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Isotope-labeling derivatization with 3-nitrophenylhydrazine for LC/multiple-reaction monitoring-mass-spectrometry-based quantitation of carnitines in dried blood spots

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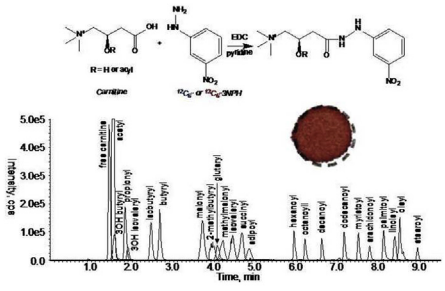
HIGHLIGHTS

- Pre-analytical derivatization with 3-nitrophenylhydrazine (3NPH) was optimized for LC/MRM-MS of carnitines.
- Use of a 2-step carnitine extraction and isotope-labeled ISs led to precise and accurate quantitation of 24 carnitines
- $^{13}C_6$-3NPH derivatives were used as ISs for calibration.
- Measurements of carnitines in DBSs on cellulose or cotton-based paper showed good stabilities after 4-h sunlight exposure.
- Concentration changes in several carnitines were observed after cycled temperature transitions during DBS storage.

GRAPHICAL ABSTRACT



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ABSTRACT

Carnitines are diagnostic biomarkers of fatty acid oxidation defects and organic acidemias. Quantitative measurements of various carnitines in dried blood spot (DBS) have potential use in remote health applications for disease diagnosis and epidemiological surveillance. To provide an improved LC/multiple-reaction monitoring (MRM)-MS method for quantitation of carnitines in DBS, 3-nitrophenylhydrazine (3NPH) was tested as a high-efficiency chemical isotope-labeling reagent for pre-analytical derivatization of 24 routinely-analyzed species. Reaction conditions were optimized and carnitine structural isomers were separated by reversed-phase LC with positive-ion MRM/MS detection, giving on-column lower LOQs of sub- to low-femtomole levels. $^{13}C_6$-3NPH was used to produce $^{13}C_6$- or $^{13}C_{12}$-labeled derivatives of the mono- and di-carboxylic carnitines in a “one-pot” reaction. These labeled analogues were used as stable isotope-labeled internal standards to compensate for possible ESI matrix effects. Combined with an optimized, two-step procedure for the extraction of carnitines from DBS, this isotope-

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LC/MRM-MS
stability testing

labeling derivatizaiton - LC/MRM-MS method provided good linearity, high precision (intra-day CVs of ≤7.8% and inter-day CVs of ≤8.8%) and high accuracy (three levels of standard substances spiked in, with recoveries of 86.9%–109.7%) quantitation of carnitines in three sets of DBSs on cellulose or cotton filter paper. This method was then applied to determine the concentration changes of the analytes in the DBSs under two stability-testing regimes: 1) a one-time 4-h sunlight exposure and 2) a set of cycled temperature transitions (−20 °C for 2 days, 40 °C for 2 days, and back to −20 °C for 2 additional days). All of the carnitines showed good stabilities under the first testing condition. Under the second testing condition, free carnitine showed concentration *increases* of 9.3%–16.1%; acetyl carnitine, 3-OH butyryl carnitine, and malonyl carnitine showed concentration *decreases* of 12.2%–17.3%, 12.9%–17.1% and 10.7%–15.3%, respectively, and other 20 acyl carnitines showed concentration changes of <10% in three sets of DBSs on cellulose or cotton filter paper. These preliminary stability-testing results indicate a need to more systematically investigate the effects of various environmental conditions on the chemical stabilities of carnitines in DBS specimens if this sampling method is to be used in remote health applications.

Abbreviations

3NPH	3-nitrophenylhydrazine
DBS	dried blood spot
CIL	chemical isotope labeling
CoA	coenzyme A
IEM	inborn error of metabolism
SIS	stable isotope-labeled internal standard
ILCD	isotope labeling chemical derivatization
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
DC	di-carboxylic
MRM	multiple-reaction monitoring
DP	declustering potential
UPLC	ultrahigh-performance liquid chromatography
LLOD	lower limit of detection
LLOQ	lower limit of quantitation

1. Introduction

Carnitines are quaternary ammonium compounds that are responsible for transporting fatty acyl groups across the inner mitochondrial membrane from the cytosol into the mitochondrial matrix under the regulation of carnitine palmitoyltransferase I and carnitine palmitoyltransferase II in fatty acid oxidation [1]. Carnitines also play a role in stabilizing coenzyme A (CoA) and acyl-CoA levels in cells through its ability to receive or donate an acyl group [2]. Abnormalities in the concentrations of some acyl carnitines in blood and urine are diagnostic markers for dozens of metabolic disorders including fatty acid oxidation defects and organic acidemias (also known as organic acidurias) [3], and for multiple inherited diseases of peroxisomal and mitochondrial oxidation processes [4]. Moreover, carnitines exert substantial antioxidant action and regulate immune functions in humans. Therefore, measurements of free and acyl carnitines in biological samples have biochemical, physiological, and clinical significances.

Carnitine profile analysis in plasma or serum is well established as a test for the diagnosis and monitoring of treatments of fatty acid oxidation defects and organic acidemias [3]. Measurements of free and acyl carnitines in dried blood spot (DBS) specimens is routine for the screening of newborns for inborn errors of metabolism (IEMs) [5]. These are typically done by methanol extraction of carnitines from DBS, followed by butylation [3], and then FIA-MS/MS using precursor ion scanning on *m/z* 85 (a characteristic MS/MS fragment ion of carnitines). The FIA-MS/MS technique, however, cannot distinguish between isomeric carnitines and sometimes only generates semi-quantitative profiles for some acyl carnitine biomarkers [6]. LC/MS based methods are the predominant analytical technique for separation and more quantitative measurements of various carnitines. Hydrophilic interaction liquid chromatography (HILIC)/MRM-MS has been used for determination of free and acyl carnitines in blood and urine [7–10], with high detection sensitivity due to the high-percentage organic solvents in the HILIC mobile phases favoring ESI. HILIC, however, has limited capacities for resolving the structural isomers of acyl carnitines [8–10]. Due to the large difference in polarity of various carnitines, ranging from hydrophilic free carnitine, hydroxyl (OH)- and dicarboxylic (DC) short-chain acyl carnitines to hydrophobic long-chain and very-long chain acyl carnitines, it is challenging to have good retention and good peak shape for both hydrophilic and hydrophobic carnitines on a single RPLC column. Ion pairing RPLC with heptafluorobutyric acid has been used to enhance LC retention of very hydrophilic carnitines for their quantitation [11,12].

Another often-used strategy in RPLC for the analysis of free and acyl carnitines is to incorporate pre-analytical derivatization to improve the chromatographic retention of highly hydrophilic carnitines and to better resolve structural isomer species for their detection and quantitation. Fluorescent labeling with 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole has been used for the LC determination of various carnitines in human plasma [13], and 4′-bromophenacylbromide was used for the LC determination of acyl carnitines in the urine of IEM patients [14]. Minkler proposed the use of pentafluorophenacyl trifluoromethanesulfonate to derivatize up to more than 60 carnitines for their “second-tier” analysis by LC/MRM-MS [15–18]. In 2015, Giesbertz et al. reported the use of butylation for the analysis of carnitines including some odd-carbon species by LC/MRM-MS [19].

Matrix effects (i.e., ionization suppression or enhancement) often encountered in ESI are a major factor that affects the precision and accuracy of bioanalysis using LC/MS techniques [20]. The use of stable isotope-labeled internal standard (SIS) is well recognized as an ideal means for reliable quantitation in LC/MS based measurements by compensating for ionization suppression or enhancement resulting from the components co-eluting with an analyte [20]. In almost all of the LC/MS or LC/MRM-MS methods for quantitation of various carnitines published thus far, with or without pre-analytical chemical derivatization, only a few selected deuterium-labeled analogues of carnitines were used as internal standards, due to the lack of SISs for all of the targeted carnitines. An

alternative strategy to overcome the hurdle of matrix effects of ESI in LC/MS is the use of isotope labeling chemical derivatization (ILCD), i.e., the use of a stable heavy isotope (such as ^{13}C) -labeled chemical tagging reagent which reacts with standard substances of the targeted analytes to produce isotope-labeled derivatives which can be used as internal standards in LC/MS-based measurements. At present, this strategy has been used to provide relative or absolute quantitation of endogenous metabolites such as amino acids/amines [21] and hydroxyl compounds [22] by using $^{13}C_2$-dansyl chloride; carboxylic acids by using $^{13}C_2$-dimethylaminophenacyl bromide [23] or deuterium $(D)_4$-2-dimethylaminoethylamine [24]; short-chain fatty acids by using $^{13}C_6$-3-nitrophenylhydrazine (3NPH) [25]; and *cis*-diol containing brassinosteroids by using D_5-4-phenylaminomethyl-benzeneboronic acid [26].

The purpose of this current study is to evaluate the use of $^{12}C_6/^{13}C_6$-3NPH as a pair of pre-analytical ILCD reagents to provide an improved LC/MRM-MS method for the precise and accurate quantitation of 24 routinely-analyzed free and acyl carnitines. We also report the use of this new method to determine the stabilities of carnitines, including several pairs of structural isomers, in DBS specimens onto three sets of cellulose or cotton-filter paper collection cards. In the past, the long-term [27] and short-term [28] stabilities of selected amino acids and carnitines in DBS specimens were analyzed by FIA-MS/MS. Here, we use a high-accuracy LC/MRM-MS method described in this study to determine the stabilities of an expanded panel of carnitines, i.e., the 24 routinely-analyzed species, in DBS samples under environmental conditions that might be encountered in remote health applications.

2. Materials and methods

2.1. Solvents, reagents and standard substances

LC/MS grade methanol, acetonitrile, isopropanol, water, formic acid and TFA; AR grade 3NPH·HCl and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).HCl and HPLC grade pyridine were obtained from Sigma-Aldrich (St. Louise, MO, USA). $^{13}C_6$-3NPH.HCl was custom-synthesized by IsoSciences (King of Prussia, PA, USA). L-Carnitine (C0) chloride, isobutyryl L-carnitine (C4) chloride, butyryl L-carnitine (C4) chloride, and myristoyl L-carnitine (C_{14}) chloride were also obtained from Sigma-Aldrich. Acetyl L-carnitine (C2) chloride, L-propionyl carnitine (C3) chloride, L-octanoyl carnitine (C8), L-lauroyl carnitine (C12) chloride, and L-palmitoyl carnitine (C16) chloride were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Malonyl carnitine (C3DC), succinyl carnitine (C4DC) chloride, methylmalonyl DL-carnitine (C4DC) chloride (mixture of diastereomers), 3-hydroxybutyryl carnitine (C4OH), isovaleryl L-carnitine (C5) chloride, 2-methylbutyryl L-carnitine (C5) chloride, L-glutaryl carnitine (C5DC), 3-hydroxyisovaleryl carnitine (C5OH), hexanoyl carnitine (C6), adipoyl L-carnitine (C6DC) trifluoroacetate, decanoyl carnitine (C10), stearoyl carnitine (C18) chloride, oleoyl L-carnitine (C18:1) chloride, linoleoyl carnitine (C18:2), and arachidonoyl carnitine (C20:4) chloride were acquired from Toronto Research Chemicals Inc. (Toronto, ON, Canada). The authentic compounds of these carnitines were used, without further purification, as the reference standards for quantitative analysis.

2.2. DBS preparation

Whole blood – collected and pooled from healthy female adult donators – was obtained from BioreclamationIVT (Westbury, NY, USA) within 48 h after blood collection. Its hematocrit was measured to be 49% ± 1%. Three sets of World Health Organization-approved and United States Food and Drug Administration-cleared blood collection cards, including Whatman 903 Protein Saver (lot No. 7027015 W142), Whatman FTA DMPK-C (lot No. ET70110315), and PerkinElmer 226 Spot Saver (lot No. 101535/1005002), were used to prepare the DBS specimens from the whole blood. The blood-absorbing matrix of the 903 and DMPK-C cards was cellulose filter paper, while the 226 cards had cotton filter paper as the absorbing matrix. To prepare the DBSs, liquid blood was agitated with a magnetic stirrer at 300 rpm over ice, and 30-μL blood aliquots were precisely pipetted and blotted onto the filter paper on each spot. The spotted blood was air-dried for at least 3 h before the cards were sealed in zip-lock plastic bags and stored in a −80 °C freezer. The use of human blood and DBS specimens was approved by the research ethics committee of University of Victoria.

2.3. Ultrahigh-performance liquid chromatography (UPLC)/MRM-MS

An Agilent 1290 liquid chromatograph coupled to a Sciex 4000 QTRAP mass spectrometer *via* a Turbo-V ion spray ESI source was used. The MS instrument was operated in the positive-ion MRM mode for quantitative measurements. The Q1 to Q3 ion transitions (2 pairs per analyte; one as qualifier and the other as quantifier), the declustering potentials (DPs) in the ion source region, and the collision energies (CEs) were optimized by direct infusion of 10-μM solutions of individually $^{12}C_6$-3NPH-derivatized carnitines, delivered at 8 μL/min using a syringe solvent pump. An Agilent Eclipse Plus RRHD C_{18} column (2.1 mm I.D. x 150 mm, 1.8 μm) was used for the LC separation, with 0.1% (v/v) formic acid in water (solvent A) and 0.1% formic acid in acetonitrile:isopropanol (1:1, v/v) (solvent B) as the mobile phases for binary-solvent gradient elution. The optimized gradient was 18%–25% B in 5 min; 25%–50% B in 1 min, and 50%–90% B in 4 min, at a flow rate of 0.3 mL/min. The column was washed with 100% B for 2 min before column equilibration for 4 min at 18% B between injections. The column temperature was 55 °C and the LC autosampler temperature was 5 °C. The ESI operating parameters included ion spray voltage, 5200 V; heater temperature, 500 °C; source gas 1 pressure, 35 arbitrary units; source gas 2, 25 arbitrary units; and curtain gas flow, 22 arbitrary units.

2.4. Optimization of 3NPH derivatization

Optimization of the 3NPH derivatization was carried out by reacting 50 μL of 50 μM of each carnitine in a mixed solution with 50 μL of varying concentrations of 3NPH·HCl (25, 50, 100, 150, or 200 mM), 50 μL of varying concentrations of EDC.HCl (25, 50, 100, 150, or 200 mM), and 50 μL of pyridine solution (0.5%, 1%, 1.5%, 3%, 4.5%, or 6%), in aqueous methanolic solutions as the reaction media (methanol concentrations were 30%, 40%, 50%, 60%, 70%, or 80%). The derivatizing reactions were allowed to proceed at different reaction temperatures (10, 20, 30, 40, 50, or 60 °C) and for different time periods (10, 20, 30, 40, 50, or 60 min). Chromatographic peak areas were measured by UPLC/MRM-MS under the optimized LC and MRM conditions to compare the reaction efficiencies for each set of the reaction conditions tested.

2.5. Optimization for extraction of carnitines in DBS

The entire blood spot was punched out of each DBS sample using a 12-mm I.D. handheld stainless steel hole-punch, cut into small pieces, and put into a 2-mL capless Eppendorf tube. The carnitines in the DBSs were extracted either in a single step or in two steps, using different solvents. For the single-step extractions, 1 mL of methanol, acetonitrile, methanol:acetonitrile (1:1, v/v), 80%

aqueous acetonitrile, or 80% aqueous methanol:acetonitrile (1:1, v/v), with or without 0.02% TFA (v/v), were tested and compared as the extraction solvents. After the addition of each solvent, the tubes were capped and vortex-mixed on a digital mixer at 3000 rpm for 15 s to 2 min, sonicated in an ice-water bath for 2–10 min, and then centrifuged at 21,000 x g and 10 °C for 10 min in an Eppendorf 5420R centrifuge. For the two-step extractions, 200 μL of water or 0.02% TFA in water was added to each tube, followed by vortex-mixing for 15 s at 3000 rpm. After a 5-s spin-down in the centrifuge, 800 μL of acetonitrile or methanol:acetonitrile (1:1. v/v), with or without 0.02% TFA in each solvent, was then added. The tubes were vortex-mixed again for 15 s, followed by sonication for 2–10 min in an ice-water bath, before centrifugation at 21,000 x g for 10 min. After the centrifugal clarifications following the one- or two-step extraction protocols, 400 μL of each supernatant was precisely transferred to another Eppendorf tube and dried in a speed-vacuum concentrator. Each residue was resuspended in 75 μL of 60% aqueous methanol and then subjected to the 3NPH derivatization and analyzed by UPLC/MRM-MS, using the same procedure as described for the quantitation in section *2.6.3* below. The peak areas were measured and compared to find the optimal extraction conditions for the subsequent quantitative analyses.

2.6. Quantitation of carnitines in DBS

2.6.1. Standard solution

Stock solutions of 24 carnitines were prepared by dissolving each reference standard in methanol to produce the same 2.4 mM concentration. Equal volumes of these stock solutions were mixed and the resulting solution was diluted stepwise at a dilution ratio of 1–3 (v/v) with 60% aqueous methanol to produce a series of working standard solutions, with concentrations ranging from 0.1 nM to 100 μM for each standard.

2.6.2. Generation of $^{13}C_6$-3NPH derivatized internal standards

One-hundred (100) μL of a standard solution that contained all 24 carnitines at a concentration of 10 μM–50 μM for each standard, depending on the endogenous concentrations of individual carnitines in whole blood, was added to an Eppendorf tube containing 1.0 mg of $^{13}C_6$-3NPH·HCl. After 50 μL of a 100-mM EDC/1.5% pyridine solution in 60% aqueous methanol was added, the mixture was allowed to react at 30 °C for 30 min. During the incubation period, the tube was taken out and vortex mixed at least twice to make sure there were no unreacted carnitines in the tube. After the incubation period, the solution was diluted to 100 mL with 40% aqueous methanol and was used as the SISs for the UPLC/MRM-MS quantitation. This internal standard solution could be used for at least 6 months when it was stored in a −20 °C freezer.

2.6.3. Sample preparation and quantitation of carnitines in DBS

The carnitines in three sets of the DBS samples were extracted using an optimized two-step procedure, based on the optimization of the sample preparation described above. Briefly, the dried blood was punched out of the collection cards as an entire spot and then cut into small pieces which were placed in a 2-mL capless Eppendorf tube. 200 μL of 0.02% TFA in water was added and the tube was vortex mixed for 15 s at 3000 rpm, followed by sonication for 2 min in an ice-water bath. 800 μL of methanol:acetonitrile:TFA (50:50:0.02, v/v/v) was then added, and the mixture was vortex mixed for 15 s and sonicated for 3 min before centrifugal clarification at 21,000 x g and 10 °C in an Eppendorf 5420R centrifuge for 10 min. 400 μL of the clear supernatant was transferred to another 2-mL Eppendorf tube and was dried in a speed-vacuum concentrator. The residue was resuspended in 75 μL of 60% aqueous methanol.

For derivatization, 75 μL of each standard solution, or each of the sample solutions prepared as described above, was mixed with 37.5 μL of 100-mM 3NPH·HCl solution in 60% aqueous methanol and 37.5 μL of mixed 100-mM EDC.HCl/1.5% pyridine in 60% aqueous methanol. The mixture was allowed to react at 30 °C for 30 min before 100 μL of water and 50 μL of the SIS solution were sequentially added followed by vortex-mixing. The resulting sample solutions were centrifuged at 21,000 x g for 5 min. A 10-μL aliquot of each standard solution or the clear supernatant of each sample solution was injected for UPLC/(+)ESI-MRM-MS runs. All the data files were recorded with the Sciex's *Analyst* software suite and were processed with the Sciex's *MultiQuant* 1.2 software suite. The calibration curves were constructed as linear regressions of the analyte-to-SIS peak area ratios (As/Ai) measured from the injected standard solutions and the molar concentrations (nM) within the appropriate concentration range for each carnitine. The concentrations of the carnitines in DBSs were determined from the calibration curves of individual carnitines with the measured As/Ai ratios from the sample solutions. Statistical analyses were performed with the SYSTAT software suite (San Jose, CA, USA).

2.7. Validation of the quantitation method

2.7.1. Analytical sensitivity

To estimate the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) of each carnitine, serially diluted standard solutions at the same dilution ratio of 1–3 (v/v) were derivatized with $^{12}C_6$-3NPH and analyzed by UPLC/MRM-MS, in the same way as described in 2.6.3. The lowest concentration that would yield a signal-to-noise ratio of at least 3, while giving a CV of the measured peak area ratios for individual analytes of ≤15% for triplicate injections (n = 3), was defined as the LLODs. The lowest concentrations which would yield a signal-to-noise ratio of at least 10, while giving a CV of the measured peak area ratios for the individual analytes of ≤10% for triplicate injections (n = 3), and where inclusion of these lowest concentrations in a least-square regression with internal standard calibration for each analyte generated a correlation coefficient (R^2) of at least 0.999, was defined as the LLOQ.

2.7.2. Matrix effects

To determine if there are significantly unfavorable influences of possible matrix effects on the quantitation of carnitines in DBS using the proposed new ILCD-UPLC/MRM-MS method, 75-μL aliquots of the serially diluted standard solutions, as described in *2.6.1*, were added to the aliquots of the metabolite extract residues. Each aliquot was obtained by drying 400 μL of the clear supernatants pooled from the optimized extractions of carnitines in 10 spots for each DBS set. The solutions were then derivatized with $^{12}C_6$-3NPH. After the derivatization, 100 μL of water and 50 μL of the SIS solution were added. The resulting solutions were analyzed by UPLC/MRM-MS to determine the linearity of each analyte in the presence of the sample matrix. The linearity was compared with that of each analyte prepared in the same way, but with matrix-free neat-solvent standard solutions.

2.7.3. Precision and accuracy of quantitation

The intra-day precision was determined as the %CV of the measured concentrations of individual analytes when the sample preparation and quantitation for each DBS set was performed every 3 h, for six times (n = 6) within the same day. The inter-day precision was determined as the %CV when the sample preparation and quantitation was performed once per day for each DBS set for five continuous days (n = 5). The quantitation accuracy was determined as the standard-substance spiking recoveries of each carnitine at

three spiking levels, equivalent to 100%, 250%, and 500%, of their endogenous concentrations as measured in the DBSs. Both the total concentrations (with spiked-in reference standards) and the endogenous concentrations of the individual carnitines in each set of DBS samples were calculated from their self-calibration curves, which had been prepared during the accuracy tests, along with the sample analyses. The % recoveries were calculated as (total concentration - endogenous concentration)/spiked-in concentration $\times 100$. Four replicates ($n = 4$) for each sample set and for each spiking level were tested to determine the recoveries.

2.8. Stability testing

Two stability-testing experiments were performed: *(1)* a one-time sunlight exposure, by flattening and placing the DBS cards under direct sunlight for 4 h (the air temperature was 21 °C on that day), and *(2)* a 6-day/2-temperature cycling scheme, done by sealing the DBS cards in individual plastic bags containing two packs of Ruby-indicating drying silica gel (6 g/bag) (Sigma-Aldrich) and placing the sealed bags at −20 °C for 2 days, then at 40 °C for 2 days, and back to −20 °C for 2 additional days. After each stability experiment, samples for quantitation were collected and processed as soon as possible, using the analytical method developed and validated in this study. When the quantitation was performed, the DBSs that had been stored at −80 °C for each set were used as the control samples. The carnitine concentrations were measured over four replicate spots of each sample set and the concentrations in the DBSs subjected to the stability testing were reported as a % of the corresponding concentration measured in the control.

3. Results and discussion

In this study, 24 carnitines routinely analyzed during IEM screening and for the diagnostic analysis of fatty acid oxidation defects and organic acidemias [3] in blood or in DBSs were chosen as the targets for the analytical method development and for the short-term stability testing. These carnitines could be reproducibly quantified in the whole blood that was used to prepare the DBS samples in this study.

3.1. Derivatization of carnitines with 3NPH

The large difference in polarity of these 24 carnitines, ranging from very hydrophilic free carnitine (C0) to highly hydrophobic stearyl carnitine (C18), made it difficult to have good retention of all the 24 carnitines on a single RPLC column, without chemical derivatization. In addition, due to the lack of available stable isotope-labeled analogues to be used as SISs for all of the targeted carnitines, the accurate measurements of these carnitines in DBS, which has a complicated matrix than plasma or serum, is challenging due to the ESI matrix effects resulting from their co-eluting components during the UPLC-MRM/MS runs. To provide an improved method for appropriate retention of various carnitines on a single RPLC column, to produce good separations of different carnitine structural isomers, and – most importantly – to have SISs for all the targeted carnitines for their accurate quantitation, $^{12}C_6/^{13}C_6$-labeled 3NPH was tested as a pre-analytical chemical isotope-labeling derivatization reagent. The reaction of 3NPH with carnitines, with the presence of EDC as a condensing reagent and pyridine as a weakly basic catalyst, follows a reaction mechanism of nucleophilic addition − elimination by converting the mono- or di-carboxylic groups in each carnitine molecule to their structurally stable 3-nitrophenylhydrazides.

The optimized MRM transitions, including the quantifier ions, declustering potentials (DPs), and collision energies (CEs) for the $^{12}C_6$-3NPH derivatives of the 24 carnitines involved in this study are given in Supplementary Material Table S1. Unlike the 3NPH-derivatized organic acids, which were preferentially detected in the negative-ion mode [25,29], all of the 3NPH-derivatized carnitines were more favorably detected in the (+) ion mode, due to the existence of a positively-charged quaternary ammonium group in the each derivatized molecule.

Various reaction conditions for the derivatization, including the concentrations of 3NPH, EDC, pyridine and methanol in the reaction, as well as the reaction temperatures and time periods were optimized. Fig. 2A and B, respectively, show how the concentrations of 3NPH and EDC in the reaction media influenced the reaction efficiencies as measured by the chromatographic peak areas of the individual derivatives. Based on these plots, 25 mM of 3NPH and 25 mM of EDC were chosen as the optimal reaction conditions. The effects of reaction temperature and reaction time on the derivatization are shown in Fig. 1C and D. From these plots, the combination of 30 °C for 30 min was selected as the optimal reaction temperature and time period. Other factors, such as pyridine concentration in a range of 0.25%–1.0%, and methanol concentration ranging from 50% to 80% in the reaction media, did not lead to significant differences in the measured peak areas of the individual carnitines (data not shown). For consistency, 0.375% pyridine and 60% methanol were used in all of the subsequent quantitative measurements.

3.2. Chromatographic separation of structural isomers of carnitines

Among the 24 carnitines, there are three pairs of structural isomers, i.e., isobutyryl carnitine *versus* butyryl carnitine, 2-methylbutyryl carnitine *versus* isovaleryl carnitines, and methylmalonyl carnitine *versus* succinyl carnitine. The different isomeric carnitines are involved in different disorders of fatty acid defects or organic acidemias [3]. Therefore, it is necessary to be able to separate the structural isomers of carnitines from each other. After comparisons of several reversed-phase C_{18} LC columns in combination with different mobile phases, an Agilent Eclipse RRHD C_{18} column (2.1 × 150 cm, 1.8 μm) with a mobile phase composed of 0.1% FA in water (solvent A) and 0.1% FA in acetonitrile-isopropanol (1:1, v/v) (solvent B) for binary-solvent gradient elution was used. After a few optimization steps, good separations between the different pairs of the structural isomers were achieved, with the elution gradient described in the section "2.3 *UPLC/MRM-MS*". The representative LC/MRM-MS chromatograms of the 24 derivatized carnitines in a mixed standard solution (A) and their detection in a DBS sample solution (B) are shown in Fig. 2. Baseline separation of all the structural isomers among the carnitines was achieved. The 24 targeted carnitines had retention times between 1.5 and 9 min with sharp peak shapes, except for the di-carboxylic carnitines, which eluted from the column with retention times between 3.6 and 5.0 min. These di-carboxylic carnitines showed both broader peaks and more tailing than did the mono-carboxylic carnitines eluting within the same retention-time range, probably due to the fact that the two carboxylic groups in di-carboxylic carnitines reacted with two 3NPH molecules, which induced stronger polar-polar interactions between the derivatives and the residual free-silanol groups (Si-OH) on the surface of the column-packing resin.

3.3. One-pot reaction for generation of ^{13}C-labeled internal standards

To generate SISs for all of the targeted carnitines for more accurate quantitation, we used $^{13}C_6$-3NPH as a single ILCD reagent to react with a mixed standard solution of the 24 carnitines under the optimized reaction conditions to produce the $^{13}C_6$-labeled

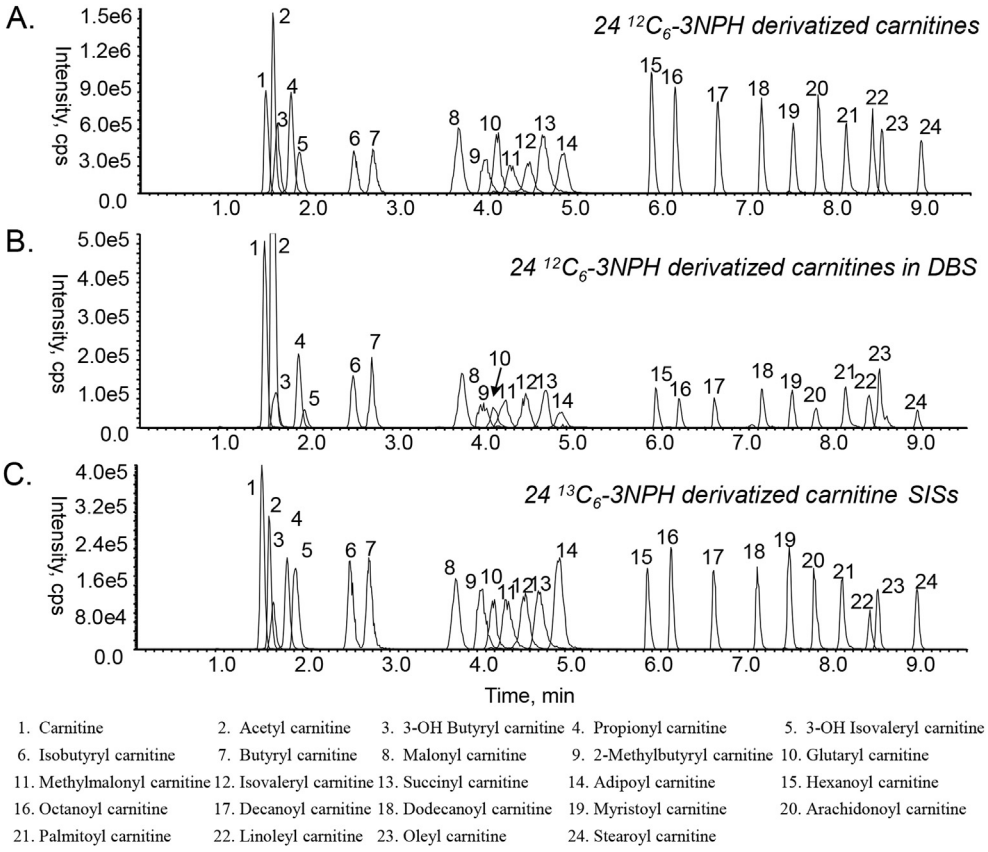

Fig. 1. Representative UPLC/MRM-MS chromatograms of the 3NPH derivatives of 24 carnitines detected with MRM in a standard solution (**A**) and in a DBS sample (**B**). Figure 1C is a typical UPLC/MRM-MS chromatogram showing the 24 $^{13}C_6$-3NPH derivatized carntines.

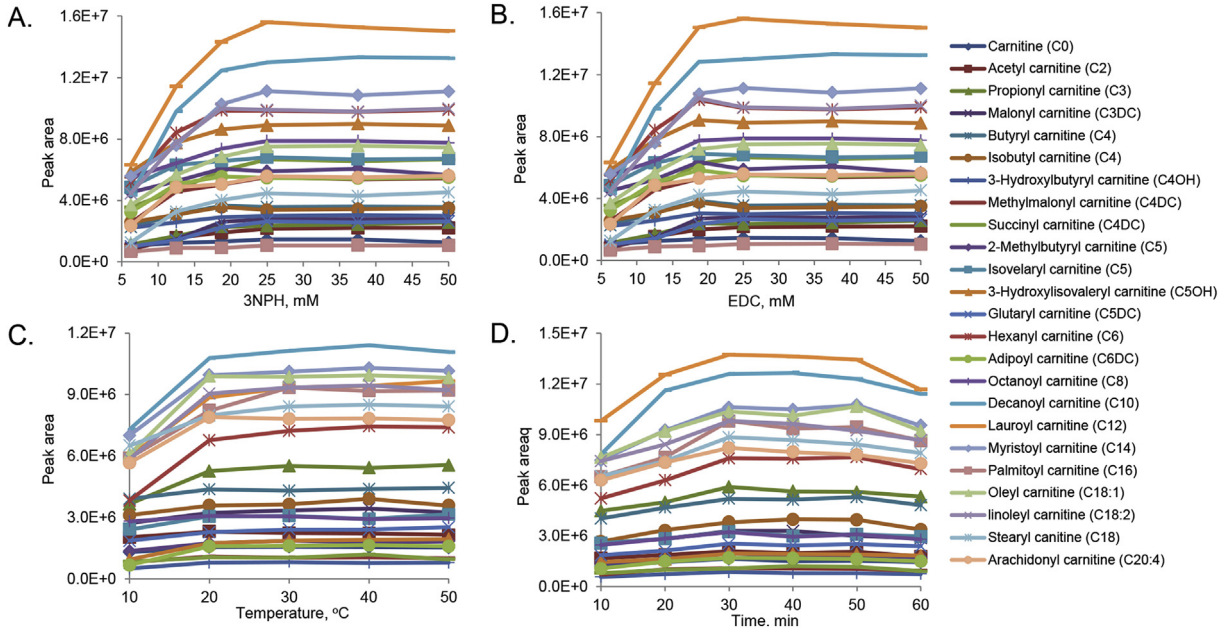

Fig. 2. Effect of different reaction conditions on 3NPH derivatization of 24 carnitines, including 3NPH concentration (**A**), EDC concentration (**B**), reaction temperature (**C**), and reaction time (**D**).

derivatives of the mono-carboxylic carnitines and the $^{13}C_{12}$-labeled derivatives of the di-carboxylic carnitines (e.g., methylmalonyl carnitine) in a "one-pot" reaction. After reaction, the resulting solution was diluted with 40% aqueous methanol and was used as the SIS solution. This SIS solution contained 10 nM–50 nM of each individual carnitine, with the concentrations partially balanced

with their endogenous concentrations measured in DBSs. Fig. 1C is the UPLC/MRM-MS chromatogram that shows the 24 $^{13}C_6$-3NPH derivatized carntines. As tested during this study, this SIS solution could be used for at least 6 months when it had been stored at −20 °C.

The plots in Supplementary Material Fig. S1 show the analyte-to-SIS peak area ratios (As/Ai) of 8 representatives of the 24 3NPH derivatized carnitines, which were measured from injections of a mixed standard solution and three sample solutions from each DBS set, and run every 6 h for two days, while these solutions were maintained at 5 °C in the autosampler. The minimal %CVs (<7.2%, n = 9, in all cases) of the measured peak ratios and the fast LC/MRM-MS runs (RTs of <9 min for all the derivatives) suggests that this proposed ILCD-LC/MRM-MS method had a potential for high-throughput analysis of a large cohort of samples.

Paired isotope labeling has already been demonstrated as a useful technique for chemical derivatization-LC/MS of endogenous metabolites such as amino acids [21] and organic acids [23,25] in various biological samples to provide relative or absolute quantitation. In our experience, in order to achieve reliable quantitation by ILCD-LC/MRM-MS with the use of $^{12}C_6/^{13}C_6$-3NPH, where a post-derivatization quenching step is not needed (as is the case in this study and in our previous study on the UPLC/MRM-MS quantitation of short-chain fatty acids [30]), two prerequisites must be met: (1) the derivatization needs to be complete (i.e., 100% reaction efficiency), without any unreacted compounds in the reacted mixture, and (2) the reaction needs to unidirectional and non-reversible. Otherwise, there could be back-exchange between the light isotope-labeled derivatives and the heavy isotope-labeled derivatives after they were mixed and prior to the LC/MS/MS runs.

This might change the measured analyte-to-SIS peak area ratios and cause unreliable quantitation, especially when low-abundance compounds are analyzed. To determine if there was back-exchange between the light and heavy forms of the 3NPH derivatives of the carnitines, we used 75 μL of 60% aqueous methanol as a blank sample to perform the derivatization with natural 3NPH, and after the derivatization, mixed the reactant with 50 μL of the $^{13}C_6$-3NPH-derivatized SIS solution to mimic the complete analytical workflow and in order to have exactly the same compositions of reagents, solvents, and SISs as used in the preparation of the calibration curves and in the sample analyses. This solution was maintained at 5 °C in the autosampler and was injected every 6 h for a total of 48 h. We observed no natural-form of the 3NPH-derivatived carnitines over this time period, indicating no back-exchange between the two forms of the derivatives existed (data not shown).

3.4. Optimized sample preparation for quantitation

To extract the different carnitines in DBSs on cellulose or cotton filter paper, we evaluated the extractions from the three sets of collection cards using different solvents including methanol, acetonitrile, methanol:acetonitrile (1:1,v/v), and their 80% aqueous solutions (except for 80% aqueous methanol which was not good enough to completely remove proteins during the carnitine extraction steps), with or without 0.02% TFA in each, and with the extractions performed as a single step or in two steps. Fig. 3 shows a comparison of the measured peak areas of 8 representatives of the 24 carnitines extracted from the three sets of DBS specimens with five of these different solvent systems. The optimal extraction of all the targeted carnitines among the extraction experiments were with a two-step extraction procedure, using 200 μL of 0.02% TFA in water to completely resuspend dried blood from the cellulose or cotton filter paper as the first step (with vortex-mixing and water-bath sonication), and using 800 μL of 0.02% TFA in methanol-acetonitrile (1:1, v/v) as the second step. This method was used for the subsequent quantitative measurements.

3.5. Quantitative performance

3.5.1. Sensitivity, linearity and matrix effects

Table 1 lists the LLODs, the LLOQs, the linearity, and the linear ranges of concentrations for the 24 carnitines determined using the new UPLC/MRM-MS method. These parameters indicated good analytical sensitivities at sub- to low femtomole levels on-column, and excellent linearity of MRM-MS detection across wide concentration ranges of the individual carnitines – concentration ranges which covered the endogenous concentrations of these analytes in DBS. The linearity was also determined in the target sample matrix by spiking the standard substances into the DBSs. As shown in Table 1, the linearity obtained between the matrix-free standard solutions and the matrix-matched sample solutions, using the 903sample set as an example, only displayed differences in the slopes of the linear-regression equations in a range of −5.8% to 8.3%, for all of the 24 carnitines, indicating that there was no significant systematic influence of the ESI matrices on the quantitation when the $^{13}C_6$- or $^{13}C_{12}$-labeled derivatives were used for internal standard calibration.

3.5.2. Precision and accuracy of quantitation

The precision of this derivatizaiton-UPLC/MRM-MS method for quantitation of the 24 carnitines in the three sets of DBSs was measured. Supplementary Material Table S3 lists the intra-day % CVs, which were calculated from the molar concentrations of the individual carnitines determined for each DBS set six times within the same day, and the inter-day %CVs, which were calculated from the molar concentrations of the individual carnitines which were determined once per day for five consecutive days. The intra-day and the inter-day CVs were ≤7.8% (n = 6) and ≤8.9% (n = 5), respectively, in all cases.

To assess the quantitation accuracy, standard substance spiked-in recovery tests were carried out at three spiking levels, i.e., by pipette-spotting the individual standard substances at 100%, 250% or 500% of the endogenous concentrations of each carnitine onto four replicate spots (n = 4) from each DBS set, followed by the analyte extraction, derivatization, and UPLC/MRM-MS quantitation. Table 2 lists the measured endogenous concentrations (nM) of the 24 carnitines in each sample set, and the corresponding recoveries compared to the spiked-in amounts of the standard substances. These recoveries ranged from 86.9% (CV = 5.8%) to 107.9% (CV = 4.6%) at the 100% spiking level, from 89.0% (CV = 2.1%) to 108.8% (CV = 6.3%) at the 250% spiking level, and from 87.7% (CV = 4.4%) to 109.7% (CV = 5.5%) at the 500% spiking level for all the 24 carnitines measured in the three sets of DBS samples, with n = 4 for each spiking level and for each sample set.

The measured intra-day and inter-day CVs and the spiked-in recoveries demonstrated the good precision and high accuracy of quantitation using this new analytical method. Statistics using ANOVA did not show significant differences (P > 0.05) in the measured recoveries at each spiking level among the three DBS sets on either cellulose or cotton filter paper, indicating that the blood-absorbing matrices did not induce any systematic bias in the quantitation when the optimized sample preparation procedure and the UPLC/MRM-MS method were used.

3.6. Short-term stabilities of 24 carnitines in DBS on cellulose and cotton filter paper-based collection cards

As a proof-of-concept study, the developed method was used to measure the short-term stabilities of the 24 carnitines on three sets of DBS samples prepared on cellulose-based 903 and DMPK-C

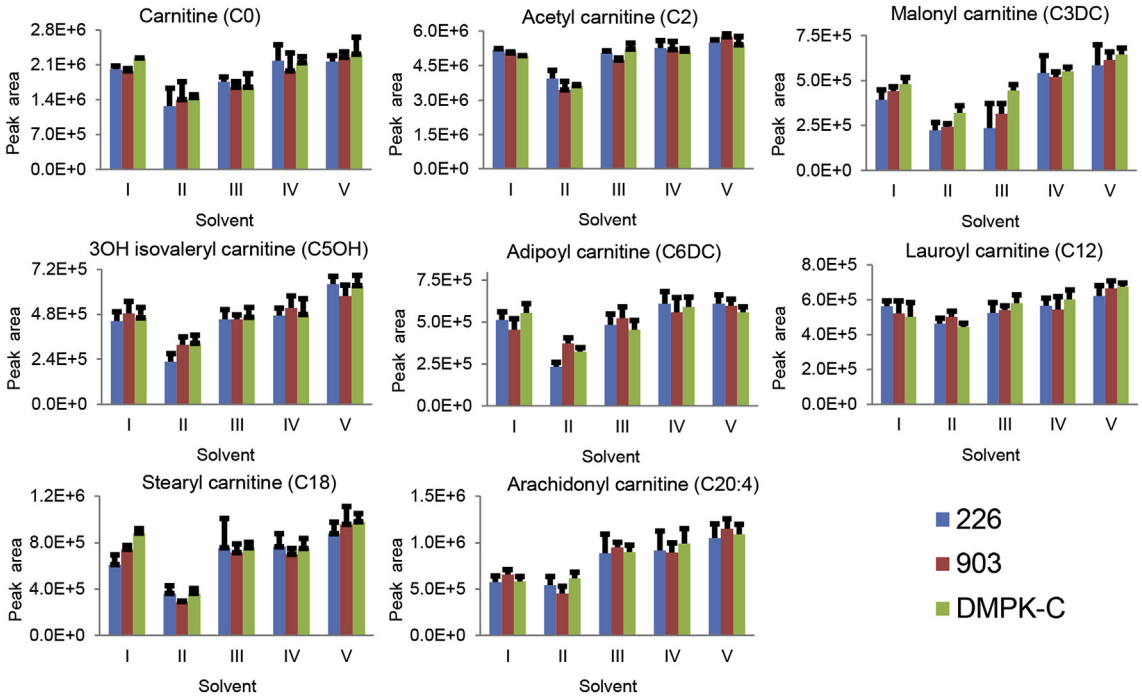

Fig. 3. Comparison of one-step or two-step extractions of carnitines using five different solvents. (**I**) 1 mL of methanol in a single step, (**II**) 1 mL of 80% acetonitrile in a single step, (**III**) 200 μL of 0.02% TFA in water and 800 μL of 0.02% TFA in acetonitrile in two steps, (**IV**) 200 μL of water and 800 μL of methanol:acetonitrile (1:1, v/v) in two steps, and (**V**) 200 μL of 0.02% TFA in water and 800 μL of 0.02% TFA in methanol:acetonitrile (1:1, v/v) in two steps.

Table 1
List of LLOD, LLOQ, linearity, and linear ranges of concentration for 24 routinely-analyzed carnitines.

Compound	LLOD	LLOQ	Matrix-free linearity		Matrix-matched linearity		Slope Difference (%)	Linear range
	(fmol)	(fmol)	C, nM	R^2	C, nM	R^2		(nM)
Carnitine (C0)	1.0	4.0	As/Ai = 0.0441C-0.0013	0.9999	As/Ai = 0.0422C-0.0487	0.9999	4.3	0.2–1420
Acetyl carnitine (C2)	0.3	1.0	As/Ai = 0.0524C-0.0007	0.9998	As/Ai = 0.0547C-0.0126	0.9998	−4.4	0.5–360
Propionyl carnitine (C3)	0.4	1.7	As/Ai = 0.0440C-0.0016	0.9999	As/Ai = 0.0465C-0.0099	0.9998	−5.7	0.087–360
Malonyl carnitine (C3DC)	0.8	0.8	As/Ai = 0.0235C-0.0013	0.9999	As/Ai = 0.0237C-0.0061	0.9999	−0.8	0.087–360
Butyryl carnitine (C4)	0.3	1.0	As/Ai = 0.0451C-0.0030	0.9998	As/Ai = 0.0448C-0.0082	0.9999	0.7	0.087–360
Isobutyl carnitine (C4)	.3	1.0	As/Ai = 0.0441C-0.0016	0.9990	As/Ai = 0.0423C-0.0031	0.9997	4.1	0.12–1420
Methylmalonyl carnitine (C4DC)	2.2	6.9	As/Ai = 0.0456C-0.0021	0.9997	As/Ai = 0.0452C-0.0016	0.9999	0.9	0.12–1420
Succinyl carnitine (C4DC)	0.4	1.7	As/Ai = 0.0180C-0.0005	1.0000	As/Ai = 0.0173C-0.0012	0.9998	3.9	0.087–360
3-Hydroxylbutyryl carnitine (C4OH)	0.4	0.4	As/Ai = 0.0132C-0.0093	0.9997	As/Ai = 0.0133C + 0.0018	0.9999	−0.8	0.022–360
2-Methylbutyryl carnitine (C5)	0.4	0.4	As/Ai = 0.0440C-0.0033	0.9993	As/Ai = 0.0409C-0.0018	0.9994	7.0	0.022–360
Isovaleryl carnitine (C5)	3.1	1.2	As/Ai = 0.0418C-0.0016	0.9996	As/Ai = 0.0412C-0.0010	1.0000	1.4	0.12–720
Glutaryl carnitine (C5DC)	0.6	1.9	As/Ai = 0.0436C-0.0010	0.9996	As/Ai = 0.044C-0.0028	0.9997	−0.9	0.035–720
3-Hydroxylisovelaryl carnitine (C5OH)	0.5	2.2	As/Ai = 0.0204C-0.0005	0.9996	As/Ai = 0.0214C-0.0008	0.9992	−4.7	0.087–360
Hexanoyl carnitine (C6)	0.2	0.6	As/Ai = 0.0468C-0.0021	0.9994	As/Ai = 0.0464C-0.0010	0.9994	0.9	0.087–360
Adipoyl carnitine (C6DC)	0.2	0.6	As/Ai = 0.0460C-0.0010	0.9999	As/Ai = 0.0475C-0.0004	0.9995	−3.3	0.087–360
Octanoyl carnitine (C8)	0.4	1.2	As/Ai = 0.0455C-0.0012	0.9994	As/Ai = 0.0422C-0.0106	1.0000	7.3	0.087–360
Decanoyl carnitine (C10)	0.2	0.6	As/Ai = 0.0604C-0.0006	0.9996	As/Ai = 0.0554C-0.0017	1.0000	8.3	0.087–360
Lauroyl carnitine (C12)	0.4	1.2	As/Ai = 0.0453C-0.0018	0.9996	As/Ai = 0.0481C-0.0014	0.9991	−5.8	0.087–360
Myristoyl carnitine (C14)	0.3	1.0	As/Ai = 0.0357C-0.0016	0.9990	As/Ai = 0.0347C-0.0036	0.9999	2.9	0.087–360
Palmitoyl carnitine (C16)	0.4	1.7	As/Ai = 0.0576C-0.0020	0.9998	As/Ai = 0.0582C-0.0037	0.9994	−1.0	0.087–360
Oleyl carnitine (C18:1)	2.3	6.9	As/Ai = 0.0680C-0.0012	0.9996	As/Ai = 0.0664C-0.0840	0.9994	2.4	0.035–720
Linoleyl carnitine (C18:2)	0.3	1.2	As/Ai = 0.0822C-0.0197	1.0000	As/Ai = 0.0819C-0.0197	0.9994	0.4	0.021–160
Stearyl canitine (C18)	0.9	4.0	As/Ai = 0.0819C-0.0197	0.9994	As/Ai = 0.0851C-0.0611	1.0000	−3.9	0.087–360
Arachidonyl carnitine (C20:4)	0.5	2.0	As/Ai = 0.0615C-0.0069	0.9999	As/Ai = 0.0648C + 0.010	0.9990	−5.4	0.087–360

cards, and cotton-based 226 cards under two sets of the stressed environmental conditions.

3.6.1. One-time sunlight exposure of DBS

The molar concentrations of each carnitine were measured in each set of DBS samples which underwent one-time sunlight irradiation for 4 h, and in the corresponding controls for each DBS set. Fig. 4A are the bar graphs + CVs (n = 4), representing the % concentrations of individual carnitines measured in the DBSs on the three sets of collection cards (226, 903, and DMPK-C). The relative concentrations of all the carnitines ranged from 92.4% to 107.0% for the 226 card, 90.8%–109.1% for the 903 card, and 90.8%–108.0% for the DMPK-C card. These concentrations indicated the good stabilities to sunlight exposure of all of the carnitines on the three types of cellulose- or cotton-filter paper collection cards.

Table 2
Concentrations and quantitation accuracy of 24 carnitines in three sets of DBS.

Compound	DBS set	Concentration (n = 4)		Standard substance-spiking recovery % (n = 4)					
		C (μM)	CV%	100% spiking	CV%	250% spiking	CV%	500% spiking	CV%
Carnitine (C0)	903	26.5	3.8	93.3	2.1	103.5	8.0	99.2	2.5
	226	28.5	2.5	102.7	4.5	100.3	2.9	99.2	2.2
	DMPK-C	29.9	2.6	96.0	2.5	105.3	3.4	104.7	2.0
Acetyl carnitine (C2)	903	11.0	6.0	104.3	7.9	99.0	5.6	95.8	6.4
	226	10.7	2.1	97.6	6.7	91.5	7.8	100.5	7.5
	DMPK-C	10.6	6.8	104.5	8.4	105.7	2.4	102.6	3.3
Propionyl carnitine (C3)	903	0.84	3.6	102.8	3.4	96.3	3.7	99.6	5.0
	226	0.81	2.6	95.2	4.2	100.3	3.6	94.3	4.4
	DMPK-C	0.81	3.9	102.9	6.1	96.3	2.8	96.5	3.5
Malonyl carnitine (C3DC)	903	0.015	3.4	107.9	2.6	108.1	3.5	104.7	6.0
	226	0.012	5.6	105.6	3.1	106.2	2.7	88.9	4.8
	DMPK-C	0.014	1.1	101.8	4.2	108.8	6.3	96.8	6.3
Butyryl carnitine (C4)	903	0.28	2.9	99.2	3.0	103.2	2.7	105.5	4.0
	226	0.26	5.1	99.5	0.9	98.9	5.5	102.3	6.3
	DMPK-C	0.25	4.0	95.6	5.4	100.2	7.2	98.7	4.1
Isobutyl carnitine (C4)	903	0.27	2.9	99.2	3.0	103.2	2.6	105.6	3.3
	226	0.25	3.3	98.2	7.2	105.2	5.2	97.2	5.2
	DMPK-C	0.29	2.0	92.1	3.1	94.3	1.7	91.1	6.0
Methylmalonyl carnitine (C4DC)	903	0.39	4.4	90.8	5.0	90.5	5.2	98.1	7.8
	226	0.35	9.2	87.8	8.3	89.0	2.1	88.0	3.4
	DMPK-C	0.37	5.1	92.6	2.6	91.6	2.6	92.4	8.6
Succinyl carnitine (C4DC)	903	0.011	4.2	100.6	9.9	98.7	4.0	106.7	4.8
	226	0.011	9.1	97.0	3.7	102.8	5.2	105.6	8.3
	DMPK-C	0.010	5.0	103.7	7.4	102.3	9.5	99.5	2.8
3-Hydroxylbutyryl carnitine (C4OH)	903	0.18	3.0	97.2	2.0	95.3	4.3	96.5	3.5
	226	0.16	3.2	102.8	4.2	89.6	3.6	103.2	5.1
	DMPK-C	0.19	4.8	96.2	6.2	99.5	6.6	97.1	1.8
2-Methylbutyryl carnitine (C5)	903	0.052	4.7	101.1	3.0	96.0	9.5	98.9	0.8
	226	0.050	6.3	98.1	2.8	97.1	2.0	98.5	2.8
	DMPK-C	0.051	3.4	93.8	3.8	90.9	3.6	87.7	4.4
Isovaleryl carnitine (C5)	903	0.061	8.2	98.2	3.0	93.9	5.2	99.1	3.6
	226	0.059	4.9	96.5	6.0	97.3	3.2	96.5	4.2
	DMPK-C	0.059	4.6	97.3	3.8	97.6	0.4	98.5	5.2
Glutaryl carnitine (C5DC)	903	0.035	6.4	98.7	9.1	102.3	8.6	105.5	4.0
	226	0.033	2.5	95.2	7.4	92.5	6.2	95.2	6.0
	DMPK-C	0.033	4.1	91.2	2.5	96.5	7.7	95.5	3.9
3-Hydroxylisovelaryl carnitine (C5OH)	903	0.18	4.9	103.5	3.2	104.4	2.6	102.1	5.1
	226	0.17	1.0	100.0	7.7	104.4	2.9	95.8	3.3
	DMPK-C	0.19	5.2	101.9	3.6	100.5	5.5	99.8	4.4
Hexanoyl carnitine (C6)	903	0.086	5.0	99.5	3.6	104.2	1.5	109.7	1.5
	226	0.088	6.1	92.9	3.1	95.0	5.2	95.2	6.6
	DMPK-C	0.083	4.2	95.5	8.1	100.2	6.3	102.2	4.5
Adipoyl carnitine (C6DC)	903	0.036	2.2	99.4	4.8	107.4	5.0	106.4	4.6
	226	0.034	5.2	95.2	3.5	103.8	2.3	100.2	5.5
	DMPK-C	0.036	3.4	97.5	2.3	99.1	5.0	96.9	1.6
Octanoyl carnitine (C8)	903	0.050	5.6	93.1	2.0	93.4	3.6	92.5	2.7
	226	0.052	4.8	95.2	8.8	98.1	6.0	96.6	3.3
	DMPK-C	0.055	3.4	86.9	5.8	96.2	1.5	95.2	5.4
Decanoyl carnitine (C10)	903	0.113	4.8	92.7	2.2	101.4	4.2	106.3	2.4
	226	0.106	2.2	98.1	3.9	96.8	6.1	107.1	3.3
	DMPK-C	0.118	1.6	95.2	5.6	100.0	2.6	100.5	8.1
Lauroyl carnitine (C12)	903	0.098	5.3	101.4	7.9	108.2	8.9	97.4	8.6
	226	0.090	6.2	94.8	6.1	105.6	5.5	98.8	5.4
	DMPK-C	0.102	3.6	98.2	5.8	104.5	4.6	96.2	3.5
Myristoyl carnitine (C14)	903	0.23	7.1	96.5	4.4	95.1	3.0	100.2	2.5
	226	0.24	8.9	94.4	6.1	93.9	6.1	107.4	6.7
	DMPK-C	0.23	3.5	91.2	3.0	96.6	5.2	102.5	6.2
Palmitoyl carnitine (C16)	903	0.77	5.7	95.7	8.5	88.2	4.9	90.4	5.2
	226	0.72	7.4	94.8	2.2	89.0	8.3	98.4	8.2
	DMPK-C	0.73	8.8	95.0	3.4	95.9	3.1	99.4	7.4

(*continued on next page*)

Table 2 (*continued*)

Compound	DBS set	Concentration (n = 4)		Standard substance-spiking recovery % (n = 4)					
		C (μM)	CV%	100% spiking	CV%	250% spiking	CV%	500% spiking	CV%
Oleyl carnitine (C18:1)	903	1.78	5.1	101.7	9.3	98.6	11.2	91.7	3.3
	226	1.73	4.3	99.8	6.9	97.2	5.9	105.5	3.1
	DMPK-C	1.88	2.2	97.6	2.0	105.4	8.6	99.9	2.5
Linoleyl carnitine (C18:2)	903	0.093	6.9	92.8	4.4	93.1	4.0	92.7	6.4
	226	0.080	12.5	87.9	3.9	82.9	2.6	98.4	5.9
	DMPK-C	0.078	1.4	94.3	4.2	94.5	1.4	96.9	7.8
Stearyl canitine (C18)	903	0.50	6.32	99.6	6.69	103.1	6.4	104.8	6.9
	226	0.49	3.83	95.7	2.2	100.1	5.8	102.8	5.2
	DMPK-C	0.47	5.26	98.4	5.1	98.5	4.5	100.2	3.9
Arachidonyl carnitine (C20:4)	903	0.064	5.4	97.5	2.5	98.5	3.5	102.5	5.9
	226	0.065	4.8	98.4	3.0	99.5	4.5	98.9	3.3
	DMPK-C	0.062	6.2	96.5	2.1	97.4	7.5	103.2	6.6

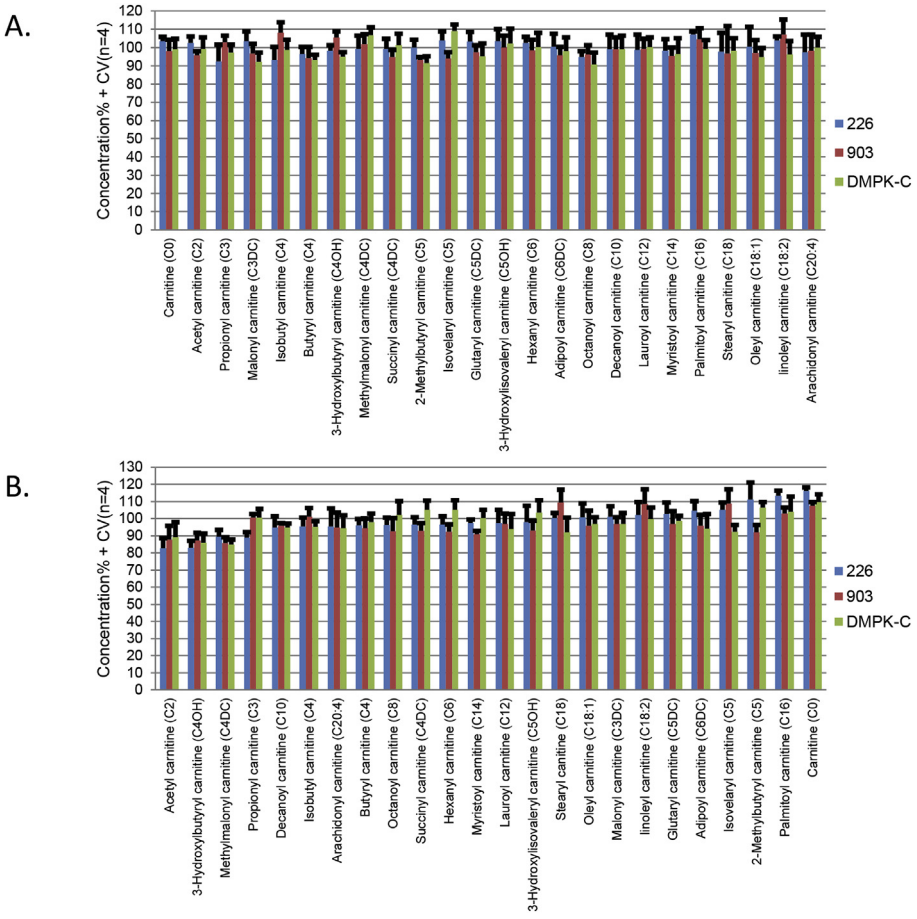

Fig. 4. Concentration changes of 24 carnitines in three sets of DBSs on cotton-based 226 and cellulose-based 903, and DMPK-C collection cards. (**A**) after one-time sunlight exposure for 4 h, and (**B**) after a 6-day cycle of temperature transitions during sample storage (−20 °C for 2 days, then at 40 °C for 2 days and at −20 °C again for 2 days), all under drying gel silica/reduced humidity.

3.6.2. Cycled temperature transitions of DBS storage

To mimic another DBS storage condition often incurred in a remote health workflow, the three sets of DBSs were cycled between two storage temperatures for a total of 6 days (i.e., −20 °C for 2 days, 40 °C for 2 days and then back to −20 °C for 2 days), under drying silica gel-reduced humidity. The bar graphs in Fig. 4B indicate the concentration changes (%s + CVs) of 24 individual carnitines in each DBS set after the cycled temperature transitions. ANOVA did not show statistical differences ($p < 0.01$) between the measured concentrations of all of the 24 carnitines among the three types of cellulose or cotton based collection cards. The concentrations of free carnitines increased by 16.1%, 9.3%, and 9.5%, respectively, in the DBSs averaged over four replicate spots of each set on the 226, 903, and DMPK-C cards. At the same time, short-chain acyl carnitines including acetyl (C2), 3-hydroxylbutyryl (C4OH) and methylmalonyl (C4DC) carnitine showed slight concentration

decreases in all the three sets of DBSs. Acetyl carnitine showed concentration reductions of 17.3%, 12.2%, and 10.7%; 3-hydroxylbutyryl carnitine showed concentration reductions of 17.1%, 12.9%, and 13.9%; and methylmalonyl carnitines showed concentration reductions of 10.7%, 14.4% and 15.3%, in the DBSs on the 226, 903, and DMPK-C cards, respectively. The other 20 carnitines showed concentration changes of <10% in all the three sets of DBSs, indicating the good stabilities of the other carnitines under this test condition.

The concentration changes observed for free carnitine and the three short-chain acyl carnitines, however, implied that the sample storage temperature could be a major factor that affects the chemical stabilities of these species, and that high temperatures during DBS sampling, transportation, and storage should be avoided in a remote health workflow.

4. Concluding remarks

In this work, a new quantitation method based on isotope-labeling derivatization-UPLC/(+)ESI-MRM-MS for the targeted analysis of carnitines in DBS samples was developed with 3NPH as a high-efficiency pre-analytical derivatizing reagent. The use of $^{13}C_6$-3NPH to generate $^{13}C_6$- or $^{13}C_{12}$-labeled derivatives of the targeted carnitines as stable isotope-labeled internal standards to compensate for matrix effects, in combination with the optimized two-step analyte extraction procedure, ensured the high precision and high accuracy of the quantitation.

This method was successfully applied to the measurement of 24 routinely-analyzed carnitines in three sets of DBSs on three sets of World Health Organization-certified and United States-Food and Drug Administration-cleared cellulose and cotton-based collection cards, after a 4-h sunlight exposure or after a 6-day cycle of temperature transitions. The measured concentrations indicated good stabilities of the targeted carnitines in all of the DBSs on cellulose or cotton filter paper under the sunlight-exposure condition. Under the cycled storage temperature transitions, 20 of the 24 carnitines showed concentration changes of <10%. The concentration changes of free carnitine and 3 short-chain hydroxyl or di-carboxylic carnitines – increases for free carnitine and decreases for others – in the DBSs under the cycled storage temperature transitions indicates that high temperatures are a major factor that affects the chemical stabilities of carnitines in DBS specimens, and that a more systematic investigation of the chemical stabilities of various carnitines in DBS specimens used in remote health applications is needed. It should be also mentioned that, although 24 routinely-analyzed carnitines were targeted in this study, the developed derivatization-LC/MRM-MS method should also be applicable to the analysis of other carnitines in DBSs or other biological samples.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.01.045.

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