of SLOS patients and by tandem mass spectrometry coupled to the APTDCI technique need to be done to improve the dynamic range and background noise.

## Conclusions

The advantages of the ADI techniques and the ability to ionize more lipophilic compounds, such as cholesterol, make the proposed APTDCI-MS method a useful tool that could be potentially used to screen the SLO syndrome, determining the DHCs and Chol levels and the DHCs/Chol ratios in proband patients. The method is also able to detect sitosterol, a diagnostic marker of sitosterolemia, a rare autosomal recessively inherited disorder of sterol transport characterized by hypercholesterolemia and hypersitosterolemia;[37] moreover, it is potentially able to differentiate other metabolic disorders of sterols.

This is the first report in which sterols are quickly analyzed directly on dried plasma/blood spots, without sample preparation, providing qualitative and quantitative results acceptable for the screening of SLOS in less than 3 min; moreover, the closed ionization chamber guarantees the operator's safety.

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