LC-MS/MS Analysis of 25 Underivatized Acylcarnitines for Differential Diagnosis

By Samantha Herbick

Abstract

Acylcarnitines are important indicators in the diagnosis of many metabolic disorders. This rapid, 9-minute acylcarnitines LC-MS/MS analysis allows testing of 25 underivatized acylcarnitines, including several isobaric compounds that are essential for differential diagnosis. A Raptor ARC-18 column (100 x 2.1 mm, 2.7 µm) used under the established conditions provided chromatographic separation of key isobars that shared MRM transitions.

Related Products

- *Raptor ARC-18 column*
- *Raptor ARC-18 EXP guard column cartridge*
- *2.0 mL, 9 mm screw-thread vial*
- *2.0 mL, 9 mm short-cap, screw-vial closure*

Introduction

Carnitine (Figure 1) is a quaternary ammonium compound that is required for fatty acid β-oxidation. In the body, it can exist either in free form or bind with fatty acids to form acylcarnitines. It can also vary in chain length from shorter acetyl chains to longer palmitoyl chains (Figure 2) [1].

Figure 1: Chemical structure of carnitine.

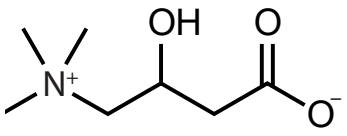
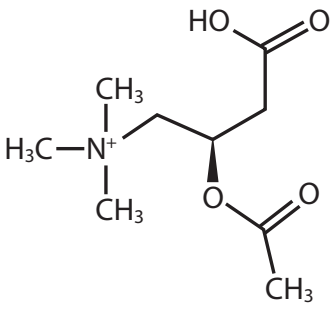
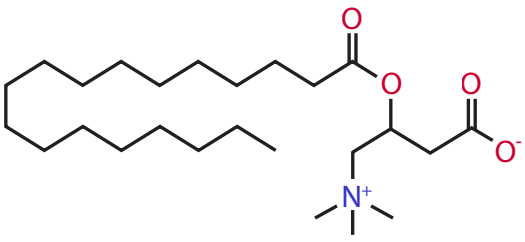


Figure 2: Acylcarnitines exist in two forms: short chain and long chain.

A. Short-Chain Acylcarnitine



B. Long-Chain Acylcarnitine



Many different organic acid and fatty acid oxidation disorders can be diagnosed using acylcarnitine profiles in blood or plasma samples. Acylcarnitines are especially important in newborn testing, which has become mandatory in many places [2]. It is important for testing to be done as soon as possible because if proper treatment is prescribed early, it can greatly reduce the risk of long-term effects. Some of these disorders present later in life, but in most cases, accurate newborn screening and early treatment can minimize their impact.

Restek previously developed a rapid screening method for acylcarnitines using a Raptor HILIC-Si EXP guard column cartridge [3]. That method can improve productivity by allowing combined screening of acylcarnitines and amino acids without derivatization, but it does not provide chromatographic separation of several compounds that are used for differential diagnosis. While high-throughput screening methods are valuable, the method developed here takes a different approach and focuses on the chromatographic separation of several key acylcarnitines that are used for diagnosing different disorders [2]. In addition, while methods have been developed using either underivatized or derivatized sample preparation techniques [4], this method was developed without derivatization in order to simplify and speed up sample preparation.

Experimental

Plasma Samples

For plasma, 100 µL of sample was added to a microcentrifuge tube along with 5 µL of internal standard (5 ng/mL of internal standard working stock containing the D3 internal standards shown in Table I). Next the sample was vortexed for 10 seconds. After vortexing, the samples were incubated for 10 minutes at ambient temperature. Following incubation, 300 µL of methanol was added to each sample, and the samples were vortexed again for 10 seconds. The samples were then centrifuged for 10 minutes at 4000 rpm. Finally, 100 µL of the supernatant was aliquoted into a vial containing 900 µL of mobile phase A (0.1% formic acid in water) and vortexed for 10 seconds before being injected into the LC-MS/MS for analysis. While a 10x final dilution was appropriate for the instrument used here, a different dilution factor may be necessary for instruments with lower or higher sensitivity.

Calibration Standards and Quality Control Samples

Preliminary experiments determined that plasma would not be a suitable matrix for the standards and QC samples used for method evaluation due to the endogenous levels of acylcarnitine. For this reason, multiple surrogate matrices were investigated and 100 µg/mL of bovine serum albumin (BSA) in water was found to be the best surrogate matrix. Calibration and QC stock solutions were then made across a 25–5000 ng/mL concentration range in 1000 µL of BSA, and 100 µL of each solution was added to a microcentrifuge tube and processed following the same sample preparation procedure that was previously described for the plasma samples. Note that not all compounds were included when evaluating method performance. Instead, C5-valeryl-L-carnitine was chosen as a representative analyte for the quantitative and standard addition experiments due to its mid-range polarity and presence in a group of isomers.

Instrument Conditions

Analysis of acylcarnitines by LC-MS/MS was performed on a Shimadzu Nexera UHPLC paired with a SCIEX 4500 MS/MS using the following instrument conditions and the analyte transitions presented in Table I.

Column:	Raptor ARC-18 (2.7 µm, 100 mm x 2.1 mm ID [cat.# 9314A12])
Guard column:	Raptor ARC-18 EXP guard column cartridge (2.7 µm, 5 mm x 2.1 mm [cat.# 9314A0252])
Column temp.:	35 °C
Injection volume:	3 µL
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile:isopropanol (90:10)
Time (min)	%B
0.00	2
1.00	2
4.00	12
6.00	100
7.50	100
8.00	2
9.00	stop
Flow rate:	0.6 mL/min
Ion mode:	Positive ESI

Table I: Ion transitions for acylcarnitines LC-MS/MS analysis.

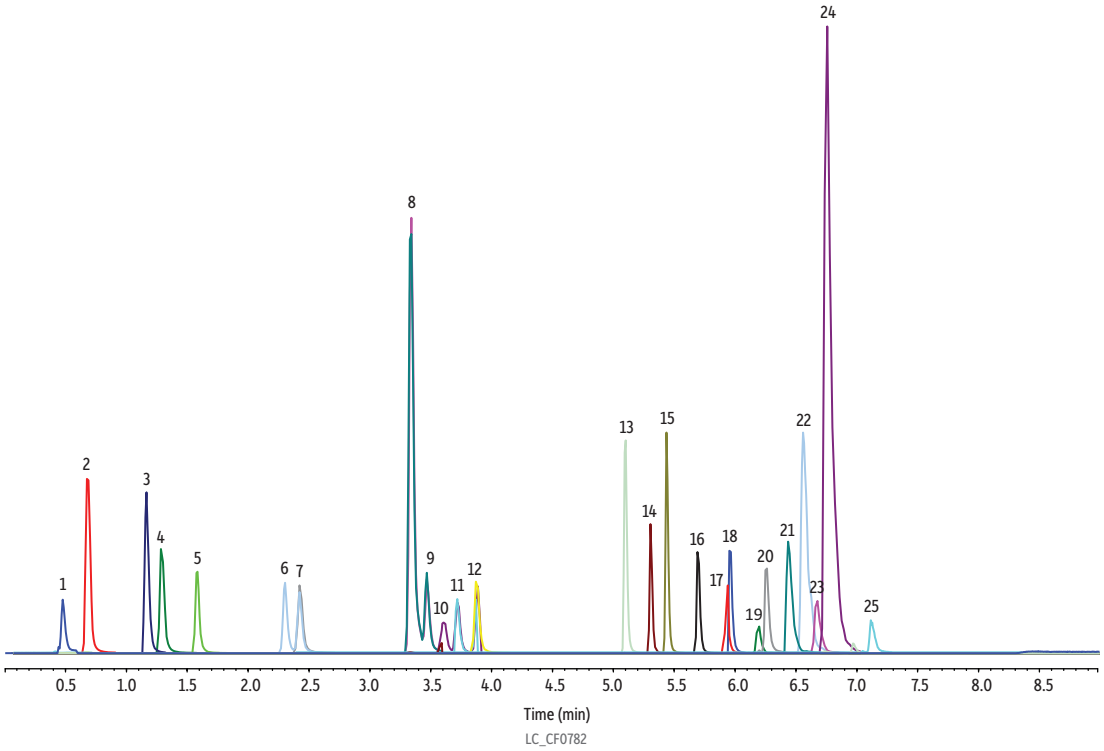
Analyte	Precursor Ion	Product Ion
C0-L-carnitine	162.2	85.1
C0-L-carnitine-D3	165.2	85.1
C2-Acetyl-L-carnitine	204.2	85.1
C2-Acetyl-L-carnitine-D3	207.2	85.1
Methyl-malonyl-L-carnitine	262.3	85.1
Methylmalonyl-L-carnitine-D3	265.3	85.1
C3-Propionyl-L-carnitine	218.1	85.1
C3-Propionyl-L-carnitine-D3	221.1	85.1
3-Hydroxyisovaleryl-L-carnitine	262.4	85.1
3-Hydroxyisovaleryl-L-carnitine-D3	265.1	85.1
C4-Isobutyryl-L-carnitine	232.1	85.1
C4-Isobutyryl-L-carnitine-D3	235.1	85.1
C4-Butyryl-L-carnitine	232.2	85.1
C4-Butyryl-L-carnitine-D3	235.2	85.1
3-Methylcrotonyl-L-carnitine	244.1	85.1
3-Methylcrotonyl-L-carnitine-D3	247.3	85.1
C5:1-Tigyl-L-carnitine	244.2	85.1
2-Methylbutyryl-L-carnitine	246.1	85.1
C5-Isovaleryl-L-carnitine	246.1	85.1
C5-Isovaleryl-L-carnitine-D3	249.2	85.1
C5-Valeryl-L-carnitine	246.2	85.1
C5-Valeryl-L-carnitine-D3	249.1	85.1
C6-Hexanoyl-L-carnitine	260.3	85.1
C6-Hexanoyl-L-carnitine-D3	263.2	85.1
C7-Heptanoyl-L-carnitine	274.2	85.1
C8-Octanoyl-L-carnitine	288.4	85.1
C8-Octanoyl-L-carnitine-D3	291.2	85.1
C10-Decanoyl-L-carnitine	316.3	85.1
C10-Decanoyl-L-carnitine-D3	319.2	85.1
C12-Lauroyl-L-carnitine	344.5	85.1
C12-Lauroyl-L-carnitine-D3	347.3	85.1
C14:2-Tetradecadienoyl-L-carnitine	368.5	85.1
C14:1 Tetradecanoyl-L-carnitine	369.8	85.1
C14-Myristoyl-L-carnitine	372.4	85.1
C14-Myristoyl-L-carnitine-D3	375.3	85.1
C16:1 Palmitolelyl-L-carnitine	398.4	85.1
C16-Palmitoyl-L-carnitine	400.5	85.1
C16-Palmitoyl-L-carnitine-D3	403.3	85.1
C18:2 Linoleoyl-L-carnitine	424.1	85.1
C18:1 Oleoyl-L-carnitine	426.4	85.1
C18-Oleoyl-L-carnitine-D3	429.4	85.1
C18 - Stearoyl-L-carnitine	429.1	85.1
C18 - Stearoyl-L-carnitine-D3	431.9	85.1

Results and Discussion

Chromatographic Performance

Simultaneous LC-MS/MS analysis of 25 underivatized acylcarnitines was achieved in a fast 9-minute cycle time on a Raptor ARC-18 column as demonstrated in Figure 3. This method uses a quick and simple sample preparation procedure and provides the chromatographic separation of multiple isobars that is essential for differential diagnosis.

Figure 3: 25 Underivatized acylcarnitines were chromatographically separated, allowing for positive identification of compounds that are critical for differential diagnosis (internal standards not shown).



Peaks	t_R (min)	Precursor Ion	Product Ion
1. C0-L-Carnitine	0.40	162.2	85.1
2. C2-Acetyl-L-carnitine	0.59	204.2	85.1
3. Methyl-malonyl-L-carnitine	1.15	262.3	85.1
4. C3-Propionyl-L-carnitine	1.29	218.1	85.1
5. 3-Hydroxyisovaleryl-L-carnitine	1.60	262.4	85.1
6. C4-Isobutyrl-L-carnitine	2.31	232.1	85.1
7. C4-Butyryl-L-carnitine	2.42	232.2	85.1
8. 3-Methylcrotonyl-L-carnitine	3.31	244.1	85.1
9. C5:1-Tigyl-L-carnitine	3.49	244.2	85.1
10. 2-Methylbutyryl-L-carnitine	3.61	246.1	85.1
11. C5-Isovaleryl-L-carnitine	3.71	246.1	85.1
12. C5-Valeryl-L-carnitine	3.87	246.2	85.1
13. C6-Hexanoyl-L-carnitine	5.08	260.3	85.1
14. C7-Heptanoyl-L-carnitine	5.27	274.2	85.1
15. C8-Octanoyl-L-carnitine	5.41	288.4	85.1
16. C10-Decanoyl-L-carnitine	5.67	316.3	85.1
17. C14:2-Tetradecadienoyl-L-carnitine	5.90	368.5	85.1
18. C12-Lauroyl-L-carnitine	5.94	344.5	85.1
19. C14:1-Tetradecanoyl-L-carnitine	6.17	369.8	85.1
20. C14-Myristoyl-L-carnitine	6.25	372.4	85.1
21. C18:2 Linoleoyl-L-carnitine	6.42	424.1	85.1
22. C16:1 Palmitolelyl-L-carnitine	6.58	398.4	85.1
23. C16-Palmitoyl-L-carnitine	6.69	400.5	85.1
24. C18:1 Oleoyl-L-carnitine	6.77	426.4	85.1
25. C18 - Stearoyl-L-carnitine	7.13	429.1	85.1

Column	Raptor ARC-18 (cat.# 9314A12)
Dimensions:	100 mm x 2.1 mm ID
Particle Size:	2.7 µm
Pore Size:	90 Å
Guard Column:	Raptor ARC-18 EXP Guard Column Cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9314A0252)
Temp.:	35 °C
Standard/Sample	
Diluent:	Water, 0.1% formic acid
Conc.:	100 ng/mL
Inj. Vol.:	3 µL
Mobile Phase	
A:	Water, 0.1% formic acid
B:	90:10 Acetonitrile:isopropanol, 0.1% formic acid

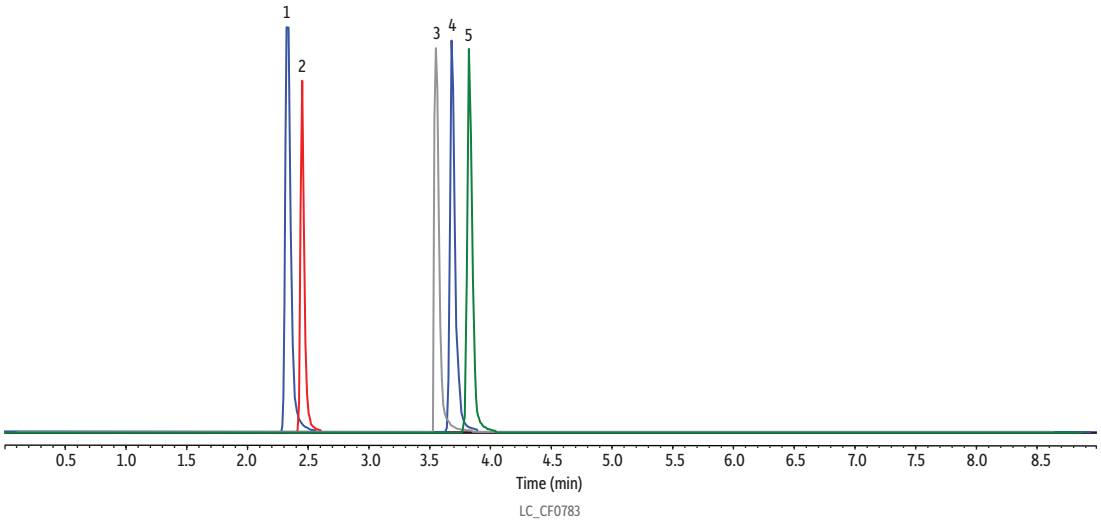
Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	98	2
1.00	0.6	98	2
4.00	0.6	88	12
6.00	0.6	0	100
7.50	0.6	0	100
8.00	0.6	98	2
9.00	0.6	98	2

Detector	MS/MS
Ion Source:	Electrospray
Ion Mode:	ESI+
Mode:	MRM
Instrument	UHPLC
Sample Preparation	A 100 ng/mL standard mix of all of the acylcarnitines was prepared in plasma. A 100 µL aliquot was taken from the standard and mixed with 300 µL of methanol, vortexed for 10 seconds, and then centrifuged for 10 minutes at 4000 rpm. 100 µL of the supernatant was added to a 2 mL vial (cat.# 24619) containing 900 µL of Mobile Phase A (0.1% formic acid in water), capped with a short screw cap (cat.# 24498), and injected for LC-MS/MS analysis.

Key Separations

There are many isomeric separations that are crucial for differential diagnosis. Some of these include the separation of C4-butyryl-L-carnitine from C4-isobutyryl-L-carnitine for the diagnosis of butyryl-CoA dehydrogenase deficiency versus isobutyryl-CoA dehydrogenase deficiency. This separation can be seen in Figure 4 along with the separation of C5-valeryl-L-carnitine, C5-isovaleryl-L-carnitine, and 2-methyl-butyryl-L-carnitine. The separation of these critical isobars is easily achieved on a Raptor ARC-18 column in a 9-minute run, which allows higher sample throughput compared to the 20+ minute runs observed in some literature for this type of isobaric separation.

Figure 4: Separation of C4-butyryl-L-carnitine and C4-isobutyryl-L-carntine as well as C5-valeryl-L-carnitine, C5-isovaleryl-L-carnitine, and 2-methyl-butyryl-L-carnitine.



Peaks	t_R (min)	Precursor Ion	Product Ion
1. C4-Isobutyryl-L-carnitine	2.33	232.1	85.1
2. C4-Butyryl-L-carnitine	2.47	232.1	85.1
3. 2-Methylbutyryl-L-carnitine	3.64	246.1	85.1
4. C5-Isovaleryl-L-carnitine	3.74	246.1	85.1
5. C5-Valeryl-L-carnitine	3.90	246.2	85.1

Column	**Raptor ARC-18 (cat.# 9314A12)**
Dimensions:	**100 mm x 2.1 mm ID**
Particle Size:	**2.7 µm**
Pore Size:	**90 Å**
Guard Column:	**Raptor ARC-18 EXP Guard Column Cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9314A0252)**
Temp.:	**35 °C**
Standard/Sample Diluent:	**Water, 0.1% formic acid**
Conc.:	**100 ng/mL**
Inj. Vol.:	**3 µL**
Mobile Phase A:	**Water, 0.1% formic acid**
B:	**90:10 Acetonitrile:isopropanol, 0.1% formic acid**

Time (min)	Flow (mL/min)	%A	%B
0.00	0.6	98	2
1.00	0.6	98	2
4.00	0.6	88	12
6.00	0.6	0	100
7.50	0.6	0	100
8.00	0.6	98	2
9.00	0.6	98	2

Detector	**MS/MS**
Ion Source:	**Electrospray**
Ion Mode:	**ESI+**
Instrument	**UHPLC**
Notes	**All analytes spiked at 100 ng/mL**

Accuracy and Precision

Accuracy and precision were evaluated using QC samples that were analyzed over a total of three days, and the results are presented in Table II. Method accuracy was demonstrated by recovery values being within 15% of the nominal concentrations for all QC samples. Precision was shown by the %RSD values being ≤14% for all QC samples.

Table II: Interday accuracy and precision for underivatized C5-valeryl-L-carnitine in quality control samples prepared in BSA.

	Avg. Conc. (ng/mL)	Avg. Accuracy (%)	% RSD
QC 25 ng/mL	27.7	110.9	13.5
QC 150 ng/mL	146.5	97.8	3.1
QC 600 ng/mL	679.0	113.0	3.8
QC 1500 ng/mL	1500.0	100.0	2.0
QC 3000 ng/mL	2900.0	96.6	0.0

Column Robustness

Column robustness was tested by making 250 injections onto the same column of a 100 ppb acylcarnitines standard prepared in BSA. First injection and last injection retention times were consistent for all 25 of the monitored acylcarnitines as demonstrated in Table III below. Stable retention times help ensure accurate selectivity and identification over longer column lifetimes.

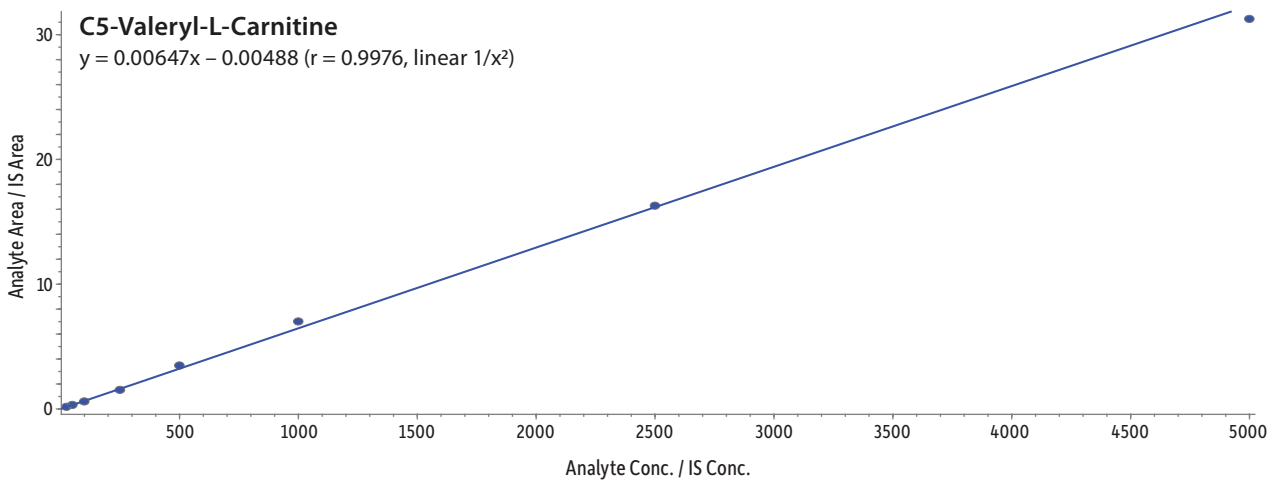
Table III: Retention times in this acylcarnitines LC-MS/MS analysis were highly consistent over 250 injections for all compounds, demonstrating good robustness.

Analyte	Inj. 1 (min)	Inj. 250 (min)	% Difference
C0-L-carnitine	0.40	0.40	0.0
C2-Acetyl-L-carnitine	0.59	0.59	0.0
Methyl-malonyl-L-carnitine	1.15	1.15	0.0
C3-Propionyl-L-carnitine	1.29	1.28	0.8
3-Hydroxyisovaleryl-L-carnitine	1.60	1.58	1.3
C4-Isobutyryl-L-carnitine	2.34	2.31	1.3
C4-Butyryl-L-carnitine	2.49	2.43	2.4
3-Methylcrotonyl-L-carnitine	3.32	3.31	0.3
C5:1-Tigyl-L-carnitine	3.32	3.31	0.3
2-Methylbutyryl-L-carnitine	3.63	3.63	0.0
C5-Isovaleryl-L-carnitine	3.73	3.72	0.3
C5-Valeryl-L-carnitine	3.89	3.89	0.0
C6-Hexanoyl-L-carnitine	5.07	5.07	0.0
C7-Heptanoyl-L-carnitine	5.28	5.28	0.0
C8-Octanoyl-L-carnitine	5.41	5.41	0.0
C10-Decanoyl-L-carnitine	5.67	5.67	0.0
C14:2-Tetradecadienoyl-L-carnitine	5.92	5.92	0.0
C12-Lauroyl-L-carnitine	5.94	5.94	0.0
C14:1 Tetradecanoyl-L-carnitine	6.18	6.18	0.0
C14-Myristoyl-L-carnitine	6.25	6.25	0.0
C18:2 Linoleoyl-L-carnitine	6.40	6.41	0.2
C16:1 Palmitolelyl-L-carnitine	6.56	6.57	0.2
C16-Palmitoyl-L-carnitine	6.69	6.69	0.0
C18:1 Oleoyl-L-carnitine	6.77	6.78	0.1
C18 - Stearoyl-L-carnitine	7.14	7.15	0.1

Linearity

Using $1/x^2$ weighted linear regression, C5-valeryl-L-carnitine showed acceptable linearity with R^2 of 0.9952 or greater (Figure 5).

Figure 5: C5-Valeryl-L-carnitine showed good linear response over the entire calibration range.



Accuracy of Surrogate Matrix

Fortified plasma samples were prepared from three different lots of plasma and analyzed using calibrators prepared in BSA to test the accuracy of using BSA as a surrogate matrix for underivatized acylcarnitines analysis. First, the endogenous concentration of C5-valeryl-L-carnitine was quantitated for each lot. Then, standard addition was used to test the accuracy of the surrogate matrix. For standard addition, the samples were fortified with an additional 1000 ng/mL. All three lots of plasma showed acceptable results that were less than 11% different from the expected values for both intra- and interday repeatability studies as demonstrated in Table IV and Table V below.

Table IV: Intraday repeatability for standard addition spiking of C5-valeryl-L-carnitine in human plasma.

Plasma Lot	Expected (ng/mL)	Average (ng/mL)	% Difference	% RSD
Lot 1	1006	983	2.3	2.3
Lot 2	1005	1100	9.0	3.4
Lot 3	1002	1072	6.8	3.3

Table V: Interday repeatability for standard addition spiking of C5-valeryl-L-carnitine in human plasma

Plasma Lot	Expected (ng/mL)	Average (ng/mL)	% Difference	% RSD
Lot 1	1006	989	1.7	2.6
Lot 2	1006	1120	10.7	2.7
Lot 3	1004	1109	9.9	2.3

Conclusion

The acylcarnitines LC-MS/MS analysis developed here provides a quick, efficient approach for the preparation and analysis of underivatized acylcarnitines in plasma samples. Separation of critical pairs was achieved in a fast 9-minute run, allowing high-throughput analysis that can support differential diagnosis. Method performance testing demonstrated acceptable method precision, accuracy, and linearity, and the standard addition experiment showed that BSA is a suitable surrogate matrix.

References

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[4] V.R. De Jesús, D.H. Chace, T.H. Lim, J.V. Mei, W.H. Hannon, Comparison of amino acids and acylcarnitines assay methods used in newborn screening assays by tandem mass spectrometry, Clinica Chemica Acta 411 (2010) 684-689. DOI: https://doi.org/10.1016/j.cca.2010.01.034

This method has been developed for research use only; it is not suitable for use in diagnostic procedures without further evaluation.

ordering notes

Certificates of analysis for new Restek LC columns are now provided electronically. To view and download, visit www.restek.com/documentation then enter your cat.# and serial #.

Raptor ARC-18 LC Columns (USP L1)

- Ideal for high-throughput LC-MS/MS applications with minimal sample preparation.
- Well-balanced retention profile for better detection and integration of large, multiclass analyte lists.
- Sterically protected to endure low-pH mobile phases without sacrificing retention or peak quality.
- Part of Restek's Raptor LC column line featuring 1.8, 2.7, and 5 µm SPP core-shell silica.

Stationary Phase Category: C18, octadecylsilane (L1)
Ligand Type: Sterically protected C18
Particle: 1.8 µm, 2.7 µm, or 5 µm superficially porous particle (SPP or "core-shell" particle) silica
Pore Size: 90 Å
Carbon Load: 7% (1.8 µm); 7% (2.7 µm); 5% (5 µm)
End-Cap: no
Surface Area: 125 m^2/g (1.8 µm); 130 m^2/g (2.7 µm); or 100 m^2/g (5 µm)
Recommended Usage:
pH Range: 1.0–8.0
Maximum Temperature: 80 °C
Maximum Pressure: 1034 bar/15,000 psi* (1.8 µm); 600 bar/8700 psi (2.7 µm); 400 bar/5800 psi (5 µm)
* For maximum lifetime, recommended maximum pressure for 1.8 µm particles is 830 bar/12,000 psi.
Switch to an ARC-18 column when:
• You are analyzing large, multiclass lists by LC-MS/MS.
• Strongly acidic (pH 1–3) mobile phases are required.

ID	Length	qty.	cat.#
2.7 µm Particles Raptor ARC-18			
2.1 mm	100 mm	ea.	9314A12

Raptor EXP Guard Column Cartridges

- Free-Turn architecture lets you change cartridges by hand without breaking inlet/outlet fluid connections—no tools needed.
- Patented titanium hybrid ferrules can be installed repeatedly without compromising high-pressure seal.
- Auto-adjusting design provides ZDV (zero dead volume) connection to any 10-32 female port.
- Guard column cartridges require EXP direct connect holder (cat.# 25808).
- Pair with EXP hand-tight fitting (cat.# 25937–25938) for tool-free installation.

Description	Particle Size	Length	ID	qty.	cat.#
Raptor ARC-18 EXP Guard Column Cartridge	2.7 µm	5 mm	2.1 mm	3-pk.	9314A0252

Maximum cartridge pressure: 600 bar/8700 psi (2.7 µm)
Intellectual Property: optimizetech.com/patents

ordering notes

Certificates of analysis for new Restek LC columns are now provided electronically. To view and download, visit www.restek.com/documentation then enter your cat.# and serial #.

2.0 mL, 9 mm Short-Cap, Screw-Thread Vials (vial only)

Fit all 2.0 mL, 12 x 32 mm, screw-thread 9 mm/425 vial-based autosamplers.

21142

Description	Type	Volume	Color	Size	qty.	Similar to Part #	cat.#
Short-Cap Vial w/White Graduated Marking Spot	9-425 Screw-Thread	2.0 mL	Amber	12 x 32 mm	100-pk.	Agilent 5182-0716	21142

Ideal for Agilent 7673, 7683, 7693 & other autosamplers that process 12 x 32 mm crimp-top vials. | Compare to Target DP, Robo, Sun1, ABC, and R.A.M. Vials.

2.0 mL, 9 mm Short-Cap, Screw-Vial Closures (Polypropylene, preassembled)

24498

Description	Type	Cap Size	Color	Septa Material	qty.	cat.#
Short Screw Caps	Screw-Thread	9-425	Blue	PTFE/Silicone/PTFE	1000-pk.	24498

Choose preslit caps (available for some vials) to reduce the risk of needle bending, release vacuum from high-volume injections, and improve injection reproducibility when greater than 20% of vial volume is withdrawn.

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Questions? Contact us or your local Restek representative (www.restek.com/contact-us).

www.restek.com

Lit. Cat.# CFAN4029-UNV