Application Note

Life Science Research

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LC/MS/MS Method for Quantification of Acylcarnitines and Carnitine Intermediates

Standardized workflow demonstrated in plasma and fecal matrices

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Abstract

Acylcarnitines are key intermediates in fatty acid oxidation and amino acid metabolism. They are important biomarkers for mitochondrial function, inflammatory diseases, and microbiome-related disorders. In life science and pharmaceutical research, acylcarnitine profiling is now increasingly applied to understand basic disease states as well as support mechanism-of-action studies, metabolic safety assessment, and translational biomarker strategies. This application note describes a standardized LC/MS/MS method for quantifying 32 acylcarnitines (C0 to C20) in plasma and fecal samples without derivatization. The method uses reversed-phase chromatography and an Agilent 6495D triple quadrupole LC/MS and is designed to align with the standardized omics platforms from Agilent, supporting harmonized data generation across targeted metabolomics and integrated multi-omics workflows. It delivers excellent linearity, reproducibility, and recovery, supporting high-throughput targeted metabolomics workflows. The workflow is designed to enable scalable, decision-quality data generation for disease research, drug discovery, drug metabolism and pharmacokinetics (DMPK), and investigational new drug (IND), enabling studies requiring robust and reproducible metabolic measurements.

Introduction

Acylcarnitines are essential intermediates in mitochondrial fatty acid β-oxidation and amino acid metabolism. They facilitate the transport of fatty acids into mitochondria for energy production and act as buffers for excess acyl groups in cells. Because of these roles, acylcarnitines are widely studied as biomarkers for metabolic disorders, mitochondrial dysfunction, and inflammatory diseases.[1-3] Thus, in basic research and drug development, disruptions in acylcarnitine profiles are routinely evaluated as indicators of altered energy metabolism in disease states and potential mitochondrial liabilities associated with small molecules and biologic therapeutics.

Recent research has linked altered acylcarnitine profiles to conditions such as type 2 diabetes, cardiovascular disease, and inflammatory bowel disease (IBD).[4-6] These disease-associated signatures are increasingly leveraged in translational research to bridge preclinical models with human pharmacology and clinical outcomes. Further, these metabolites are also emerging as indicators of gut microbiome activity, reflecting host-microbe interactions in energy metabolism.[7] In translational research and drug development programs targeting metabolic, inflammatory, or gastrointestinal diseases, fecal acylcarnitine profiling provides a complementary, noninvasive readout of target engagement and pathway modulation.

Despite their importance, accurate quantification of acylcarnitines remains challenging. Many existing methods require derivatization, which adds complexity and variability. Additionally, isobaric and isomeric species often coelute, and matrix interferences can lead to inaccurate quantification.[8-9] Such challenges can hinder comparability across studies and delay decision-making in contexts where consistency and reliability are essential. These constraints underscore the importance of a standardized, high-throughput approach that delivers reliable separation, wide coverage, and consistent results without the need for derivatization.

This application note describes a standardized LC/MS/MS method for quantifying 32 acylcarnitines (C0 to C20) in plasma and fecal samples. The method uses reversed-phase chromatography and an Agilent 6495D triple quadrupole LC/MS. It offers excellent linearity, recovery, and reproducibility, making it suitable for targeted metabolomics workflows in research areas such as mitochondrial function, inflammation, and microbiome health. The method is optimized for workflows spanning early research and discovery, DMPK, translational biomarker development, and clinical research where high-throughput, derivatization-free acylcarnitine analysis can support confident decision making.

Experimental

Sample preparation

Two workflows were developed for plasma and fecal matrices. For plasma, 20 µL of pooled plasma was mixed with 20 µL of labeled internal standards (20 ng/mL in methanol) and 60 µL of isopropanol containing 0.3% formic acid. Samples were vortexed for 5 minutes and centrifuged at 10,000 × g for 10 minutes at 4 °C to precipitate proteins. A 20 µL aliquot of the supernatant was diluted with 80 µL of water before LC/MS/MS analysis. Calibration curves ranged from 0.01 to 500 ng/mL in MeOH:H_2O (8:2, v/v).

For fecal samples, approximately 120 mg of certified human fecal material was weighed into 2 mL bead tubes containing ceramic beads. Samples were extracted with ice-cold methanol:water (8:2, v/v) containing internal standards to a final concentration of 100 mg/mL. After homogenization, the slurries were incubated overnight at –80 °C, vortexed, and centrifuged at 20,000 × g for 20 minutes at 4 °C. A 100 µL aliquot of the supernatant was dried under vacuum and further reconstituted by first adding 20 µL of methanol, followed by an addition of 80 µL water (final volume of 100 µL), mixing at each step for 15 minutes (Eppendorf Thermo mixer at 1500 rpm at 6 °C) prior to injection. Calibration curves ranged from 0.1 to 500 nmol/L. It is important to note that adding the methanol as a first step to reconstitute the samples was essential for the solubilization of the longer chain acylcarnitines.

LC/MS/MS analysis

Chromatographic separation was performed using an Agilent 1290 Infinity II Bio LC System coupled to the 6495D triple quadrupole LC/MS with iFunnel technology. The method employed reversed-phase chromatography on an Agilent ZORBAX Eclipse Plus C18 column to achieve a robust separation of 32 acylcarnitines, including key isomeric species such as C4, C5, and C6. The LC gradient and mobile phase composition are summarized in Table 1, while MS source parameters are provided in Table 2.

The system operated in positive electrospray ionization mode with dynamic multiple reaction monitoring (dMRM) for targeted quantification. Two transitions were monitored for each analyte, along with one transition for the corresponding internal standard. The product ion at *m/z* 85.1 served as the primary quantifier for most compounds, except for L-carnitine (C0) and deoxycarnitine, which used *m/z* 43.1 and 45.1, respectively. Table 3 summarizes all dMRM transitions for each compound with all parameters used for the MS analysis. For absolute quantitation, labeled internal standards were used. Due to their low availability, some of the compounds used standards that are closest in retention time and/or chain length as the internal standards. Each related labeled internal standard used for the quantitation analysis is listed in Table 4.

Data acquisition and processing were performed using the Agilent MassHunter software. Method development utilized the Compound Optimizer and Source Optimizer tools within MassHunter Acquisition 12.2 to ensure optimal sensitivity and reproducibility. Quantitative analysis was carried out using MassHunter Quantitative Analysis 12.0, which provided calibration-at-a-glance visualization for a rapid review of linearity and recovery across all analytes.

Collectively, this workflow configuration enabled high sensitivity and selectivity across a broad dynamic range, ensuring accurate quantification without derivatization.

Table 1. LC conditions for the standardized acylcarnitine quantification method.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
Column Temperature	15 °C
Autosampler Temperature	4 °C
Injection Volume	2 µL
Needle Wash	S1: 5 s ACN:MeOH:H_2O (1:1:1, v/v) S2: 5 s Milli-Q water
Mobile Phase	A: Water containing 15 mM ammonium acetate + 0.3% formic acid B: MeOH:H2O (95:5, v/v) + 15 mM ammonium acetate + 0.3% formic acid
Flow Rate	0.4 mL/min
Total Run Time	21 minutes

Gradient Program:

Time [min]	A [%]	B [%]	Flow mL/min]
0.00	98.00	2.00	0.300
1.50	98.00	2.00	0.200
1.60	98.00	2.00	0.300
1.70	93.50	6.50	0.300
3.00	93.50	6.50	0.300
3.10	92.50	7.50	0.300
7.00	92.50	7.50	0.300
7.10	85.00	15.00	0.300
13.00	85.00	15.00	0.300
13.10	15.00	85.00	0.300
15.00	0.00	100.00	0.300
18.00	0.00	100.00	0.300
18.10	98.00	2.00	0.300
20.00	98.00	2.00	0.300
20.10	98.00	2.00	0.200

Table 2. MS source parameters for the Agilent 6495D triple quadrupole LC/MS.

Parameter	Value
Sheath Gas Temperature, Flow	300 °C, 11.0 L/min
Gas Temperature, Flow	250 °C, 18.0 L/min
Nebulizer	35.0 psi
Capillary	3,000 V (+)
Nozzle Voltage	0 V (+)
iFunnel Mode	Fragile
Detector Gain	2

Results and discussion

The standardized LC/MS/MS method provided comprehensive coverage, enabling the quantification of 32 acylcarnitines from C0 to C20 in a single run (Figure 1). This included short-, medium-, and long-chain species, as well as critical isomeric forms. Broad acylcarnitine coverage in a single analytical method is particularly valuable for evaluating mitochondrial function, fatty acid oxidation, and systemic metabolic perturbations across disease research and drug discovery and development stages. Robust chromatographic separation was achieved without ion-pairing reagents, simplifying the workflow and reducing contamination risk. The elimination of ion-pairing reagents improves method robustness and instrument uptime, key considerations for high-throughput environments like DMPK and bioanalytical laboratories operating under tight timelines. Notably, isomeric species such as butyryl- and isobutyryl-carnitine (C4), as well as valeryl-, isovaleryl-, and 2-methylbutyryl-carnitine (C5), were baseline-resolved (Figure 2), ensuring accurate quantification of compounds that are often misidentified in less selective methods. Thus, accurate resolution of isomeric acylcarnitines is critical for mechanistic interpretation and reduces the risk of false biomarker signals in translational studies.

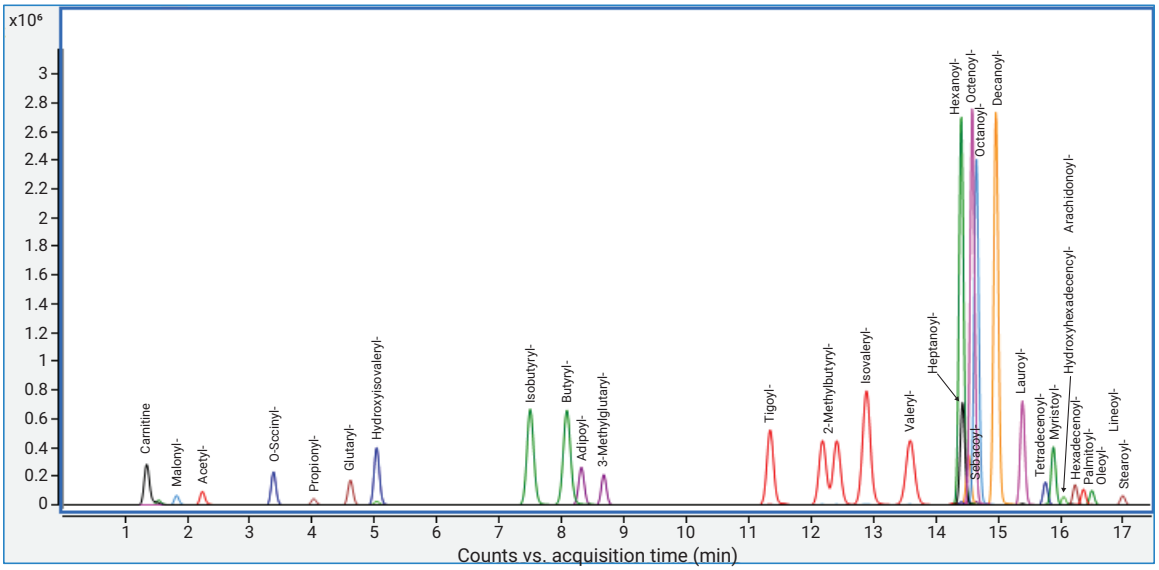

Figure 1. Chromatographic separation of 32 acylcarnitines (C0 to C20) using the Agilent ZORBAX Eclipse Plus C18 column and the standardized LC/MS/MS method.

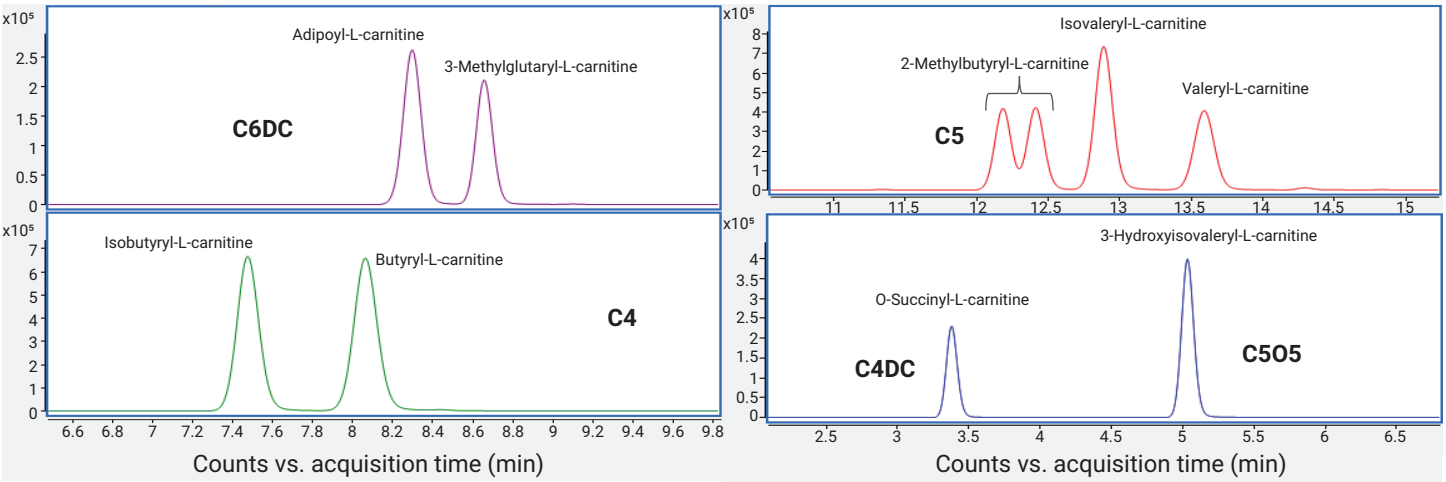

Figure 2. Baseline resolution of key isomeric acylcarnitines (carbon length chain C4, C5, and C6 species) achieved without ion-pairing reagents.

Linearity and sensitivity

Individual unlabeled and labeled standards of acylcarnitines were prepared in methanol and stored at −20 °C. The final calibration curve ranged from 0.01 to 500 ng/mL in MeOH:H_2O (8:2, v/v). Labeled standards were added to each sample and calibrant levels for absolute quantitation (at a final concentration of 20 ng/mL). Calibration curves for all analytes exhibited excellent linearity, with correlation coefficients (R^2) greater than 0.99 across the tested ranges (0.01 to 500 ng/mL for plasma and 0.1 to 500 nmol/L for fecal samples). This wide dynamic range supports quantitative measurements across diverse scenarios in complex biological matrices, from basic research to translational samples. MassHunter Quantitative Analysis 12.0 generates calibration-at-a-glance figures allowing for quick processing of all calibration curves and customizable visualization to fit any view and subset of compounds. Representative calibration plots are shown in Figure 3.

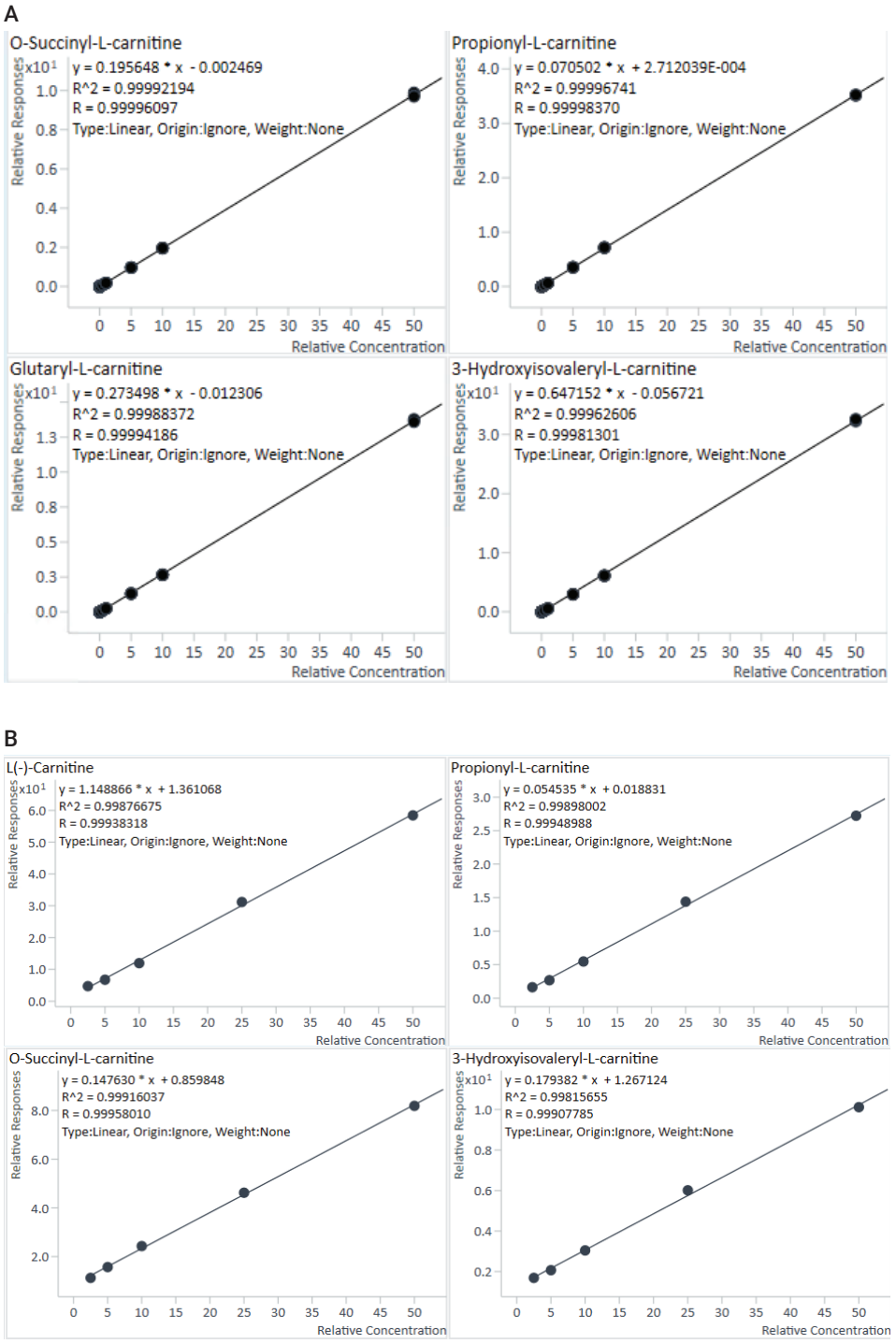

Figure 3. Representative calibration curves for selected acylcarnitines showing excellent linearity (R^2 > 0.99) across the tested concentration range in (A) plasma and (B) fecal samples.

Precision and recovery

The method demonstrated strong reproducibility, with relative standard deviations (RSD) below 10% for most analytes across replicate injections. Recovery studies were also evaluated by adding labeled and unlabeled standards before and after extraction. The response of each standard was calculated by R = (A/B)×100 (where A is the response of standards spiked before extraction and B is the response of the standards spiked after the extraction). The recovery values ranged from 80% to 122%, confirming the efficiency of the simple sample preparation for both plasma and fecal matrices (Table 3). These performance metrics meet expectations for quantitative bioanalysis, confidently supporting cross-study and cross-cohort comparisons. These results indicate that the method is suitable for high-throughput workflows without compromising accuracy where consistent precision and recovery are particularly important for longitudinal studies, dose-response experiments, and biomarker qualification efforts.

Table 3. Summary of recovery and linearity for acylcarnitines in plasma matrix.

Class	Compound Name	Calibration Curve Range (ng/mL)	R^2	Extraction Recovery (%)
C0	L(-)-Carnitine	1–100	0.997	117.8
C2	Acetyl-L-carnitine	0.05–500	0.999	111.6
C3	Propionyl-L-carnitine	0.05–500	0.999	95.4
C3DC	Malonyl-L-carnitine	0.1–100	0.999	91.3
C4	Butyryl-L-carnitine	0.01–500	0.999	92.0
C4	Isobutyryl-L-carnitine	0.05–500	0.999	91.2
C4DC	O-Succinyl-L-carnitine	0.01–500	0.999	89.7
C5	2-Methylbutyryl-L-carnitine	0.05–500	0.998	90.6
C5	Isovaleryl-L-carnitine	0.05–500	0.999	90.0
C5	Valeryl-L-carnitine	0.05–500	0.998	90.0
C5:1	Tigloyl-L-carnitine	0.01–500	0.997	90.2
C5DC	Glutaryl-L-carnitine	0.01–100	0.999	90.5
C5OH	3-Hydroxyisovaleryl-L-carnitine	0.05–100	0.998	90.1
C6	Hexanoyl-L-carnitine	0.05–100	0.994	89.6
C6DC	3-Methylglutaryl-L-carnitine	0.05–500	0.999	90.4
C6DC	Adipoyl-L-carnitine	0.05–500	0.999	90.5
C7	Heptanoyl-L-carnitine	0.05–500	0.998	90.1
C8	Octanoyl-L-carnitine	0.05–100	0.996	90.6
C8:1	trans-2-Octenoyl-L-carnitine	0.05–100	0.994	90.8
C10	Decanoyl-L-carnitine	0.05–100	0.998	88.8
C10DC	Sebacoyl-L-carnitine	0.05–500	0.998	90.7
C12	Lauroyl-L-carnitine	0.05–100	0.996	89.8
C14	Myristoyl-L-carnitine	0.05–100	0.996	89.0
C14:1	trans-2-Tetradecenoyl-L-carnitine	0.05–500	0.997	90.5
C16	Palmitoyl-L-carnitine	0.01–100	0.995	90.8
C16:1	trans-2-Hexadecenoyl-L-carnitine	0.01–100	0.997	89.9
C16OH	[(3R)-3-Hydroxyhexadecanoyl]-L-carnitine	0.05–500	0.997	94.7
C18	Stearoyl-L-carnitine	0.05–500	0.997	89.2
C18:1	Oleoyl-L-carnitine	0.01–100	0.992	99.8
C18:2	Linoleoyl-L-carnitine	5–500	0.996	118.1
C20:4	Arachidonoyl-L-carnitine	10–500	0.990	95.7
Other	Deoxycarnitine hydrochloride	0.1–100	0.997	93.9

Throughput and ease of use

The total run time, including column re-equilibration, was 21 minutes, allowing for efficient sample processing in targeted metabolomics studies. This throughput enables scalable deployment in drug discovery and translational environments where large compound libraries or study cohorts must be evaluated. The elimination of derivatization steps further reduces sample preparation time and minimizes variability, making the method easy to implement in routine laboratory workflows. Derivatization-free workflows are particularly attractive where simplicity and robustness reduce operational risk and improve reproducibility.

Biological application

To demonstrate applicability, the method was used to profile acylcarnitines in fecal samples from a small cohort of individuals with IBD compared to healthy controls. Preliminary data revealed a trend toward decreased levels of L-carnitine (C0) and several acylcarnitines in IBD samples (Figure 4). Although exploratory, this application illustrates how the method can support hypothesis generation and biomarker discovery in disease-focused and translational research programs. While statistical significance could not be assessed due to the limited sample size, these findings highlight the potential of acylcarnitine profiling as a tool for biomarker discovery in gut health and inflammation research. Such approaches are increasingly relevant for research programs targeting metabolic, inflammatory, and microbiome-associated diseases.

Overall, the method combines high sensitivity, selectivity, and reproducibility with a streamlined workflow, making it an ideal solution for targeted metabolomics applications in mitochondrial function, fatty acid oxidation disorders, and microbiome studies. These attributes directly support decision-making by enabling reliable metabolic phenotyping, safety assessment, and translational biomarker evaluation.

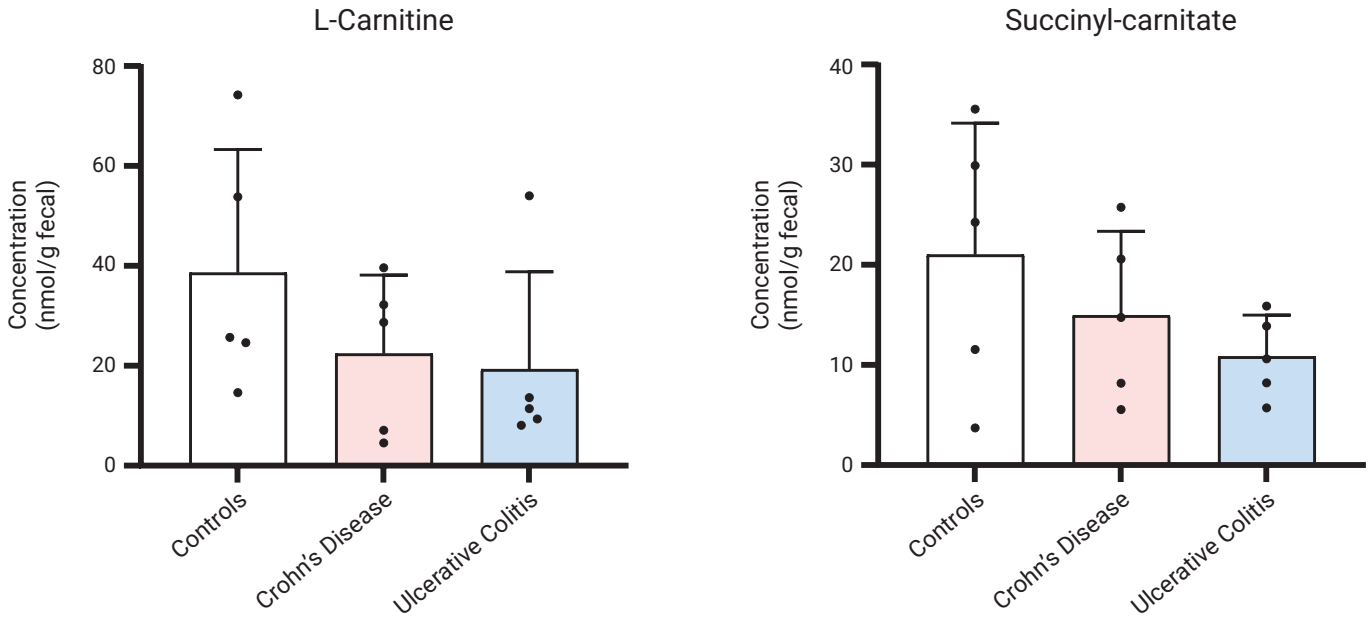

Figure 4. Relative concentrations of selected acylcarnitines in fecal samples from healthy controls and individuals with inflammatory bowel disease (IBD). Concentrations were plotted using GraphPad Prism software.

Conclusion

This standardized LC/MS/MS workflow provides a robust, high-throughput solution for acylcarnitine analysis in plasma and fecal samples. It simplifies workflows by eliminating derivatization and ensures accurate quantification with excellent reproducibility. The method is well aligned with basic research needs, and translationally supports drug discovery, DMPK, biomarker development, and clinical research applications. The method integrates seamlessly into the standardized omics platform from Agilent, supporting research in mitochondrial function, inflammation, and microbiome health. By delivering scalable, decision-quality data, this approach enables confident evaluation of metabolic pathways and biomarkers across the drug development continuum.

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