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Short communication

A routine method for cholesterol and 7-dehydrocholesterol analysis in dried blood spot by GC–FID to diagnose the Smith–Lemli–Opitz syndrome

Monica Gelzo[a,1], Stefano Clericuzio[a,1], Rosalba Barone[a,1], Oceania D'Apolito[b], Antonio Dello Russo[a], Gaetano Corso[a,b,*]

[a] Department of Biochemistry and Medical Biotechnologies, University Federico II of Naples, Naples, Italy
[b] Clinical Biochemistry, Department of Biomedical Sciences, University of Foggia, Foggia, Italy

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ABSTRACT

This work was aimed to implement a fast and simple method to quantify cholesterol (CHOL) and 7-dehydrocholesterol (7-DHC) in dried blood spot (DBS) to diagnose the Smith–Lemli–Opitz syndrome (SLOS), an inborn error of CHOL biosynthesis. We developed and validated a GC–FID method for separation and quantification of underivatized CHOL and 7-DHC using a DBS disc of 6 mm with a run time of 9 min. Correlation coefficients (r) of calibration curves ranged from 0.998 to 0.999 for CHOL and from 0.997 to 0.998 for 7-DHC. Within-day and between-day imprecision (CV%), accuracy (%), carry-over, and extraction efficacy (%) were also evaluated for validation. CHOL and 7-DHC were analyzed in DBS and plasma samples from 8 SLOS patients and 30 unaffected subjects. In SLOS patients, 7-DHC/CHOL ratios in DBS and plasma samples ranged from 0.035 to 1.448 and from 0.012 to 0.926, respectively. Results from calibration curves, quality controls and patient samples reveal that the method is suitable to analyze DBS to screen patients affected by SLOS.

1. Introduction

The Smith–Lemli–Opitz syndrome (SLOS; OMIM 270400) is an autosomal recessive multiple malformation syndrome, due to an inborn error of cholesterol (CHOL) synthesis, with a very broad phenotypic spectrum. Infants affected by the severe form typically die in the perinatal or prenatal period while the milder variant causes distinct behavioral and learning problems, growth failure, and intellectual disability [1–3]. The SLOS is caused by deficiency of sterol delta-7-reductase (DHCR7; EC 1.3.1.21) that catalyzes the last step of CHOL biosynthesis, resulting in low CHOL and increased 7- and 8-dehydrocholesterol (7-DHC, 8-DHC) plasma levels [4–6]. The worldwide incidence of SLOS is variable and difficult to estimate accurately. As recently reported, SLOS incidence ranges from 1:20,000 to 1:70,000 and is more common in Northern European individuals [7]. The carrier rate for the most frequently occurring mutation (IVS8-1G>C) in the gene sterol delta-7-reductase (*DHCR7*) is approximately 1:100 for the Caucasian population in North America (1%) and possibly as high as 1:50 to 1:30 in Central European populations (2–3.3%). Based on the allele frequencies and the proportion of this mutation observed in various patient populations, the expected incidence of SLOS in those populations was calculated and reported to be from 1:1590 to 1:17,000. The discrepancy between the expected incidence and prevalence can be explained only in part by the neonatal and infancy deaths of the most severely affected children with SLOS and the under ascertainment of mild and atypical cases [8].

The diagnosis of SLOS is performed by analyzing CHOL, 7-DHC and 8-DHC in blood, tissues or cells by GC–MS, the reference method [3]. Some methods have been used in the past to analyze sterols from dried blood spots (DBS) by GC–MS [9,10], by TOF-secondary ion mass spectrometry (TOF-SIMS) [11], and by an ambient MS technique [12]. However, to date, a fast screening method to analyze sterols in DBS for the diagnosis of SLOS is lacking. The use of DBS specimens has achieved the same level of precision and reproducibility of standard methods that collect whole blood in vacuum tubes and capillary pipettes [13]; moreover, DBS is simple to perform and can be collected in a variety of environments. It has also the advantage that it can be archived on a long-term basis and used in retrospective diagnosis, thus, it can elucidate unexplained cases of sudden infant death [14]. Due to these advantages, DBS use in clinical and research settings has expanded to include testing for many diseases [15] and monitoring of biomarkers and therapeutic agents [16]. Obviously, the molecules of interest must remain stable when dried and must be released from the paper during the analysis. In particular, some studies have been performed on stability and measurability of lipids in the dried matrix [10,17–20].

* Corresponding author at: Department of Biomedical Sciences, University of Foggia, Via L. Pinto 1, 71122 Foggia, Italy. Tel.: +39 0881 588055; fax: +39 0881 588037.
E-mail address: g.corso@unifg.it (G. Corso).
[1] These authors contributed equally to this work.

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Considering the importance of 7-DHC for diagnostic purpose, this study was aimed to develop and validate a fast GC–FID method to quantify CHOL, 7-DHC, and their ratio in DBS for SLOS detection.

2. Experimental

2.1. Materials and reagents

All solvents of HPLC grade were obtained from J.T. Baker (Deventer, Netherlands). Potassium hydroxide (KOH) was purchased from Merck (Merck KGaA, Darmstadt, Germany). Sodium chloride (NaCl, 0.9%) was from Bieffe Medital (Grosotto, Sondrio, Italy). 5α-Cholestane as internal standard (IS), standards of sterols, and butylated hydroxytoluene (BHT) were purchased from Sigma–Aldrich Ltd. (St. Louis, MO, USA). Stock standard solutions were prepared in chloroform/methanol (2:1) to provide solutions of 0.4 g/L for IS, and 1 g/L for 7-DHC, and stored at −20 °C until use. Whatman 903® filter paper cards (Whatman GmbH, Dassel, Germany), treated with BHT as previously described [20], were used to prepare blood spots.

2.2. Samples, calibrators and quality controls preparation

2.2.1. Samples

Blood samples were collected in EDTA-tubes from 30 unaffected subjects and from 8 SLOS patients; the samples were selected from those already analyzed at the clinical laboratory of Department of Biochemistry and Medical Biotechnologies – University Federico II, Naples. A portion of the whole blood (0.5–1.0 mL) was used to prepare DBS as previously described [20]. Briefly, the DBS were prepared by spotting 20 μL of whole blood onto filter paper cards treated with BHT, the cards were dried for 3 h at room temperature and stored protected from light at 4 °C until the analysis. The remaining portion of the whole blood was centrifuged at 2500 rpm for 10 min to separate plasma and erythrocytes. Plasma samples were stored protected from light at −20 °C until the analysis.

2.2.2. Calibrators

A negative plasma pool was prepared from unaffected subjects, and the CHOL concentration, accurately measured by an enzymatic method, was of 110 mg/dL. A positive plasma pool was prepared by enriching the negative plasma pool with 7-DHC to obtain a final concentration of 40.5 mg/dL. Calibrators were obtained by serial dilutions of the positive plasma pool, using NaCl solution, to the final concentrations ranging from 1.72 to 110 mg/dL for CHOL, and from 0.63 to 40.5 mg/dL for 7-DHC.

2.2.3. DBS quality controls

To prepare DBS for quality controls (QC), erythrocytes were isolated from two unaffected subjects with a blood group 0 Rh negative, washed using NaCl, and mixed in 1:1 ratio with the negative plasma pool and with two different positive plasma pools to obtain whole blood for negative QC (neg-QC) and two levels of positive QC, positive QC-low (pos-QC-L) and positive QC-high (pos-QC-H). DBS for each QC specimen were prepared as above described for patient blood samples. Washed erythrocytes alone (12.4 μL), obtained from unaffected subjects, were analyzed three times to obtain the average concentration of CHOL in erythrocyte membranes. The expected concentrations of CHOL and 7-DHC in the QC blood were calculated as the average between the concentrations in plasma and washed erythrocytes corrected by used volume.

2.3. Sterol extraction and GC–FID analysis

Calibrators and patient plasma were analyzed using 12.4 μL of sample with the addition of IS (10 μg). The sterols in QC and patient dried blood samples were quantified from a 6 mm disc punched from the DBS. The sterol extraction and GC–FID analysis were performed as previously described with slight modifications [20]. Briefly, after alkaline hydrolysis of esters the sample was mixed with distilled water and extracted with hexane (1 mL × 3 times). The upper organic layers were pooled and evaporated under nitrogen flow. The dry residue was reconstituted in 50 μL of dichloromethane, and 1 μL of the resulting solution was injected into GC–FID.

GC–FID (HP-5890; Agilent Laboratories, CA, USA) was equipped with a SAC-5 capillary column (30 m length, 0.25 mm I.D., 0.25 μm film thickness; Supelco, Germany), using N_2 as gas flow (20 mL/min) with a linear velocity of 25 cm/s. The total run time was 9 min using a constant oven temperature of 295 °C, injector and detector temperatures were set at 300 °C.

2.4. Method validation

The method was validated for selectivity, linearity, imprecision, accuracy, extraction efficacy, and carry-over.

The selectivity of the method was evaluated by comparing the DBS extracts obtained from unaffected subject before and after spiking different standards of sterols such as coprostanol, cholestanol, desmosterol, lathosterol, and sitosterol. The samples were extracted and analyzed for potential interfering of other sterols on the relative retention time of 7-DHC and 8-DHC.

Calibration curves were prepared by analyzing the calibrators at seven concentration levels, and in five separate days. The linearity of each calibration curve was evaluated by linear regression analysis, plotting the peak area ratios (y) of analytes to IS versus the nominal concentration (x) of analytes.

The lower limit of quantification (LOQ) is defined as the lowest concentration that provides an area S/N ratio higher than 10, with imprecision (CV%) and accuracy (%) less than 20%.

Imprecision and accuracy of the method were evaluated by analyzing the three QC DBS levels (Neg-QC, pos-QC-L and pos-QC-H). Within-day imprecision and accuracy were calculated analyzing 5 times each level of QC samples in the same analytical run. For evaluation of between-day imprecision and accuracy, each level of QC samples was analyzed once a day for 15 working days during a period of 6 weeks.

To evaluate the sterol extraction method from DBS, SLOS patient samples ($n = 3$) were extracted four times with 1 mL of hexane and each fraction was dried in separate tubes. The extracts were resuspended and analyzed by GC–FID to calculate the percentage of recovery of each extraction step.

Carry-over was evaluated by three consecutive chromatographic runs of pos-QC-H followed by three runs of pure solvent (dichloromethane). The percentage of carry-over was calculated for IS, CHOL and 7-DHC as ratio between the average areas of each sterol obtained from solvent and pos-QC-H.

3. Results and discussion

3.1. Gas chromatography

Fig. 1 shows typical GC–FID chromatograms of underivatized sterols obtained using our method from DBS of an unaffected subject (A), from a SLOS patient (B), and from a pos-QC-H (C). The typical sterol profile in the DBS of an unaffected subject shows two main peaks corresponding to IS (peak #2) and CHOL

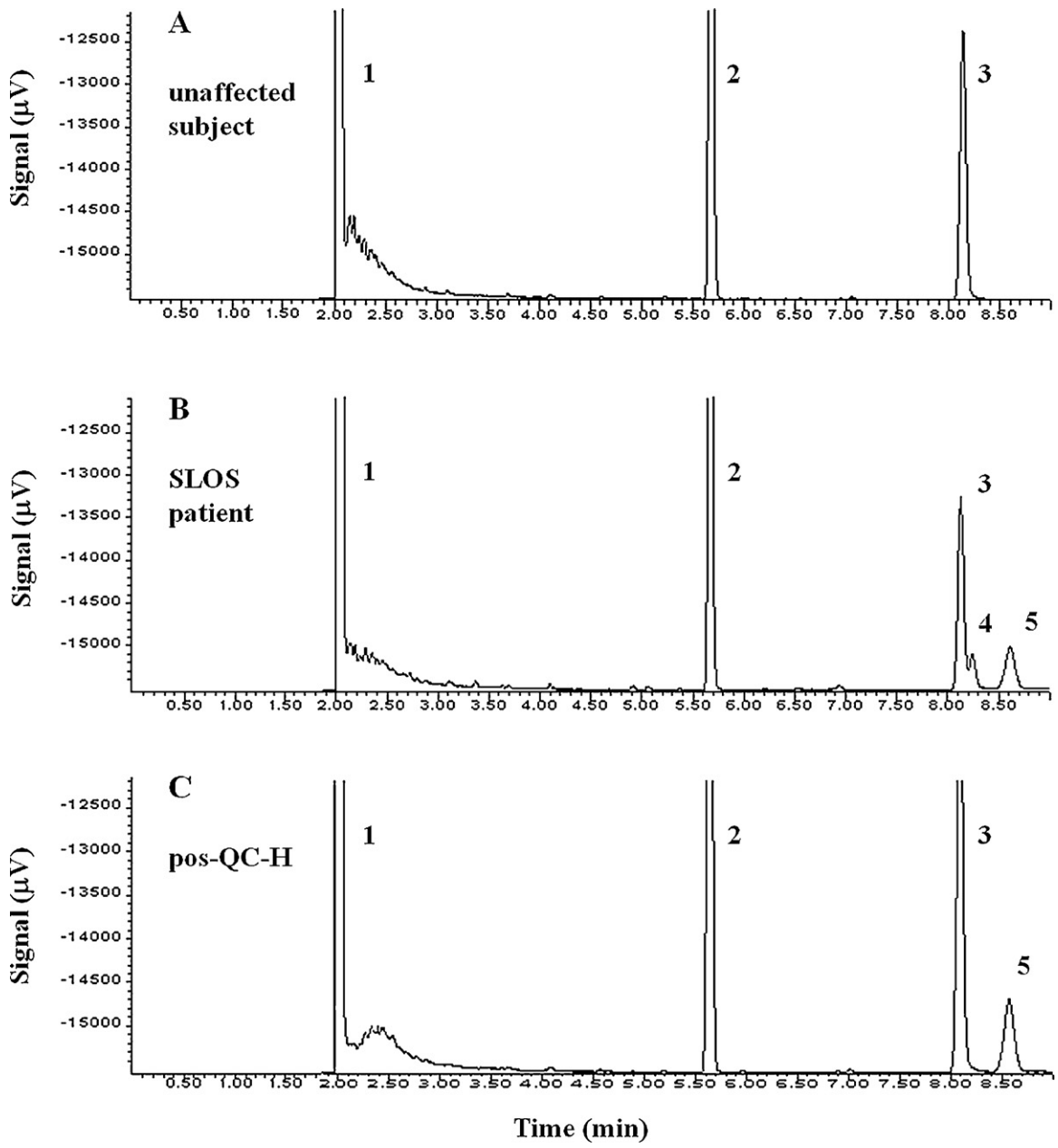

Fig. 1. Typical GC–FID chromatograms of sterols in DBS from an unaffected subject (A), SLOS patient (B), and pos-QC-H (C). 1: solvent; 2: IS; 3: cholesterol; 4: 8-DHC; 5: 7-DHC.

(peak #3), while the typical SLOS profile shows additional peaks corresponding to 8-DHC (peak #4) and 7-DHC (peak #5). The relative retention time (RRT ± SD) of each peak compared to IS (5.67 min) was 1.43 ± 0.0011 for CHOL, 1.45 ± 0.0015 for 8-DHC, and 1.52 ± 0.0016 for 7-DHC. The RRT of 7-DHC was also confirmed by injecting a negative sample spiked with a pure standard solution. Even though CHOL and 8-DHC levels are biased because not completely resolved, the presence of 8-DHC represents a qualifier marker of sterol profile for SLOS diagnosis.

3.1.1. Selectivity

The GC analysis of underivatized sterols was performed using a capillary column specially tested for sterols. The RRT of coprostanol, cholestanol, desmosterol, and lathosterol were 1.36, 1.47, 1.53, and 1.58, respectively, instead the sitosterol eluted in the subsequent run at RT of 0.91 min. Analytical interferences were not observed for coprostanol, lathosterol, and sitosterol. Instead cholestanol, a marker of cerebrotendinous xanthomatosis, co-eluted at the RRT of 8-DHC, and desmosterol, a marker of desmosterolosis, co-eluted at the RRT of 7-DHC. In case of the occurrence in a real sample of an isolated peak at the RRT of 7-DHC or 8-DHC a plasma sample should be re-analyzed by GC–MS.

3.2. Method validation

3.2.1. Calibration curves

The parameters of calibration curves and the correlation coefficients (r) for CHOL and 7-DHC are reported in Table 1. The LOQ for 7-DHC was evaluated analyzing 12.4 µL of a SLOS plasma sample diluted at the concentrations of 0.72, 0.36, and 0.18 mg/dL, the area S/N ratios were of 14.9 (CV = 1.9%, accuracy = −3.9%), of 10.3 (CV = 6.1%, accuracy = 27.8%), and of 3.7 (CV = 22.7%, accuracy = 38.9%), respectively. Based on these results, the LOQ for 7-DHC should be of 0.36 mg/dL even if the accuracy is over 20%.

Table 1
Parameter ranges of calibration curves of CHOL and 7-DHC.

Analyte	*n*	Concentration (mg/dL)	Slope (range)	Intercept (range)	*r* (range)
CHOL	5	1.72–110.0	0.0076/0.0091	−0.0050/0.0463	0.9986/0.9998
7-DHC	5	0.63–40.5	0.0066/0.0092	−0.0106/0.0029	0.9980/0.9997

Table 2
Within- and between-day imprecision and accuracy study of CHOL, 7-DHC, and 7-DHC/CHOL in the three QC levels.

	Analyte	Expected value (mg/dL)	Within-day ($n=5$)			Between-day ($n=15$)		
			Measured value (mg/dL)	Imprecision (CV%)	Accuracy (%)	Measured value (mg/dL)	Imprecision (CV%)	Accuracy (%)
Neg-QC	CHOL	100.0	101.7	4.3	1.7	100.2	7.1	0.2
Pos-QC-L	CHOL	82.1	90.0	6.9	9.7	88.0	7.1	7.3
	7-DHC	2.02	2.07	4.4	2.6	1.89	13.5	−6.1
	7-DHC/CHOL	0.025	0.023	6.3	−6.2	0.021	11.9	−13.3
Pos-QC-H	CHOL	56.9	60.3	9.5	5.9	60.3	6.5	5.9
	7-DHC	6.05	5.79	4.3	−4.3	5.25	14.8	−13.1
	7-DHC/CHOL	0.106	0.097	9.0	−8.9	0.086	11.2	−19.2

3.2.2. Imprecision and accuracy

As shown in Table 2, the within-day imprecision (CV%) and accuracy (%) for CHOL were less than 9.5% and 9.7%, respectively; for 7-DHC were estimated to be less than 4.4% and −4.3%, respectively; for 7-DHC/CHOL were less than 9.0% and −8.9%, respectively. The between-day imprecision and accuracy for CHOL were less than 7.1% and 7.3%, respectively; for 7-DHC were less than 14.8% and −13.1%, respectively; for 7-DHC/CHOL were less than 11.9% and −19.2%, respectively. The between-day variability observed in QC for 7-DHC reflects the total error coming from different factors, such as the variability of spot preparation, the analytical variability, and the instability of compound during the DBS storage.

As previously reported [20], in this study we confirm that the use of paper treated with antioxidant BHT for QC DBS preparation permitted us to analyze a single lot of QC up to a period of at least 56 days. The reduction of 7-DHC/CHOL during this storage time was less than 20% and it is in agreement with our previous results [20]. In addition, the use of BHT-impregnated filter paper, also for real samples, would be very useful to improve the stability of DHC and could avoid false negative results.

During the preparation of QC DBS, we observed a wider diffusion of QC blood in filter paper (larger DBS circles) compared to patient blood. The effect of this diffusion was evaluated by comparing CHOL levels from DBS of patients and from DBS of neg-QC specimens. CHOL concentration of QC obtained from 6 mm disc ($n=3$), which theoretically contains 12.4 μL of whole blood, was 56.0 ± 0.67 mg/dL and significantly lower than that obtained from the analysis of the entire DBS circle ($n=3$), corresponding to 20 μL, in which the CHOL concentration was 97.5 ± 1.3 mg/dL. This difference was not observed in DBS from patient blood. The results demonstrate that DBS discs of 6 mm from QC samples contain less volume of blood than that of patient samples. This difference was taken into account to calculate accurately the expected QC concentrations. Using the ratio between the concentration values, the volume of QC blood contained in a disc of 6 mm was of 7.1 μL.

3.2.3. Extraction efficacy and carry-over

Table 3 summarizes the extraction efficacy obtained by this method to extract blood sterols from filter paper analyzing in triplicate a DBS from a SLOS patient. The peak areas of each extraction step demonstrate that the first two extractions are able to recover more than 98% of IS and of CHOL, and 100% of DHC.

The carry-over was evaluated for IS, CHOL, and 7-DHC as the average percent of peak area ratio between the solvent and pos-QC-H injected before the solvent. The carry-over was 0.13% and 0.23% for IS and CHOL, respectively, while for 7-DHC it was zero.

3.2.4. Patient sample analysis

This method was used to analyze DBS and plasma samples from 30 unaffected subjects and 8 SLOS patients. In SLOS patients, both CHOL and 7-DHC plasma levels were not statistically different from that obtained in DBS (CHOL in plasma/DBS: 71.0/50.4 mg/dL; $p=0.231$; and 7-DHC in plasma/DBS: 11.3/10.1 mg/dL; $p=0.733$). The 7-DHC/CHOL ratios obtained from SLOS patients in DBS and plasma as average (range) were 0.360 (0.035–1.448) and 0.256 (0.012–0.926), respectively ($p=0.596$; Table 4). The 7-DHC/CHOL ratios in DBS and plasma from SLOS patients were significantly higher than control subjects ($p<0.05$; Table 4).

GC–FID method proposed here could have some minor limitations about the LOQ for 7-DHC. To avoid false negative results, patient samples with a peak corresponding to 7-DHC ranging from 0.18 to 0.36 mg/dL should be re-analyzed using at least two DBS (~25 μL). In our experience, at least the 95% of unaffected subjects

Table 3
Efficacy of each extraction step on sterols and IS recovered from DBS ($n=3$) of SLOS patient.

Extraction step	IS		CHOL		7-DHC		8-DHC	
	Peak area[a]	Recovery %	Peak area[a]	Recovery %	Peak area[a]	Recovery %	Peak area[a]	Recovery %
1st	360,660	85.0	110,645	85.2	16,084	88.0	9918	84.2
2nd	55,633	13.1	16,596	12.8	2198	12.0	1857	15.8
3rd	5973	1.4	1855	1.4	0	0	0	0
4th	2030	0.5	717	0.6	0	0	0	0
Total	424,296	100	129,813	100	18,282	100	11,775	100

[a] Area values correspond to the average of three experiments.

Table 4
7-DHC/CHOL ratios in DBS and plasma samples from SLOS patients ($n = 8$) and control subjects ($n = 30$).

Patient	7-DHC/CHOL	
	DBS	Plasma
SLOS 1	0.095	0.109
SLOS 2	0.277	0.308
SLOS 3	0.263	0.233
SLOS 4	0.128	0.093
SLOS 5	0.035	0.012
SLOS 6	0.471	0.269
SLOS 7	1.448	0.926
SLOS 8	0.167	0.101
Average (SD)	0.360 (0.460)[a]	0.256 (0.289)[a]
Controls (SD)	0.0002 (0.0001)	0.0002 (0.0004)

[a] $p < 0.05$ vs control subjects.

showed not detectable or detectable (~0.18 mg/dL) 7-DHC levels, and less than 5% showed levels ranging from 0.19 to 0.30 mg/dL, and the 7-DHC/CHOL ratio in SLOS patients was at least 10 fold higher than value at 95th percentile of unaffected subjects [21]. In addition, the 7-DHC/CHOL ratio is useful for diagnostic purpose, it also correlates well with clinical severity score, and the 7-DHC fraction better expresses the systemic sterol abnormality than absolute blood sterol levels, which are subject to wide physiological variations [22].

4. Conclusion

The described GC–FID method is fast and useful for the quantification of CHOL and 7-DHC from DBS. Calibration curves, quality controls, and patient samples show satisfactory analytical and clinical results. Moreover, this method overcomes some analytical problems, such as an efficient sample extraction using small volumes of solvent, GC analysis of underivatized sterols, and a fast turnaround-time (2 h). Finally, the method could be automated and miniaturized (by a commercial automated liquid sampler) with an estimated productivity of ~100 samples/day/instrumentation.

In conclusion, we believe that this GC–FID method is suitable to support screening analysis of SLOS using DBS. The method is simple and reproducible and could be applied in all laboratories provided by a GC–FID instrument to analyze a high number of samples per day.

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