Measurement of 7-dehydrocholesterol and cholesterol in hair can be used in the diagnosis of Smith-Lemli-Opitz syndrome

Yitao Luo[1,‡], Chengqiang Zhang[2,‡], Li Ma[3], Yuxiao Zhang[4], Zhengyuan Liu[5], Li Chen[6], Rui Wang[7], Yujing Luan[8*], and Yulan Rao[1*]

[1]Department of Forensic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai, PR China; [2]Department of Neonatology, Obstetrics and Gynecology Hospital Affiliated to Fudan University, Shanghai, PR China; [3]Department of Neonatology, Shanghai Children's Hospital Shanghai Jiao Tong University, Shanghai, PR China; [4]Institutes of Biomedical Sciences, Fudan University, Shanghai, PR China; [5]Department of Biochemistry and Pharmacology, University of Melbourne, Melbourne, Victoria, Australia; [6]Department of Medical Microbiology, Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, School of Basic Medical Sciences, Fudan University, Shanghai, PR China; [7]College of Forensic Medicine, Kunming Medical University, Kunming, PR China; [8]Institute of Forensic Science, Ministry of Public Security, Beijing, PR China

Abstract 7-dehydrocholesterol (7-DHC) and cholesterol (CHOL) are biomarkers of Smith-Lemli-Opitz Syndrome (SLOS), a congenital autosomal recessive disorder characterized by elevated 7-DHC level in patients. Hair samples have been shown to have great diagnostic and research value, which has long been neglected in the SLOS field. In this study, we sought to investigate the feasibility of using hair for SLOS diagnosis. In the presence of antioxidants (2,6-ditert-butyl-4-methylphenol and triphenylphosphine), hair samples were completely pulverized and extracted by micro-pulverized extraction in alkaline solution or in n-hexane. After microwave-assisted derivatization with N,O-Bis(trimethylsilyl)trifluoroacetamide, the analytes were measured by GC-MS. We found that the limits of determination for 7-DHC and CHOL were 10 ng/mg and 8 ng/mg, respectively. In addition, good linearity was obtained in the range of 50–4000 ng/mg and 30–6000 ng/mg for 7-DHC and CHOL, respectively, which fully meets the requirement for SLOS diagnosis and related research. Finally, by applying the proposed method to real hair samples collected from 14 healthy infants and two suspected SLOS patients, we confirmed the feasibility of hair analysis as a diagnostic tool for SLOS. In conclusion, we present an optimized and validated analytical method for the simultaneous determination of two SLOS biomarkers using human hair.

Supplementary key words hair analysis • gas chromatography-mass spectrometry • micro-pulverized extraction • microwave-assisted derivatization • diagnostic test
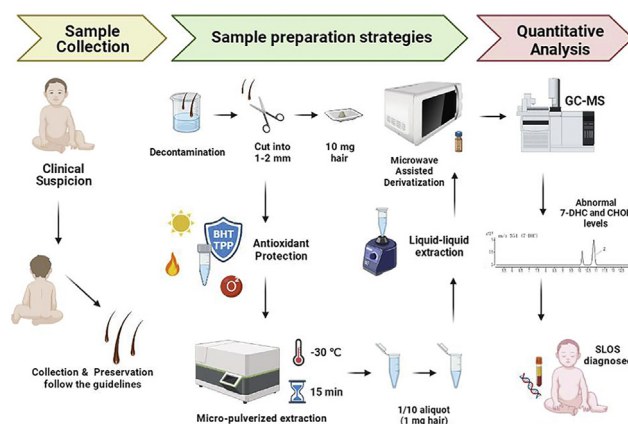
Cholesterol (CHOL) is a ubiquitous biomolecule for human and animal function serving as an utmost important precursor of steroid hormones, bile acids, and cell membrane (1). 7-dehydrocholesterol (7-DHC) is the penultimate metabolite in the Kandustch-Russell pathway for CHOL synthesis, catalyzed by an enzyme, 7-dehydrocholesterol reductase (DHCR7) (2).

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive disorder of CHOL biosynthesis, caused by the deficiency of DHCR7. Consequently, high 7-DHC plasma levels (at least 10 times higher than healthy individuals) and low CHOL plasma levels (59–1790 μg/ml of SLOS patients versus 429–2743 μg/ml of healthy children (3)) were described as a characteristic biochemical manifestation and the diagnostic criteria of SLOS (4, 5). Due to the irreplaceable role of CHOL, wide-ranging and even fatal clinical symptoms are frequently reported, including polydactyly, behavioral

---

and cognitive deficits, specific facial features, organ growth retardation, and immune and endocrine malfunction (5, 6). Various phenotypes also bring great difficulty to the clinical suspicion and diagnosis of SLOS. SLOS incidence rate ranges from 1/20,000 to 1/60,000, with the highest prevalence in the population of European descent, and the carrier frequency is 1%–2% (6, 7). With the increasing number of cases reported in Asia in recent years (8, 9), SLOS may be more common than originally thought (10).

Early intervention and treatment can improve the condition of newborns (11), so it is critical to have an efficient and simple diagnostic method. The metabolome of conventional matrices (i.e., plasma, serum) sometimes can be dynamic, resulting in a relatively unstable composition depending on maternal condition, dietary or circadian variations. In contrast to the conventional matrices (i.e., plasma, serum), hair, as a complementary matrix, can reflect the biochemical level of infants in the prenatal stage and avoid the interference of diet after birth on the analysis results (12). This is because once analytes are incorporated into hair, they can remain unchanged without fluctuations in content caused by degradation or metabolism (13). Hair can also be simply stored for a long time at room temperature and no strict anticorrosion measures are required during preservation or transportation procedure (12, 14). The sample collection of hair can be convenient (without the need of trained professionals) and less invasive (12), which is more friendly to newborns, especially those who are sick. Moreover, the analysis of hair has a much wider window of detection and could show the presence of analytes in individual's body from a few weeks up to a year (12), which is meaningful for the biochemical level monitoring of some children and adult patients.

GC-MS has been reported to be a tool for diagnosis of SLOS and other cholesterolopathies, which benefits from its excellent peak resolution, no influence of matrix effects on ionization, higher chromatographic efficiency, and relatively lower cost of instrument (15, 16). However, the published analytical methods for CHOL in hair still have a series of problems such as nonstandardized hair weighing and homogenization (17, 18), time-consuming extraction step (e.g., overnight soaking, 16 h ultrasound etc.) (17, 19, 20), lack of necessary antioxidation measures (17–20), inappropriate internal standard (IS) (17–19), relatively incomplete method validation data (17–20), and long extraction or derivatization times (up to more than 18 h) leading to low throughput (17, 18, 20).

In addition, at present, there is still a lack of analysis methods for 7-DHC in hair. In the only publication using hair as the sample for 7-DHC analysis, Serra *et al.* (17) found that the level of 7-DHC (570–780 ng/mg) in the hair of DHCR7-deficient mice (n=2) was hundreds of times higher than that of the healthy mice (<1 ng/mg) with decreased CHOL levels, which was consistent with the trend in SLOS patients' plasma. However, the levels of 7-DHC and CHOL in human hair have not been studied, and the reference values of 7-DHC and CHOL in the hair of SLOS patients and healthy individuals have yet to be established.

This study aimed at developing a reliable, simple, and efficient GC-MS method, for simultaneous determination of 7-DHC and CHOL in human hair as an aid for the diagnosing and monitoring of SLOS. The hair decontamination, weighing, and homogenization strategies were carefully compared and optimized. Micropulverized extraction (MPE) and microwave-assisted derivatization were conducted for increasing analytical throughput. Antioxidant steps were also performed to prevent the oxidative degradation of the analytes and two deuterated ISs were adopted for accuracy as recommended (16). Furthermore, this method was applied to authentic hair samples of healthy and suspected infants to describe possible differences in the two biomarkers' levels between these groups.

MATERIALS AND METHODS

Chemicals and reagents

Potassium hydroxide (KOH) and ethanol were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 7-DHC, 7-DHC-d7 (7-DHC-25,26,26,26,27,27,27-d7), CHOL, CHOL-d7 (cholest-5-en-25,26,26,26,27,27,27-d7), 2,6-Ditert-butyl-4-methylphenol (BHT), triphenylphosphine (TPP), and all other chemicals and reagents used were purchased from Merck (Darmstadt, Germany).

Neonatal hair samples were provided by Obstetrics and Gynecology Hospital Affiliated to Fudan University and Shanghai Children's Hospital, Shanghai Jiao Tong University. Informed oral consent was obtained from all volunteers' guardians in accordance with the Declaration of Helsinki principles. All related experimental and samples collection procedures in this study were approved by the ethical committee of Obstetrics and Gynecology Hospital Affiliated to Fudan University, Shanghai, China.

Preparation of standard solutions

7-DHC (1 mg/ml) and CHOL (10 mg/ml) were diluted in isopropanol to a final concentration of 800 μg/ml and 1200 μg/ml respectively as standard solutions. 7-DHC-d7 and CHOL-d7 were dissolved in isopropanol and finally diluted to the concentration of 40 μg/ml and 60 μg/ml respectively as IS. All solutions were stored at −40°C and prepared daily.

5 mg/ml BHT and 12.5 mg/ml TPP were prepared in ethanol and used as the antioxidant solution. KOH solution (1 M) was obtained by dissolving 560 mg KOH in 10 ml of 80% ethanol solution and used as an alkaline reagent in the hydrolysis step.

Instrumentation

MPE was conducted in a Bead Ruptor (JinXin, JXFSTPRP-6K, ShangHai, China) with temperature control function. Microwave-assisted derivatization was performed in a Haier MZC-2070M1 household microwave (ShangHai, China). Ultrapure water was prepared using a Millipore Milli-Q purification system (Bedford, MA). GC-MS was performed by an Agilent 7890B gas chromatograph connected with an Agilent

7000D triple quadrupole mass spectrometer (Agilent, Palo Alto, CA). The data acquisition software was Agilent Mass-Hunter Quantitative Analysis B.09.00.

Sample collection

Hair collection and storage steps followed internationally accepted guidelines (21, 22). In short, hair strands were collected from the posterior vertex of the head of the infants and stored in paper envelopes at dry place at room temperature, protected from light after collection. During the collection process, blunt scissor was carefully cleaned, and the personnel wore gloves to avoid contamination of the samples. Hair samples proportionate to the thickness of a pencil were collected close to the scalp from multiple sites within the posterior vertex region of the head (21). Newborns' hair will not be affected by factors such as cosmetic treatments and sun exposure, but visible stains (such as meconium) should be avoided during collection.

Sample preparation

Hair samples were washed successively with water, then with acetone (5 ml/15 mg, 2 min under vortex agitation), which were then dried at room temperature with gentle air flow and cut into 1–2 mm long pieces.

50 µl of antioxidant solution (5 mg/ml BHT and 12.5 mg/ml TPP in ethanol) and IS solution (40 µg/ml 7-DHC-d7, 60 µg/ml CHOL-d7, 50 µl for each kind, equal to 200 ng/mg 7-DHC-d7, 300 ng/mg CHOL-d7 in hair) were added in a 2 ml MPE tube with ceramic beads. Afterward, aliquots of 10 mg hair and 850 µl of the extraction solvent (1 M KOH in 80% ethanol) were added into the tube. The samples were placed on a Bead Ruptor system and pulverized at −30°C using the following conditions: speed: 21 m/s; time: 40 s; dwell: 30 s. This procedure was repeated 15 times to completely pulverize the hair. After pulverization, the mixture was centrifuged at 10,000 g for 5 min, and then an aliquot of 100 µl (equal to 1 mg hair sample) of the supernatant was transferred to another tube. 900 µl of n-hexane (n-hex) as the liquid-liquid extraction solvent was added. The mixture was vortexed for 3 min and then centrifuged at 10,000 g for 5 min. The organic phase was transferred into a glass tube and the residue obtained after evaporation was redissolved in 60 µl of the derivatization reagent N,O-Bis(trimethylsilyl)trifluoroacetamide. After vortexing for 10 s, the tube was sealed and microwave-assisted derivatized at medium-high power (460 W) for 3 min. Then the derivatized sample was completely transferred for GC-MS analysis and an aliquot of 1 µl was injected.

GC-MS analysis

The electron energy of GC-MS was 70 eV and the ion source temperature was 320°C. Each sample (1 µl) was injected in split mode (10:1) at 240°C and separated through a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, Palo Altro, CA) using helium as carrier gas with a flow rate of 1 ml/min. The initial temperature of GC oven was held at 240°C for 1 min and subsequently ramped at 20°C/min to 280°C and held for 17 min. 7-DHC and CHOL were determined based on peak area using two qualifier ions (m/z = 325, 351) and three qualifier ions (m/z = 129, 329, 368), respectively. All of the ions were monitored in the selected ion monitoring mode. Agilent Chemstation was applied for data collection, processing, and GC-MS control.

Method validation

Preparation of blank samples. 1/10 aliquot of 10 mg hair alkaline digested sample was considered as blank sample after being repeatedly extracted (3 times, by 900 µl n-hex) until 7-DHC and CHOL were not detected or detected below their lower limits of quantification (LLOQ). The residual levels of the two analytes were monitored by the standard spiked method, before and after the repetitive extraction as described elsewhere (23, 24).

Specificity. Specificity experiments were carried out using six blank hair samples from different healthy infant sources obtained by repeated extraction, to evaluate any possible interference from endogenous compounds.

Linearity and sensitivity. A calibration curve for each compound was prepared by spiking blank matrix with standard solutions to achieve the following final concentrations: 50, 100, 200, 400, 800, 1600, and 4000 ng/mg for 7-DHC; 30, 100, 300, 500, 1000, 3000, and 6000 ng/mg for CHOL. The two calibration curves of 7-DHC and CHOL were obtained using least-squares linear regression, with a $1/x^2$ weighting factor.

Limit of detection (LOD) and LLOQ were determined with blank matrix, spiked with different concentrations of 7-DHC and CHOL. LOD was defined as the lowest concentration of analyte having an S/N≥3. LLOQ was the lowest result of meaningful data with an S/N≥10, while maintaining CV within 20% of the nominal concentration.

Precision and accuracy. Accuracy and precision were evaluated at four levels including LLOQ (50 ng/mg for 7-DHC and 30 ng/mg for CHOL) and three QC concentrations (100, 1200, 3200 ng/mg for 7-DHC, and 100, 1800, 4800 ng/mg for CHOL). Intraday precision was assessed in replicate determinations (n = 5) of QC samples in one run. Interday precision was assessed in replicate determinations (n = 5) of the three QC samples on three independent days. Accuracy was assessed by comparing the calculated concentrations to their respective theoretical values and precision was recorded by relative standard deviation (RSD).

Extraction recovery rates. Extraction recovery were assessed by calculating the percentages of peak area ratios of extracted samples to those of nonextracted counterparts (representing 100% recovery), which were prepared by direct N,O-Bis(trimethylsilyl)trifluoroacetamide derivatization in dry, neat form at the three nominal concentrations (LQC, MQC, and HQC, n = 5).

Carryover. Carryover was evaluated by injecting five replicates of HQC sample (3200 ng/mg 7-DHC and 4800 ng/mg CHOL) followed by methanol. The calculated concentration of 7-DHC and CHOL in the methanol was used to assess the carryover.

Dilution integrity. High concentration spiked samples (10,000 ng/mg for 7-DHC and 10,000 ng/mg for CHOL) were prepared as described above. After MPE, 100 µl of supernatant were transferred and diluted 5 times and 10 times with blank hair samples, respectively, before subsequent preparations. Five replicate samples were analyzed for each dilution level, and the dilution integrity was assessed by calculating the precision and accuracy.

Stability. Stability of three QC concentrations (100, 1200, 3200 ng/mg for 7-DHC, and 100, 1800, 4800 ng/mg for CHOL) was evaluated under four different conditions. Short-term stability was determined after placing QC samples at room temperature for 3 h. Postpreparative stability was determined after placing pretreated QC samples in the autosampler (25°C) for 24 h. When evaluating the freeze-thaw stability, QC samples were frozen at −40°C for 24 h, then thawed and kept in the dark at room temperature for 30 min. The freeze-thaw cycle was repeated three times. Long-term stability was evaluated after storing QC samples at −40°C for 30 days.

Application to real samples

The 7-DHC and CHOL level in the hair samples of 14 healthy newborns (seven males, seven females) and two suspected SLOS patient (two females) were analyzed. In terms of specific clinical symptoms, these two infant girls were diagnosed with SLOS typical symptoms of syndactyly of the second and the third toes (left foot, **Fig. 1**). These patients were suspected by clinicians to be SLOS undiagnosed patients. The hair collection time of all newborns was the day of delivery to minimize the influence of external factors. Because the patients' guardian refused to provide blood, genetic test could not be conducted.

The hair samples were analyzed as described above and quantitative results were obtained using the IS method. The deuterated IS solutions were added at the same level to the standard curve samples and testing samples before sample preparation. After GC-MS detection, standard curves were obtained, with the weighted $(1/x^2)$ concentration of the standard on the x-axis, and the peak area ratio of the analyte to the corresponding deuterated IS on the y-axis. Based on the standard curves, the concentrations of the analyte in the testing samples were obtained by regression analysis.

RESULTS

Method validation

Specificity. Chromatograms of six different blank hair samples showed that no endogenous peak simultaneously existed. Representative chromatograms of blank sample, spiked sample, and suspected SLOS patient's sample are shown in **Fig. 2**. EI spectra is shown in supplemental Fig. S1.

Sensitivity. The LOD and LLOQ were 10 ng/mg and 50 ng/mg for 7-DHC and 8 ng/mg and 30 ng/mg for CHOL, respectively.

Linearity. Linearity was achieved with correlation coefficients greater than 0.99, within the concentration ranges of 50–4000 ng/mg for 7-DHC and 30–6000 ng/mg for CHOL (supplemental Fig. S2).

Accuracy and precision. Accuracy of the assay was in the range of 88.2%–111.9% for 7-DHC and 86.9%–114.2% for CHOL. The intraprecision (RSD between 3.06% and 6.34% for 7-DHC, between 1.33% and 5.38% for CHOL) and interassay precision (RSD between 2.09% and 11.92% for 7-DHC, between 5.79% and 12.66% for CHOL) were within the acceptable range, and the results are summarized in Table 1.

Apparent recoveries and carryover. Apparent recovery of the assay was in the range of 80.9%–92.3% for 7-DHC and 93.1%–95.6% for CHOL (Table 1). The data indicated that the extraction of the two substances was sufficient.

None of the two analytes showed any significant peak (≥20% of the LLOQ) in blank samples injected after HQC samples.

Dilution integrity. The accuracy observed for one-fifth or one-tenth dilution samples ranged from 87.8%–107.5% and 101.1%–111.0%% with RSD 9.8%, 3.8% for 7-DHC, 91.0%–102.9% and 92.4%–101.9% with RSD 5.0%, 4.3% for CHOL. The results suggested that reanalysis of 7-DHC and CHOL in hair samples above the calibration curve (4000 ng/mg for 7-DHC and 6000 ng/mg for CHOL) by appropriate dilution could be fulfilled.

Stability. Stability results of 7-DHC and CHOL suggested that no significant difference could be observed at room temperature under dark conditions for 3 h for QC samples. The spiked samples after three freeze and thaw cycle, kept at −40°C for 30 days, and the processed samples kept in the autosampler at 25°C for 24 h were also stable. The data was shown in Table 1.

Fig. 1. Syndactyly of the second and the third toes, clinical appearance of two suspected SLOS patients. SLOS, Smith-Lemli-Opitz syndrome.
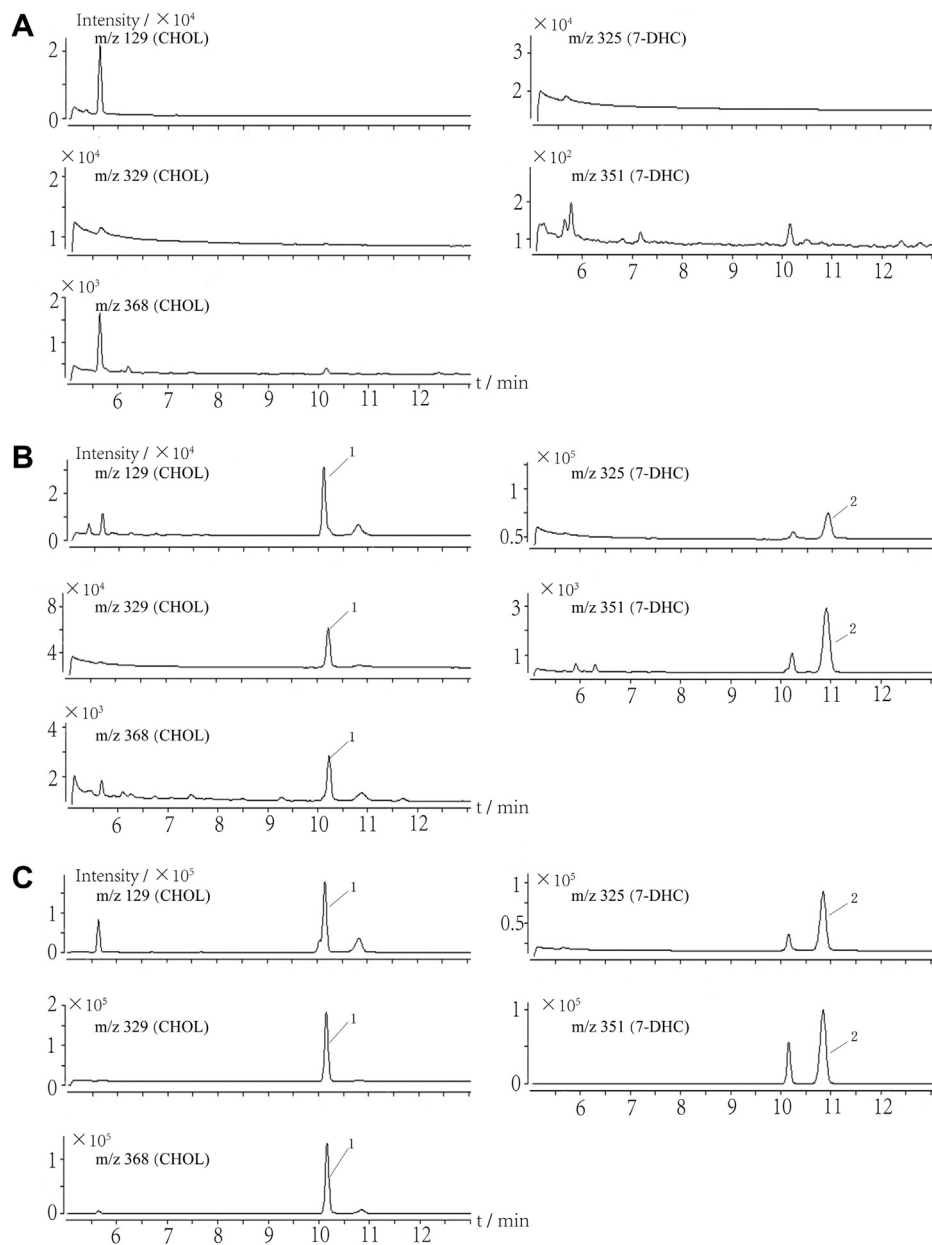
Fig. 2. Representative GC-MS/MS chromatograms of 7-DHC (m/z 325, 351) and CHOL (m/z 129, 329, 368). Results of blank sample (A); spiked sample with 7-DHC (50 ng/mg, LLOQ) and CHOL (30 ng/mg, LLOQ) (B); sample from the one SLOS patient (C); arrow 1 refers to CHOL; arrow 2 refers to 7-DHC. 7-DHC, 7-dehydrocholesterol; CHOL, cholesterol; LLOQ, lower limit of quantification.

TABLE 1.   Date of accuracy, inter-assay and intra-assay precision, recovery rate, and four kinds of stability (n = 5)

Compound		7-DHC				CHOL			
		LLOQ	LQC	MQC	HQC	LLOQ	LQC	MQC	HQC
Level (ng/mg)		50	100	1200	3200	30	100	1800	4800
Intra-assay	Accuracy (%)	103.0	91.7	100.0	97.1	95.9	91.5	111.8	92.6
	RSD (%)	3.1	3.8	6.3	3.3	5.4	3.5	2.1	1.3
Inter-assay	Accuracy (%)	102.0	92.5	105.3	99.6	96.2	92.9	107.7	97.8
	RSD (%)	2.1	4.0	11.9	5.0	5.8	10.0	8.3	12.7
Recovery rate (%)		/	80.9	81.1	92.3	/	93.4	93.1	95.6
Short-term stability (%)		/	92.0	98.7	102.1	/	94.0	104.8	96.8
Postpreparative stability (%)		/	92.5	102.8	96.1	/	97.5	105.8	88.9
Freeze-thaw stability (%)		/	97.3	103.5	101.6	/	90.3	102.9	90.4
Long-term stability (%)		/	93.1	106.1	102.3	/	91.2	108.7	99.8

n = 5 for intra-assay, recovery rate and four kinds of stability, n = 5 × 3 = 15 for inter-assay.

Application to real samples

As shown in **Fig. 3**, a certain concentration of 7-DHC is found in healthy infants' hair (concentration ranges, mean value, 120.2–260.1, 163.9 ng/mg, 120.6–288.8, 184.2 ng/mg, in the healthy female and male infants, respectively). In addition, the analytical CHOL results were 1960.5–3039.5, 2449.3 ng/mg, 2135.4–3622.1, 2866.2 ng/mg in the healthy female and male infants, respectively. For hair samples of suspected patients, the 7-DHC concentration were 376.9, 321.7 ng/mg, with CHOL levels at 1752.5, 1858.1 ng/mg, respectively.

DISCUSSION

Decontamination

According to the guidelines of Society of Hair Testing (21), hair sample decontamination is an initial washing step with an organic or aqueous solvent, to remove contaminations and interference from sweat (containing CHOL (25)), sebum, or dust. Protic solvents (e.g., H_2O, methanol) have been reported to cause swelling of hair matrix and consequently lead to unwanted partial extraction during the washing step (21). On the contrary, nonprotic solvents (e.g., dichloromethane, acetone) do not lead to the swelling of hair and are believed to remove exogenous contaminants from the surface of hair only (26).

In this study, the washing effects of three commonly used solvents for hair decontamination were compared, including methanol, water, and acetone. As shown in supplemental Fig. S3, when methanol, a protic solvent, was used, significantly more CHOL was washed off. There was little amount of CHOL eluted when another protic solvent, H_2O was used. It might be due to the difference in solubility (27). In addition, 7-DHC was not detected in all three washing solvents.

Children's hair is finer and more porous than adults' and usually there are limited amount of hair available for analysis (28, 29). Therefore, in order to avoid the inevitable side effects of extraction caused by excessive washing and retain the necessary effect of removing polar metabolites by aqueous washing step, the wash protocol of washing with H_2O and acetone was chosen in this study.

Hair amount

Another issue that should be considered is the amount of hair sample required to perform the analysis. Several advantages are provided by low amounts (e.g., 1 mg (20, 30, 31)) of hair for the analysis of high concentration substance (28, 29): a) more friendly to suspected infants and larger number of analysis can be performed; b) the hair homogenization is time- and labor-saving; and(c) less inevitable contaminants from the samples are brought to the analytical instrument. But in actual experiments, due to the insufficient precision (0.1 mg) of most commonly used analytical balance, it is difficult to accurately weigh aliquots of 1 mg hair, and predictably, it is prone to cause unacceptable deviations in the analysis results. To some extent, this defect explains why the recommended minimum hair sample weight was set to 10 mg in internationally accepted guidelines (21). Besides, the selection of low hair amounts compromises the method sensitivity and reproducibility (32), which can be critical for 7-DHC due to its low expected concentration in hair, especially for suspect infants with ambiguous biochemical level.

Taking the above factors into consideration, 10 mg hair was weighed and a one-tenth aliquot of the total volume of alkaline digested sample was transferred to subsequent analysis, to reach the final concentration of
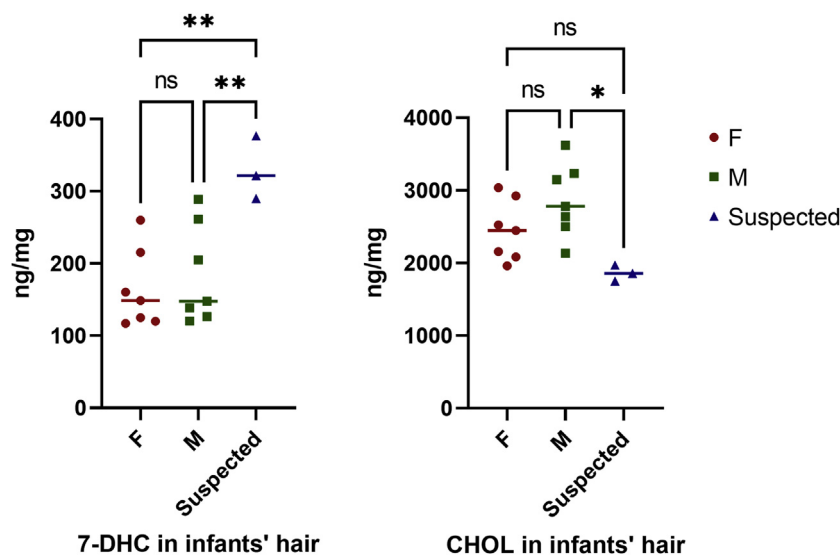

Fig. 3. Concentrations of hair 7-DHC and CHOL in seven female and seven male infants and two suspected patients. Statistical results: ns, $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$. 7-DHC, 7-dehydrocholesterol; CHOL, cholesterol.

1 mg hair (23, 24, 33). Significantly lower RSDs were obtained compared to weighing 1 mg hair directly after the optimization.

Homogenization method

Sample shredding for whole hair by milling or mincing and MPE have been adopted in previous publications. The protocol of using whole hair is time-saving and gets lower sample loss and lower risk of contamination. But some studies suggested that prior sample pretreatment can open up the hair matrix, thereby improving extraction efficiency, sensitivity, accuracy, and precision to a considerable extent (32, 34, 35).

To the best of our knowledge, the influence of the above hair homogenization methods on the analysis of high-concentration endogenous components like 7-DHC and CHOL has not been discussed. In this study, four commonly used hair pretreatments methods, including using whole hair (original length, 2–3 cm), mincing (cut by scissors, 1–2 mm), milling (1–2 mm hair pulverized by Mix Mill), MPE (1–2 mm hair pulverized by Bead Ruptor) were compared, and the results were shown in supplemental Fig. S4.

The results indicated that the worst result was achieved by milling. This result might be explained by the fact that the Mix Mill container is made of plastic and probably shows a certain extraction effect for hair samples (22). Moreover, due to the inevitable electrostatic interaction, hair loss would be caused after milling, which was even far more than the amount required by analysis (23). No significant differences were found among the other three strategies. Considering that MPE has the dual effects of grinding and extraction, which can significantly increase the analysis throughput (16 min *v.s.* at least 40 min during the alkaline digestion process of the other three methods), MPE was selected as the homogenization method.

Conditions for the extraction of 7-DHC and CHOL from hair

It is critical to choose appropriate extraction condition, including solvent, method, time, and temperature. Some studies adopted immersion hair in organic solvents or with the aid of ultrasonic extraction. Methanol is a commonly used solvent for hair extraction, which can dissolve neutral and lipophilic compounds. Methanol was shown to be better than acetonitrile for the extraction of hydroxybutyric (36). However, a mixed solution containing 0.1 M trifluoroacetic acid (TFA) and 50% acetonitrile was reported to enhance the extraction efficiency two times compared to methanol in steroid analysis method (31). Moreover, alkaline digestion is another recommended hair extraction strategy and was reported to be one of the most efficient and convenient sample preparation methods (27). Alkaline digestion at 70°C (close to the boiling point of ethanol) in water bath, as reported in the plasma analysis method (37) was found to be the best hair dissolution temperature in our preliminary experiment. However, whether KOH (used in plasma methods related to SLOS diagnosis(3)) can be replaced by NaOH (better solubility in aqu.) has not been studied. Overnight soaking (38) and ultrasound assisted extraction (17) adopted in previous hair methods were also compared. Meanwhile, since extraction can also be achieved by MPE, it was also among the list of extraction strategies we compared, as shown in supplemental Table S1.

Figure 4A, B show the effects of various combinations of solvents and method on the exaction of 7-DHC and CHOL, and the best extraction effect was achieved by the combination of 90% KOH, MPE. The results confirmed that MPE could improve the analytical throughput while achieving a satisfactory hydrolysis effect. Meanwhile, as long as the hair sample could be fully dissolved, other regular hydrolysis conditions(e.g., at 65°C for 2 h) might also provide acceptable results, which can be used as alternatives if MPE is not available.

No significant advantages were observed in the groups of overnight soaking, TFA and NaOH aqu., as shown in **Fig. 4**A, B. The unacceptable long time of overnight soaking method can also greatly reduce the throughput. So does the method using ultrasonic bath (up to 16 h (17)), although the extraction effect was improved to a certain extent with the aid of particle size reduction and cell disruption provided by ultrasonic bath (38). Moreover, the inevitable rise in water temperature might introduce uncertainties to the stability of the analytes (39). In addition, due to the extremely low solubility of CHOL and 7-DHC in water, poor extraction results were observed in the groups of NaOH aqueous and 0.1 M TFA and 50% acetonitrile.

Nevertheless, a single solvent that effectively dissolves lipophilic substances was still insufficient. This phenomenon was observed in Fig. 4. Compared to the ethanol groups (without KOH), obviously less hair fragments (visual inspection) and higher extraction effect were observed in the KOH-containing groups, which may be the consequence of sterol ester hydrolysis. The percentages of ethanol in KOH solution (70%, 80%, and 90%) were further compared. As shown in Fig. 4C, D, the best performance was achieved by 1 M KOH in 80% ethanol.

In terms of extraction methods, MPE achieved better extraction effect in Fig. 4. This phenomenon was attributed to MPE's effective combination of homogenization and extraction, significantly increasing the surface of contact between the solid hair matrix and the extracting solvent, and promoting extraction efficiency and a reduction of extraction time (36). So, compared to water bath incubation (at least 70°C, 0.5 h to fully dissolve the hair), higher analytes' stability and throughput was achieved by MPE (−30°C, 15 min).
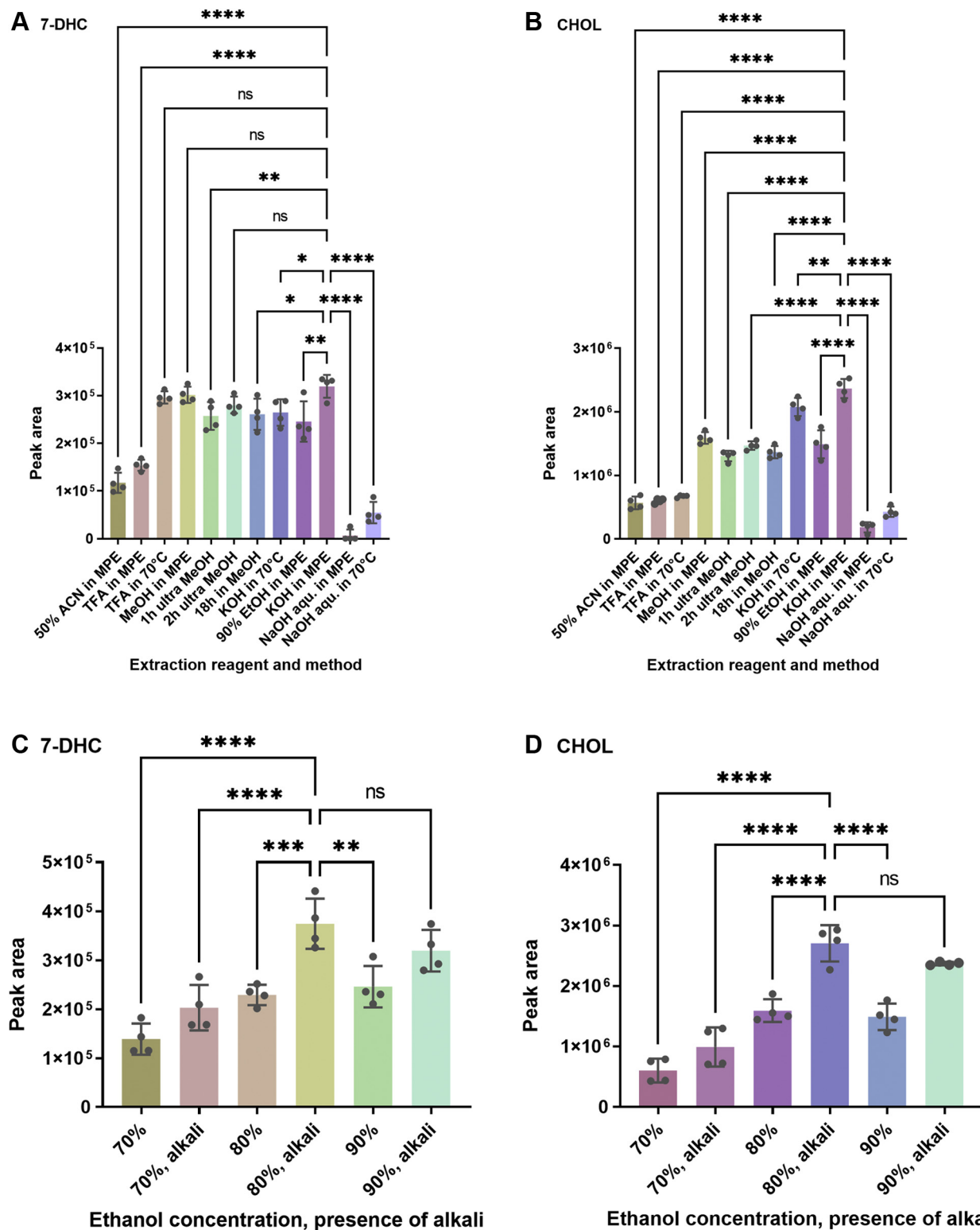
Fig. 4. Effects of different extraction solutions and methods on the extraction of (A) 7-DHC and (B) CHOL from hair and effect of different ethanol concentrations and the presence or absence of KOH on the extraction of (C) 7-DHC and (D) CHOL from hair (n = 4). Statistical results: ns, $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Range bar, standard deviation (sd). 7-DHC, 7-dehydrocholesterol; CHOL, cholesterol; KOH, potassium hydroxide; ACN, acetonitrile; TFA, trifluoroacetic acid; MeOH, Methanol; MPE, micro-pulverized extraction; aqu, aqueous solution.

The diameter of the ceramic beads, temperature, and running times of pulverization program were optimized. Specifically, three groups were compared, including < 0.5 mm group (0.5 mm, 40 ea, and 0.1 mm, 100 ea), mixed group (2 mm, 1 ea, 1.5 mm, 1 ea, 1 mm, 5 ea, 0.5 mm, 20 ea, and 0.1 mm, 50 ea), and >1 mm group

(2 mm, 2 ea, 1.5 mm, 2 ea, and 1 mm, 10 ea). The total volume of ceramic beads in the above three groups was controlled to be basically the same. As shown in supplemental Fig. 5A, B, no obvious differences between the three ceramic bead groups have been observed. The similar extraction results suggested that under sufficient grinding conditions, the difference in the diameter of ceramic beads would not significantly affect the extraction effect of high-concentration analytes. Some studies also suggested that freezing has a positive effect on hair pulverization (39), that is to say, it is friendly to thermally unstable analytes and reduces the percentage of large particles. Therefore, although increasing temperature can improve the extraction effect to a certain extent, the best performance was still observed at −30°C (supplemental Fig. 5C, D). Similarly, due to the inevitable generation of heat, the analytes were less stable in strong alkali at elevated temperature (supplemental Fig. 5E, F), which also indicated that a single MPE program run was sufficient.

In summary, the optimized conditions (mixed diameter of the ceramic beads, −30°C, a single MPE program run) would be effective and sufficient in properly MPE for the analysis of 7-DHC and CHOL in hair.

Addition of antioxidant

7-DHC has been proven to be one of the most oxidizable lipids which is prone to be oxidized during sample preparation (16, 40–42). Without appropriate protection, peroxidation products would appear and a significant loss of 7-DHC can be inevitable (43), which would then lead to the deviation of final analysis result or even false diagnosis. To some extent, the undetectable results for 7-DHC in healthy mice hair could be explained by the lack of antioxidant measures (17). Moreover, since CHOL can also be oxidized to a certain extent, the unacceptably high interlaboratory variations for CHOL analysis result reported in a previous study may be explained by the absence of effective antioxidant measures and different oxidation levels under various sample preparation conditions (16).

It should be realized that an incorrect result, either over- or under-estimation, will inevitably have a potential impact on medical recommendations and decisions regarding treatment (44). However, to the best of our knowledge, no relevant countermeasure has been adopted or discussed in published methods for the analysis of 7-DHC and CHOL in hair.

BHT, a strong and widely-used antioxidant, was applied to plasma and dried blood spot for SLOS or other CHOL precursor-related studies (45, 46). TPP is another lipophilic antioxidant, which can also significantly reduce oxidation (47). Nevertheless, previous studies suggested that the combination of BHT and TPP would have a better antioxidant performance than only one type of antioxidant in preventing loss of 7-DHC (48). In this study, different concentrations of these two antioxidants were evaluated, which ranged from the basic concentration (1 mg/ml BHT and 2.5 mg/ml TPP in ethanol) used in previous studies conducted by Liu *et al.* (48, 49), to its eight times (8 mg/ml BHT and 20 mg/ml TPP in ethanol), in order to find the most suitable concentration level for hair samples.

As shown in **Fig. 5**, the peak area of 7-DHC achieved the highest when using BHT and TPP combination at the concentrations of 5 mg/ml and 12.5 mg/ml respectively and then decreased gradually at higher concentrations. A similar pattern was also observed for CHOL. Our findings in this study also demonstrated the necessity of antioxidant protection for 7-DHC and CHOL.

Liquid-liquid extraction conditions

Three liquid-liquid extraction (LLE) reagents, n-hex (50), n-pentane (n-pen) (51), and methyl tert-butyl ether (52) that have been reported in some methods for hair analysis were evaluated in this study (supplemental Fig. 6A, B). Although methyl tert-butyl ether could enhance the extraction efficiency, the dividing line between the aqueous and organic phases (the same for n-pen) and unacceptable signal-to-noise ratio (approximately 90% reduction) were observed compared to those extracted with n-hex. The difference in solubility of ethanol and interfering components in hair can explain this phenomenon. In addition, the high volatility of n-pen (b.p. 55°C) also brought difficulties to the experiment's operability and reproducibility.

Furthermore, the number of extractions and volume of n-hex were assessed and optimized. The effect of repeating extraction twice was better than that of single extraction and similar to the results of three extractions (supplemental Fig. 6C, D). Meanwhile, higher peak area was achieved by 900, 1000, and 1000 μl of n-hex (supplemental Fig. 6E, F), therefore, the effects of above three kinds of n-hex volume for one and two times extraction were compared simultaneously. The similar results indicated that there was no significant room for improvement in LLE (supplemental Fig. 6G, H). Considering green chemistry and throughput factors, the optimal LLE strategy was chosen to be single extraction using 900 μl of n-hex.

Method validation

For method validation, a blank hair matrix without analytes must be prepared. But for endogenous substances, it seems impossible to find a blank hair without any interference from endogenous substances (53). Similar dilemmas also occurred with analysis of many other endogenous substances in hair.

Six different blank hair preparation strategies for endogenous compound quantification (surrogate analyte, standard addition, background correction, stripped matrix, solvent calibration, and artificial matrix) were compared by Voegel *et al.* (54) and Thakare *et al.*
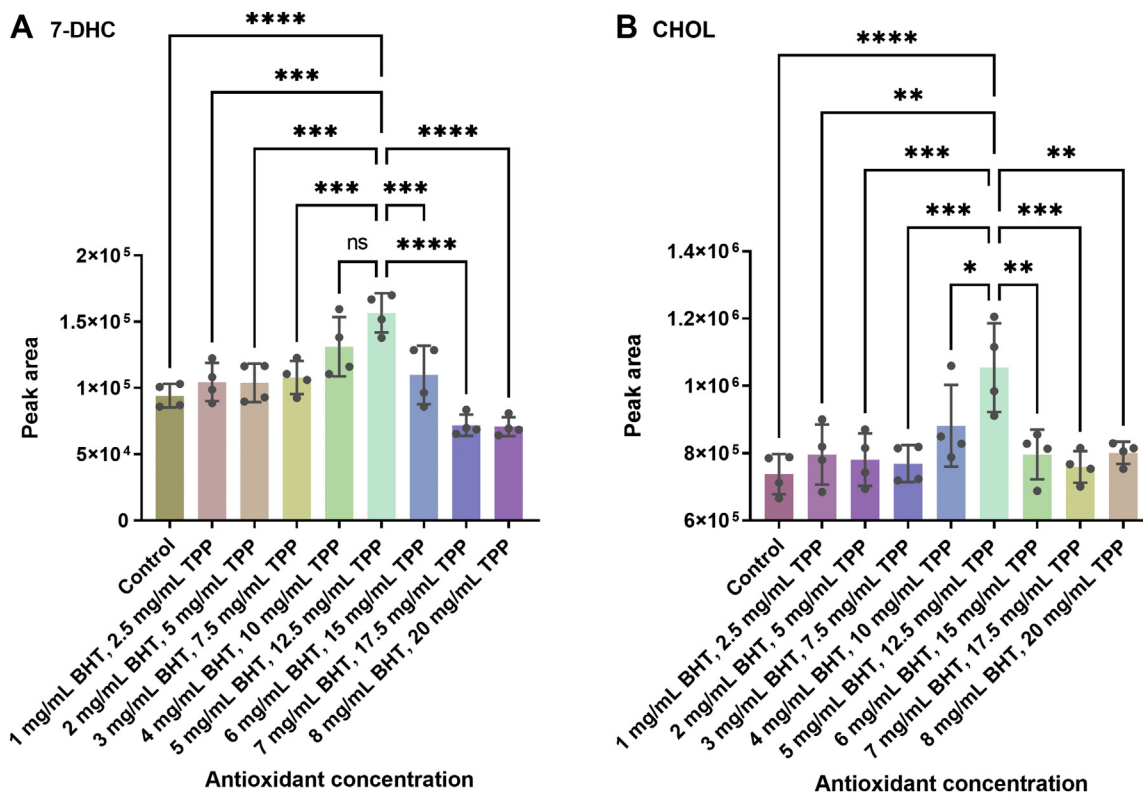
Fig. 5. Effect of antioxidant concentration on recovery rates of 7-DHC and cholesterol (CHOL) (n = 4). Statistical results: ns, $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. Range bar, standard deviation (sd). 7-DHC, 7-dehydrocholesterol.

(55). Surrogate analyte method always involves expensive isotope-labeled standards, which would greatly increase the diagnostic costs for the patients' family (55). Both standard addition method and background correction require a large amount of hair samples from suspected patients, which is not practical for infants (54). As for solvent calibration strategy, interfering substances from hair are completely absent. Artificial matrix method (e.g., melanin solution (56)) can only slightly mirror the true endogenous environment in hair. Some researchers also tried the strategy of using the tips of long hair (another strategy of stripped matrices (57)), expecting the decrease in concentration after repeated washing and sun exposure. However, we found it did not seem feasible for endogenous substances at high concentrations, and high levels of CHOL could still be detected in the hair. Therefore, a developed stripped matrix strategy was used in the present work. Although the preparation process would inevitably remove some interference, it was already the most suitable method in our opinion.

Moreover, the sensitivity of the proposed method is significantly better than that of the published methods (60 and 12 for the LOD of 7-DHC and CHOL, respectively) (17, 20). The linear range was wider than published methods and covered the reported level (17, 20) meeting the standard set forth in the FDA (58) and EMA (59) guidance for bioanalytical methods.

Application

The analysis results in this study were slightly higher than the published literature (20). The results can be explained by the fact that, the recommended sampling method (not mentioned in the previous papers (17, 18, 20)) and the concentration of endogenous substances in different hair stages may vary greatly (60). The advantages of optimized alkaline digestion (27) and the protection from frozen pulverization (39) and antioxidant((16, 40–42)) might also contribute to it.

It was found, the difference found in 7-DHC and CHOL levels for healthy and SLOS patients was obvious. However, only two suspected SLOS patients were involved in this study. Generation of pediatric reference intervals can be really difficult because of the large numbers of participants (including multiple factors such as gender, age, race, region (60), and life style (61)) required to properly define the statistical boundaries (62). Moreover, the levels of 7-DHC and CHOL in the hair of SLOS patients have not been systematically studied on a large scale (a limitation of this study, which is expected to be improved in future research). Consequently, the estimation of an accurate diagnostic threshold was interrupted by a limited number of volunteer samples and was outside the scope of this preliminary research. But it should theoretically be at least an order of magnitude away from the level in healthy

individuals' hair as there is a correlation between biochemical levels in hair and plasma (20), which can be explained by the hypothesis of how substances in blood are incorporated into hair (63). The present method has been able to accurately quantify the minimum levels of 7-DHC and CHOL in the hair of healthy infants. Therefore, predictably, it may be possible to identify SLOS patients from healthy people by analyzing hair as with traditional samples such as plasma.

CONCLUSION

A GC-MS analytical method was developed and validated for the simultaneous determination of two SLOS diagnostic biochemical indicators, 7-DHC and CHOL in human hair. The figures of merit were in good agreement with the guidelines of FDA (58) and EMA (59), which fully demonstrated the method's feasibility. As more efficient extraction methods and antioxidants protection were applied, the 7-DHC and higher concentrations of CHOL in the hair of healthy individuals were reported for the first time. This research emphasizes the importance of detecting 7-DHC and CHOL in hair as diagnostic biomarkers for SLOS, but due to the limited number of volunteers, it still needs to be extended to a larger group of SLOS patients for the establishment of the cut-off value for SLOS diagnosis using human hair. Hair from other parts of the human body and animals (healthy and SLOS individuals) could also be investigated. This innovation will also help explore many other diseases related to CHOL metabolism.

As far as we know, this is the first pilot study describing an analytical method for the simultaneous determination of 7-DHC and CHOL in human hair. It is expected that this research will fill the analytical gap and to be a powerful tool for clinical diagnosis and further research of SLOS.

Data availability

The data supporting this study are available from the corresponding author upon reasonable request. ⬛

Author contributions

Y. R., C. Z., and Y. Luo conceptualization; Y. Luo and Y. Z. methodology; Y. Luo and Y. Z. software; Y. Luo and Y. Z. validation; Y. Luo and Y. Z. formal analysis; Y. R., Y. Luo, and Y. Z. investigation; Y. R., Y. Luan, C. Z., L. M., and L. C. resources; Y. R., and Y. Luo data curation; Y. R., Y. Luo and Z. L. writing–original draft; Y. R., Y. Luo, Z. L., and L. C. writing–review and editing; Y. Luo and Y. Z. visualization; C. Z., Y. Luan., R. W. and Y. R. supervision; C. Z., Y. Luan, and Y. R. project administration; Y. Luan funding acquisition.

Author ORCIDs

Yulan Rao ⓘ https://orcid.org/0000-0002-5728-7888

REFERENCES

1. Schade, D. S., Shey, L., and Eaton, R. P. (2020) Cholesterol review: a metabolically important molecule. *Endocr. Pract.* **26**, 1514–1523
2. DeBarber, A. E., Eroglu, Y., Merkens, L. S., Pappu, A. S., and Steiner, R. D. (2011) Smith-Lemli-Opitz syndrome. *Expert Rev. Mol. Med.* **13**, e24
3. Kelley, R. I. (1995) Diagnosis of Smith-Lemli-Opitz syndrome by gas chromatography/mass spectrometry of 7-dehydrocholesterol in plasma, amniotic fluid and cultured skin fibroblasts. *Clin. Chim. Acta.* **236**, 45–58
4. Smith, D. W., Lemli, L., and Opitz, J. M. (1964) A newly recognized syndrome of multiple congenital anomalies. *J. Pediatr.* **64**, 210–217
5. Porter, F. D. (2008) Smith-Lemli-Opitz syndrome: pathogenesis, diagnosis and management. *Eur. J. Hum. Genet.* **16**, 535–541
6. Nowaczyk, M. J., and Irons, M. B. (2012) Smith-Lemli-Opitz syndrome: phenotype, natural history, and epidemiology. *Am. J. Med. Genet. C Semin. Med. Genet.* **160C**, 250–262
7. Lazarin, G. A., Haque, I. S., Evans, E. A., and Goldberg, J. D. (2017) Smith-Lemli-Opitz syndrome carrier frequency and estimates of in utero mortality rates. *Prenat. Diagn.* **37**, 350–355
8. Gao, C., Duan, J., Zhang, P., Gao, Y., Zhang, Y., Wang, Y., *et al.* (2020) [Clinical and genetic analysis of a Chinese pedigree affected with Smith-Lemli-Opitz syndrome]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi.* **37**, 1272–1275
9. Tamura, M., Isojima, T., Kasama, T., Mafune, R., Shimoda, K., Yasudo, H., *et al.* (2017) Novel DHCR7 mutation in a case of Smith-Lemli-Opitz syndrome showing 46,XY disorder of sex development. *Hum. Genome Var.* **4**, 17015
10. Nowaczyk, M. J., Waye, J. S., and Douketis, J. D. (2006) DHCR7 mutation carrier rates and prevalence of the RSH/Smith-Lemli-Opitz syndrome: where are the patients? *Am. J. Med. Genet. A.* **140**, 2057–2062
11. Donoghue, S. E., Pitt, J. J., Boneh, A., and White, S. M. (2018) Smith-Lemli-Opitz syndrome: clinical and biochemical correlates. *J. Pediatr. Endocrinol. Metab.* **31**, 451–459
12. Sallabi, S. M., Alhmoudi, A., Alshekaili, M., and Shah, I. (2021) Determination of Vitamin B3 Vitamer (Nicotinamide) and Vitamin B6 Vitamers in human hair using LC-MS/MS. *Molecules.* **26**, 4487
13. Suwannachom, N., Thananchai, T., Junkuy, A., O'Brien, T. E., and Sribanditmongkol, P. (2015) Duration of detection of methamphetamine in hair after abstinence. *Forensic Sci. Int.* **254**, 80–86
14. Gottardo, R., Fanigliulo, A., Sorio, D., Liotta, E., Bortolotti, F., and Tagliaro, F. (2012) Monitoring compliance to therapy during addiction treatments by means of hair analysis for drugs and drug metabolites using capillary zone electrophoresis coupled to time-of-flight mass spectrometry. *Forensic Sci. Int.* **216**, 101–107

15. Jezela-Stanek, A., Siejka, A., Kowalska, E. M., Hosiawa, V., and Krajewska-Walasek, M. (2020) GC-MS as a tool for reliable non-invasive prenatal diagnosis of Smith-Lemli-Opitz syndrome but essential also for other cholesterolopathies verification. *Ginekol. Pol.* **91**, 287–293

16. Lutjohann, D., Bjorkhem, I., Friedrichs, S., Kerksiek, A., Lovgren-Sandblom, A., Geilenkeuser, W. J., *et al.* (2019) First international descriptive and interventional survey for cholesterol and non-cholesterol sterol determination by gas- and liquid-chromatography-Urgent need for harmonisation of analytical methods. *J. Steroid Biochem. Mol. Biol.* **190**, 115–125

17. Serra, M., Matabosch, X., Ying, L., Watson, G., and Shackleton, C. (2010) Hair and skin sterols in normal mice and those with deficient dehydrosterol reductase (DHCR7), the enzyme associated with Smith-Lemli-Opitz syndrome. *J. Steroid Biochem. Mol. Biol.* **122**, 318–325

18. Masukawa, Y., Tsujimura, H., and Imokawa, G. (2005) A systematic method for the sensitive and specific determination of hair lipids in combination with chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **823**, 131–142

19. Son, H. H., Lee, D. Y., Seo, H. S., Jeong, J., Moon, J. Y., Lee, J. E., *et al.* (2016) Hair sterol signatures coupled to multivariate data analysis reveal an increased 7beta-hydroxycholesterol production in cognitive impairment. *J. Steroid Biochem. Mol. Biol.* **155**, 9–17

20. Ryu, H. K., Jung, B. H., Kim, K. M., Yoo, E. A., Woo, J. T., and Chung, B. C. (2006) Determination of cholesterol in human hair using gas chromatography-mass spectrometry. *Biomed. Chromatogr.* **20**, 999–1003

21. Cooper, G. A. A., Kronstrand, R., and Kintz, P. (2012) Society of Hair Testing guidelines for drug testing in hair. *Forensic Sci. Int.* **218**, 20–24

22. Salomone, A., Tsanaclis, L., Agius, R., Kintz, P., and Baumgartner, M. R. (2016) European guidelines for workplace drug and alcohol testing in hair. *Drug Test. Anal.* **8**, 996–1004

23. Dong, Z., Wang, C., Zhang, J., and Wang, Z. (2017) A UHPLC-MS/MS method for profiling multifunctional steroids in human hair. *Anal. Bioanal. Chem.* **409**, 4751–4769

24. Schaffer, M., Cheng, C. C., Chao, O., Hill, V., and Matsui, P. (2016) Analysis of cocaine and metabolites in hair: validation and application of measurement of hydroxycocaine metabolites as evidence of cocaine ingestion. *Anal. Bioanal. Chem.* **408**, 2043–2054

25. Boysen, T. C., Yanagawa, S., Sato, F., and Sato, K. (1984) A modified anaerobic method of sweat collection. *J. Appl. Physiol. Respir. Environ. Exerc. Physiol.* **56**, 1302–1307

26. Pragst, F., and Balikova, M. A. (2006) State of the art in hair analysis for detection of drug and alcohol abuse. *Clin. Chim. Acta.* **370**, 17–49

27. Horvath, A. L. (2009) Solubility of structurally complicated materials: 3. Hair. *Sci.World J.* **9**, 255–271

28. Kintz, P., Farrugia, A., Ameline, A., Eibel, A., and Raul, J. (2017) High risk of misinterpreting hair analysis results for children tested for methadone. *Forensic Sci. Int.* **280**, 176–180

29. Allibe, N., Eysseric-Guerin, H., Kintz, P., Bartoli, M., Bost-Bru, C., Grenier, F., *et al.* (2015) Amitriptyline poisoning of a baby: how informative can hair analysis be? *Forensic Sci. Int.* **249**, 53–58

30. Pantazides, B. G., Crow, B. S., Quinones-Gonzalez, J., Perez, J. W., Harvilchuck, J. A., Wallery, J. J., *et al.* (2021) Development of a clinical assay to measure chlorinated tyrosine in hair and tissue samples using a mouse chlorine inhalation exposure model. *Anal. Bioanal. Chem.* **413**, 1765–1776

31. Hobo, Y., Nishikawa, J., Miyashiro, Y., and Fujikata, A. (2020) Measurement of steroid hormones by liquid chromatography-tandem mass spectrometry with small amounts of hair. *Steroids.* **164**, 108732

32. Gomez-Gomez, A., and Pozo, O. J. (2020) Determination of steroid profile in hair by liquid chromatography tandem mass spectrometry. *J. Chromatogr. A.* **1624**, 461179

33. Boumba, V. A., Di Rago, M., Peka, M., Drummer, O. H., and Gerostamoulos, D. (2017) The analysis of 132 novel psychoactive substances in human hair using a single step extraction by tandem LC/MS. *Forensic Sci. Int.* **279**, 192–202

34. Grova, N., Wang, X., Hardy, E. M., Palazzi, P., Chata, C., and Appenzeller, B. (2020) Ultra performance liquid chromatography - tandem mass spectrometer method applied to the analysis of both thyroid and steroid hormones in human hair. *J. Chromatogr. A.* **1612**, 460648

35. Xing, X., Chen, Z., Li, J., Zhang, J., Deng, H., and Lu, Z. (2013) Study on dissolution mechanism of cortisol and cortisone from hair matrix with liquid chromatography-tandem mass spectrometry. *Clin. Chim. Acta.* **421**, 62–72

36. Chagas, A. G. D. R., Spinelli, E., Souza, T. M., Pinto Junior, J. A., and Pereira Netto, A. D. (2019) Simultaneous determination of alpha-, beta- and gamma-hydroxybutyric acids in micro-pulverized human hair by GC-MS: method development, validation and application. *Talanta.* **194**, 576–584

37. Beaumier-Gallon, G., Lanfranchi, J., Vergnes, M., Lairon, D., Pastor, J., Pauli, A. M., *et al.* (1998) Method for simultaneous measurements of traces of heptadeuterated cholesterol and cholesterol by gas chromatography–mass spectrometry: application in humans. *J. Chromatogr. B: Biomed. Sci. Appl.* **718**, 23–32

38. Gao, W., Kirschbaum, C., Grass, J., and Stalder, T. (2016) LC–MS based analysis of endogenous steroid hormones in human hair. *J. Steroid Biochem. Mol. Biol.* **162**, 92–99

39. Shi, Y., Wang, R., Yuan, S., Qiang, H., Shen, M., Shen, B., *et al.* (2020) UHPLC-MS/MS method for simultaneously detecting 16 tryptamines and their metabolites in human hair and applications to real forensics cases. *J. Chromatogr. B.* **1159**, 122392

40. Xu, L., Korade, Z., and Porter, N. A. (2010) Oxysterols from free radical chain oxidation of 7-dehydrocholesterol: product and mechanistic studies. *J. Am. Chem. Soc.* **132**, 2222–2232

41. Korade, Z., Xu, L., Shelton, R., and Porter, N. A. (2010) Biological activities of 7-dehydrocholesterol-derived oxysterols: implications for Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **51**, 3259–3269

42. Xu, L., Davis, T. A., and Porter, N. A. (2009) Rate constants for peroxidation of polyunsaturated fatty acids and sterols in solution and in liposomes. *J. Am. Chem. Soc.* **131**, 13037–13044

43. Corso, G., Rossi, M., De Brasi, D., Rossi, I., Parenti, G., and Dello Russo, A. (2002) Effects of sample storage on 7- and 8-dehydrocholesterol levels analysed on whole blood spots by gas chromatography–mass spectrometry-selected ion monitoring. *J. Chromatogr. B Anal. Tech. Biomed. Life Sci.* **766**, 365–370

44. Rola, R., Kowalski, K., Bieńkowski, T., Witwicki, J., and Studzińska, S. (2021) Evaluation of different biological matrices to assess the vitamin D status in newborns using LC-MS/MS. *Microchem J.* **168**, 106368

45. Gelzo, M., Clericuzio, S., Barone, R., D'Apolito, O., Dello, R. A., and Corso, G. (2012) A routine method for cholesterol and 7-dehydrocholesterol analysis in dried blood spot by GC-FID to diagnose the Smith-Lemli-Opitz syndrome. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **907**, 154–158

46. Helmschrodt, C., Becker, S., Schroter, J., Hecht, M., Aust, G., Thiery, J., *et al.* (2013) Fast LC-MS/MS analysis of free oxysterols derived from reactive oxygen species in human plasma and carotid plaque. *Clin. Chim. Acta.* **425**, 3–8

47. Liu, W., Yin, H., Akazawa, Y. O., Yoshida, Y., Niki, E., and Porter, N. A. (2010) Ex vivo oxidation in tissue and plasma assays of hydroxyoctadecadienoates: Z,E/E,E stereoisomer ratios. *Chem. Res. Toxicol.* **23**, 986–995

48. Liu, W., Xu, L., Lamberson, C., Haas, D., Korade, Z., and Porter, N. A. (2014) A highly sensitive method for analysis of 7-dehydrocholesterol for the study of Smith-Lemli-Opitz syndrome[S]. *J. Lipid Res.* **55**, 329–337

49. Liu, W., Xu, L., Lamberson, C. R., Merkens, L. S., Steiner, R. D., Elias, E. R., *et al.* (2013) Assays of plasma dehydrocholesteryl esters and oxysterols from Smith-Lemli-Opitz syndrome patients. *J. Lipid Res.* **54**, 244–253

50. Choi, M. H., Kim, K. R., and Chung, B. C. (2000) Determination of estrone and 17 beta-estradiol in human hair by gas chromatography-mass spectrometry. *Analyst.* **125**, 711–714

51. Choi, M. H., and Chung, B. C. (1999) GC-MS determination of steroids related to androgen biosynthesis in human hair with pentafluorophenyldimethylsilyl-trimethylsilyl derivatisation. *Analyst.* **124**, 1297–1300

52. Leporati, M., Bergoglio, M., Capra, P., Bozzetta, E., Abete, M. C., and Vincenti, M. (2014) Development, validation and application to real samples of a multiresidue LC-MS/MS method for determination of beta2 -agonists and anabolic steroids in bovine hair. *J. Mass Spectrom.* **49**, 936–946

53. van de Merbel, N. C. (2008) Quantitative determination of endogenous compounds in biological samples using chromatographic techniques. *Trac. Trends Anal. Chem.* **27**, 924–933

ASBMB

54. Voegel, C. D., Baumgartner, M. R., Kraemer, T., Wust, S., and Binz, T. M. (2021) Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS - Insights into EC baseline values and correlation to steroid concentrations. *Talanta.* **222**, 121499

55. Thakare, R., Chhonker, Y. S., Gautam, N., Alamoudi, J. A., and Alnouti, Y. (2016) Quantitative analysis of endogenous compounds. *J. Pharm. Biomed. Anal.* **128**, 426–437

56. Meng, L., Chen, S., Zhu, B., Zhang, J., Mei, Y., Cao, J., *et al.* (2020) Application of dispersive liquid-liquid microextraction and GC-MS/MS for the determination of GHB in beverages and hair. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **1144**, 122058

57. Quinete, N., Bertram, J., Reska, M., Lang, J., and Kraus, T. (2015) Highly selective and automated online SPE LC-MS3 method for determination of cortisol and cortisone in human hair as biomarker for stress related diseases. *Talanta.* **134**, 310–316

58. Food and Drug Administration. (2018) Bioanalytical Method Validation Guidance for Industry. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry

59. European Medicines Agency. (2012) Guideline on bioanalytical method validation. EMA. *Guidel.* **44**, 1–23

60. Thomas, J. L., Strickland, E. C., Lloyd, E. W., Donnelly, C. C., Rankoth, A., Pieczonka, S. M., *et al.* (2020) Endogenous GHB in segmented hair part I: inter-individual variation for group comparisons. *J. Anal. Toxicol.* **44**, 628–636

61. Zhu, M., Yuan, L., Wu, Y., Chu, L., Wang, W., Zhang, H., *et al.* (2022) Simultaneous LC-MS/MS quantification of glucocorticoids, melatonin and its metabolites in hair. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **1196**, 123217

62. Dietzen, D. J., Bennett, M. J., Lo, S. F., Grey, V. L., and Jones, P. M. (2016) Dried blood spot reference intervals for steroids and amino acids in a neonatal cohort of the national children's study. *Clin. Chem.* **62**, 1658–1667

63. Cone, E. J. (1996) Mechanisms of drug incorporation into hair. *Ther. Drug Monit.* **18**, 438–443