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Mass Spectral Library of Acylcarnitines Derived from Human Urine

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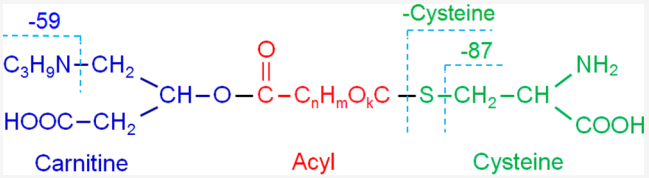
ABSTRACT: We describe the creation of a mass spectral library of acylcarnitines and conjugated acylcarnitines from the LC−MS/MS analysis of six NIST urine reference materials. To recognize acylcarnitines, we conducted in-depth analyses of fragmentation patterns of acylcarnitines and developed a set of rules, derived from spectra in the NIST17 Tandem MS Library and those identified in urine, using the newly developed hybrid search method. Acylcarnitine tandem spectra were annotated with fragments from carnitine and acyl moieties as well as neutral loss peaks from precursors. Consensus spectra were derived from spectra having similar retention time, fragmentation pattern, and the same precursor m/z and collision energy. The library contains 157 different precursor masses, 586 unique acylcarnitines, and 4 332 acylcarnitine consensus spectra. Furthermore, from spectra that partially satisfied the fragmentation rules of acylcarnitines, we identified 125 conjugated acylcarnitines represented by 987 consensus spectra, which appear to originate from Phase II biotransformation reactions. To our knowledge, this is the first report of conjugated acylcarnitines. The mass spectra provided by this work may be useful for clinical screening of acylcarnitines as well as for studying relationships among fragmentation patterns, collision energies, structures, and retention times of acylcarnitines. Further, these methods are extensible to other classes of metabolites.



The identification of small-molecule metabolites in biological samples is of importance in the diagnosis, treatment, and monitoring of human diseases. For a single metabolite mass with a given elemental composition, many isomers may exist, including structural, positional, stereoisomers as well as chromatographically inseparable enantiomers and separable diastereomers. The high degree of heterogeneity of metabolite structures and inherent difficulties in deriving chemical structures from mass spectra are major impediments in metabolite identification.

In metabolomics studies, acylcarnitines (AC) have been widely used to diagnose a variety of inherited and acquired diseases.[2−6] This is due to their biological functions of transferring long-chain fatty acids into mitochondria for energy production and removing potential toxic excesses of acyl groups.[1−6]

Although mass spectrometry (MS) analyses of underivatized and derivatized acylcarnitines in samples of newborns and patients have been performed since the 1980s,[7−22] acquiring a full profile of this chemical class from biosamples remains a major challenge. Two studies have attempted to generate such a full profile. One was published in 2011 by Zuniga and Li.[23] Their method involved a solid phase extraction (SPE) technique for selective analyte extraction, an ultra-high-performance liquid chromatography (HPLC) separation, and a targeted tandem MS analysis with information-dependent acquisition and selected reaction monitoring. The authors reported 355 unique acylcarnitines in a urine sample pooled from six healthy individuals. The authors also noted that certain very hydrophilic and hydrophobic acylcarnitines were not detectable in their experiments. This work presented valuable methods for analyzing acylcarnitines but had an important limitation in that the spectra were acquired on a low-mass resolution instrument. The other study was published in 2018 by Yu and coauthors.[14] They adopted a two-step method for acquiring all possible acylcarnitine spectra. The first step was to detect all acylcarnitine precursors based on characteristic fragments, resulting in 298 precursor ions. The second step was to acquire acylcarnitine spectra with "multirun LC−full scan MS combined with parallel reaction monitoring acquisition" using the identified precursors.[24] Due to limited sampling speed, a single run covered only 30 precursor ions, requiring 10 runs for the 298 precursor ions. With a mass spectrometer and three collision energies, the authors detected 241, 515, and 222 acylcarnitines from samples of human plasma (pooled from 36 healthy adults), human urine (24 healthy adults), and rat liver tissue, respectively. In total, they reported 733 different acylcarnitines.

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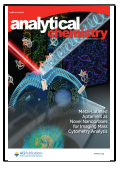

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The above-mentioned targeted studies demonstrated that many acylcarnitines are detectable in biological samples. However, currently, there is no efficient way of identifying these compounds in complex biological samples. Providing such a method through mass spectral library searching is a key objective of this work. This is done using higher-energy C-trap dissociation (HCD) mass spectra acquired with nine collision energies from an untargeted liquid chromatography-tandem mass spectrometry (LC−MS/MS) analysis of urine reference materials and a metabolite-class targeting method for making identifications. The major strategies used in this work include (1) establishing rules for detecting acylcarnitines based on the analyses of reference spectra in the NIST17 Tandem MS Library[25] and spectra retrieved by the recently developed "hybrid" search method;[26−28] (2) annotating acylcarnitine spectra by fragments of carnitine and acyl moieties as well as by neutral loss fragments; (3) excluding artifacts, such as in-source ion spectra and acylcarnitine-contaminated spectra. We provide the resulting mass spectral library for 586 acylcarnitines and 125 conjugated acylcarnitines with freely available search software.

MATERIALS AND EXPERIMENTS

Six pooled NIST standard reference materials (SRM)[29] served as the urine samples analyzed in this study. The details are provided in Table S1 of the Supporting Information pdf file.

The experimental procedure was previously reported[29,30] and is also provided in section 1 of the Supporting Information pdf file. Briefly, this involves four steps: (1) urine samples were thawed from −80 °C to room temperature; (2) diluted with methanol (1:1, v/v) and centrifuged; (3) the supernatant was collected and subjected to evaporation under a gentle nitrogen stream; and (4) the dried sample was reconstituted with 10% ACN and 0.1% formic acid.

Reverse phase separations were done on a Waters ACQUITY UPLC CSH-C18 column using a Dionex Ultimate 3000 liquid chromatograph. LC−electrospray ionization (ESI) MS and tandem spectra were recorded on an orbitrap Fusion Lumos (Thermo Scientific). The full MS scan (MS1) resolution was 120 000. To acquire HCD spectra, data dependent sampling was performed using an exclusion duration of 18 s, an isolation window of ±0.75 m/z, and a resolution of 30 000. The HCD spectra of each sample were obtained in duplicates under nine normalized collision energies (NCE, 10, 15, 20, 25, 30, 40, 50, 60, and 80). At each collision energy, 12 runs were made (2 runs per sample). The data set used in this study includes 108 individual runs in total. These runs were part of a larger series used to develop a general "recurrent spectral library".[29]

DATA ANALYSIS METHODS

Extraction of Acylcarnitine Feature Fragments and Fragmentation Patterns. *From Spectra in the NIST Metabolite Library.* Acylcarnitine feature fragments and fragmentation patterns were derived from spectra in the NIST17 Tandem MS Library, which contains mass spectra of commercially available metabolites at multiple collision energies. For this study, only protonated high-resolution acylcarnitine HCD Orbitrap library spectra were considered, which included 308 spectra of 27 acylcarnitines. These spectra were used to extract acylcarnitine fragmentation features. See Table ES1 in the Supporting Information Excel file for these acylcarnitines.

From the Hybrid Search. The NIST Hybrid Search, a recently developed algorithm applicable to unidentified spectra,[26−28] was used to detect a larger diversity of putative acylcarnitines. While only 20 acylcarnitines could be identified by a direct mass spectral library matching program in a urine run against the NIST17 Tandem MS Library, hybrid searching provided 150 candidates with match scores larger than 500 (the highest score is 999). This large number of candidates provided a diversity of examples on which to base more general rules for recognizing acylcarnitine spectra. The hybrid search algorithm performs an analysis that determines whether unmatched fragment ions can be explained as a consequence of the library and query compound differing by a chemical group or atom that has no major effect on the fragmentation mechanism. The hybrid search program used in this study is NIST *MS PepSearch*,[31] developed for batch processing of both proteomics and metabolomics data sets.

Recognition of Acylcarnitine. We characterize acylcarnitines based on four spectral feature fragments, i.e, $C_4H_5O_2^+$ (m/z, 85.0284); TMAI (trimethylamine ion $C_3H_{10}N^+$, m/z, 60.0808); neutral loss fragment TMA (trimethylamine, C_3H_9N) or (TMA + H_2O); and $C_7H_{14}NO_2^+$ (carnitine − H_2O + H^+) or precursor (P) − $C_7H_{13}NO_2$ under typical collision energies (NCE 20−40). After an acylcarnitine and its precursor m/z and retention time (RT) were determined, all spectra acquired at the same m/z and retention time (deviation < 7 seconds (s)) under other collision energies were assigned to the same acylcarnitine, without requiring the presence of all the four feature fragments in these spectra.

Determination of Acylcarnitine Ion Formula and m/z Data. We manually confirmed the formula and m/z value of each unknown acylcarnitine on the basis of its precursor and spectral fragment m/z values. Because acylcarnitine masses are relatively small (< 600 Da) and the resolving power we used is high (120 000), chemical formulas could be readily derived for almost all ions. An acceptance threshold was set at 5 ppm.

Calculation and Assignment of Ion Retention Time and Abundance. In LC−MS, each ionized compound is associated with a set of MS1 centroided peaks with associated isotopes, abundances, and retention times. This set of peaks is denoted as an ion cluster. The key properties of a cluster include monoisotopic m/z, charge state, abundance, and RT. In this work, each ion has a cluster RT, which is calculated as the time when the accumulated abundance of the most abundant isotope reaches half of its total abundance. All spectra belonging to the same ion are assigned with the same cluster RT. Two abundance values for each ion were derived in this study: the extracted ion chromatogram (XIC) and the largest peak of an ion cluster. All cluster properties of the 108 urine runs were processed. The programs performing these calculations were described previously as the NIST ProMS and NIST MSQC pipeline.[32,33]

Annotation of Acylcarnitine Fragments. Our fragmentation analysis found that acylcarnitine spectra can be annotated using three types of fragments: (A) fragments from decomposition of the carnitine moiety, such as TMAI (m/z, 60.0808) and $C_4H_5O_2^+$ (m/z, 85.0284); a list of all the relevant fragments is provided in Table S2 in the Supporting Information pdf file; (B) fragments from decomposition of the acyl moiety, including losses of CO, H_2O, C_2H_4, C_2H_2O, CH_2O_2, $C_2H_4O_2$, etc.; (C) neutral losses from the acylcarnitine

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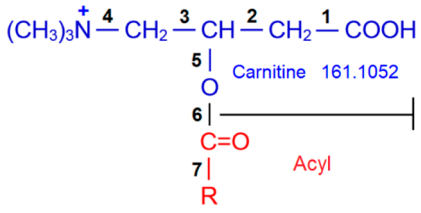
Anal. Chem. 2020, 92, 6521−6528

precursor ion, such as TMA, H_2O, CH_2O_2, $C_2H_4O_2$, and $C_7H_{13}NO_2$, or a combination of two or more neutral losses, such as TMA + H_2O, TMA + $2H_2O$, TMA + CH_4O_3, etc. For more descriptions of the annotations, see section 3 of the Supporting Information pdf file.

RESULTS AND DISCUSSION

Acylcarnitine Fragmentation Patterns, Features, and Neutral Loss Fragments. An acylcarnitine is an esterified product of carnitine and a fatty acid, which is represented in solution as a zwitterion. A protonated form is presented in Scheme 1.

Scheme 1. Representative Structure of a Protonated Acylcarnitine[a]



[a]C(=O)R is derived from its precursor fatty acid.

Under typical collision energies, such as from NCE 20 to 40, the acylcarnitine spectra usually contain fragments from carnitine and acyl moieties as well as neutral losses from both. These fragments provide not only information for recognizing acylcarnitines but also clues of their structures. A brief description of these fragments is given in the following section (see section 4 of the Supporting Information pdf file for more information).

Carnitine Moiety Related Fragments. Acylcarnitine mass spectra commonly contain three well-known feature fragments,[7,8,12] i.e., $C_4H_5O_2^+$, TMAI, and neutral loss of TMA or (TMA + H_2O). Acylcarnitine HCD spectra in the NIST17 Tandem MS Library at multiple collision energies indicate that fragments $C_4H_5O_2^+$ (m/z, 85.0284) and TMAI (m/z, 60.0808) can always be observed, except for spectra of larger mass acylcarnitines under low collision energies. For example, spectra of acylcarnitine ion $C_{23}H_{38}NO_4$ (m/z 392.279, RT around 1335 s) have observable fragments P − TMA only at NCE 10, have both $C_4H_5O_2^+$ and P − TMA at NCE 15, and have all the three fragments at NCE 20.

Fragment $C_4H_5O_2^+$ is the most significant feature peak of acylcarnitine spectra. Its abundance, under commonly used collision energies, is consistently the largest or among the most abundant peaks but may decrease at higher collision energies, such as when NCE > 80. Figure 1 shows the relationships between abundances of the major carnitine fragments and collision energies for (R)-butyrylcarnitine. The ion $C_4H_5O_2^+$ is not unique to acylcarnitine; many nonacylcarnitines can exhibit this fragment. For example, among the total 80396 HCD spectra in the NIST17 Tandem MS Library, 3874 (4.8%) spectra contain fragment $C_4H_5O_2^+$, far exceeding the number of acylcarnitine spectra.

The second feature fragment is TMAI. Its intensities at different collision energies show a non-monotonic pattern (green line in Figure 1), which is unique in comparison with the other fragments in the acylcarnitine spectra. TMAI co-maximizes with $C_4H_5O_2^+$ and then decreases with the increase

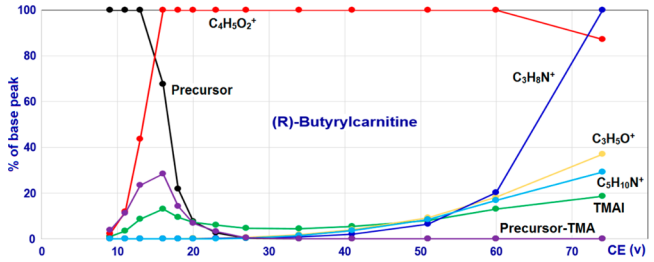


Figure 1. Intensities of the major carnitine moiety-related fragments of (R)-Butyrylcarnitine at different collision energies. Data source: NIST17 Tandem MS Library.

in collision energy. At higher collision energies, TMAI intensities gradually increase again, generated by secondary decomposition of other ions. Although this ion is a key diagnostic fragment of acylcarnitine, it also occurs in spectra of other compounds, such as betaines, choline, *N*-trimethyllysine, and their derivatives.

The third feature fragment is the neutral loss peak of TMA (mass, 59.0735) from the precursor. This fragment is relatively abundant at low collision energies and sometimes even more abundant than the ion $C_4H_5O_2^+$. A mass spectrum obtained at a lower collision energy lacking this characteristic can be confidently rejected. This fragment is not stable at higher collision energy. It maximizes as does TMAI, then, its abundance decreases quickly and disappears at higher collision energies.

A valuable usage of the neutral loss of TMA fragment is to recognize "contaminated" spectra. This is because non-acylcarnitine spectra can contain acylcarnitine fragments due to their coelution and co-fragmentation with acylcarnitines. Figure S9 (see section 7.2 in the Supporting Information pdf file) is an example, which shows that a non-acylcarnitine spectrum with a precursor at m/z 344.2283 contains abundant acylcarnitine fragments. This spectrum can be determined as non-acylcarnitine because it does not have the neutral loss peak of TMA at m/z, 285.1548 (= 344.2283 − 59.0735). Further analysis revealed that this spectrum was contaminated by a coeluting acylcarnitine at m/z 344.2076. Such co-fragmentation presents a serious concern in developing libraries derived from complex materials.

Another useful diagnostic fragment is $C_7H_{14}NO_2^+$ (= carnitine − H_2O, m/z = 144.1019), which arises from the cleavage of bond 5 in the structure in Scheme 1. Zuniga and Li discussed the formation of this fragment.[23] Generally, its abundance is low, less than 20% of the base peak. It does not appear in Figure 1 because its relative abundance is less than 4%, but it is relatively stable and can be detected even up to 70 V or higher for larger acylcarnitines.

Acyl Moiety Related Fragments. The acyl moiety peak is generated when the ester bond between carnitine and acyl moiety (bond 6 in the structure in Scheme 1) is broken. As collision energy increases, the intensity of this ion rises from undetectable to low, then high, and then decreases to undetectable at higher collision energy. For most spectra collected in this study, the acyl moiety can be detected at normalized collision energies from 20 to 40. There is a small percentage of acylcarnitines for which no acyl peaks can be detected in their spectra due to more favorable pathways to other stable ions, such as fragments of acyl − CO (from the cleavage of the bond 7 in the structure in Scheme 1), and acyl

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Anal. Chem. 2020, 92, 6521−6528

− H_2O (corresponding to the neutral loss of carnitine and a loss of H_2O from hydroxylated or carboxylated acyl moiety[23]).

The carnitine moiety has a well-defined structure, and its fragments can generally be described based on carnitine and acylcarnitine spectra in the NIST17 Tandem MS Library. In contrast, acyl structures are diverse, making it challenging to annotate their fragments. The most common neutral losses from acyl moiety are CO and C_2H_2O. Other acyl-related neutral losses vary with the individual acyl structures, for example, neutral losses of H_2O for a hydroxylated acyl and CH_2O_2 and CO_2 for a carboxylated acyl.

Retention Time Consistency of Metabolites from Different Runs. Consistent RT values of compounds are critical to link isomers across different runs. For examining the RT consistency of the urine data, a set of 92 reference chemicals was selected from the urine LC−MS/MS data (see Table ES2 in the Supporting Information Excel file). Good reproducibility and no or few isomers are the key criteria in selecting these reference chemicals. Then, RT consistencies of metabolites in all different runs were evaluated with these reference chemicals. Multiple data sets were examined, revealing one data set of highly consistent RT among its 108 replications. For this data set, the RT median standard deviations of the same compounds in different runs are less than 5 s. This data set was then used exclusively for producing consensus spectra.

Detection of Acylcarnitines. The following four criteria were used to qualify an ion as acylcarnitine. (1) The ion spectra must contain the four diagnostic fragments: (a) $C_4H_5O_2^+$, (b) TMAI, (c) P − TMA or P − TMA − H_2O, and (d) $C_7H_{14}NO_2^+$ (carnitine − H_2O + H^+) or P − $C_7H_{13}NO_2$. (2) The fragment $C_4H_5O_2^+$ is among the most abundant peaks. (3) The fragments TMAI and P − TMA are attributable to the structural element trimethylamine $(CH_3)_3N$, not elements like $-CH_2CH_2CH_2NH_2$, because the later can also form fragments $C_3H_{10}N^+$ and P − C_3H_9N, which are the same as TMAI and P − TMA in mass. The differences between their spectra are that the group $-CH_2CH_2CH_2NH_2$ can form fragments P − NH_3, P − CH_3NH_2, etc., but TMA, i.e., $(CH_3)_3N$ cannot. (4) The major fragments of an acylcarnitine spectrum can be explained by the methods described in the Annotation of Acylcarnitine Fragment. Note: fewer than 3% of spectra do not satisfy all of these rules, due either to low ion abundance or acyls having more than two oxygen atoms.

Based on these criteria and the derived ion properties (ion m/z, RT, and abundance values), 586 unique acylcarnitines were identified from 30746 spectrum replicates extracted from the 108 urine runs (6 samples, 2 replicates for each sample, and 9 collision energies). These detected acylcarnitine spectra contain clues for structure elucidation, although by themselves these clues do not generally enable the complete assignment of isomer structures.

Conjugated Acylcarnitines. During the process of recognizing acylcarnitines and filtering false positives, a set of spectra that partially satisfied the rules for acylcarnitines was further analyzed. This analysis revealed 125 unexpected species that were identified as conjugated acylcarnitines (see Table 1). These compounds have not been reported previously. Compounds in the first five rows (92 compounds) of Table 1 can be explained as products of Phase I and II biotransformation reactions.[34−40] The sixth row denotes 30 acylcarnitines that have N-containing acyls (the N atom is not in an amino acid). The seventh row denotes three Cl-containing acylcarnitines. A list of these conjugated acylcarnitines can be found from Table ES5 in the Supporting Information Excel file. Table 2 shows that these conjugates can have abundances comparable to their corresponding nonconjugated species.

Table 1. List of Conjugated Acylcarnitines and Their Possible Biotransformation Phases

no.	conjugators	counts	phases
1	cysteine	31	II
2	acetylcysteine	20	II
3	CH_4S, CH_4OS, SO_2, SO_3, and thiocysteine	14	I and II
4	taurine	4	II
5	glucuronic acid	23	II
6	N-containing groups	30	
7	Cl	3	
sum		125	

Table 2. Most Abundant Conjugated Acylcarnitines and Their Corresponding Acylcarnitines with Median Abundances from 18 Analyses of the Standard Reference Sample 3667[29]

unconjugated acylcarnitines		conjugated acylcarnitines	
acylcarnitine	abundance	conjugated acylcarnitine	abundance
C4:1	1.18×10^8	C4:1 + cysteine	4.31×10^8
		C4:1 + acetylcysteine	1.44×10^7
		C4:1 + SO_2	5.84×10^7
C5:1	5.93×10^8	C5:1 + cysteine	3.64×10^8
		C5:1 + CH_4OS	2.08×10^7
		C5:1 + acetylcysteine	7.29×10^6
C6:1	6.85×10^7	C6:1 + CH_4S	1.00×10^8
C8:1	8.74×10^9	C8:1 + cysteine	2.03×10^7
		C8:1 + acetylcysteine	2.32×10^7
C10:2	8.81×10^8	C10:2 + cysteine	1.04×10^8
		C10:2 + acetylcysteine	1.56×10^8

As an example, a spectrum of acetylcysteine (ACYS) conjugated acylcarnitine at NCE 30 is shown in Figure 2. This spectrum has two acylcarnitine characteristic peaks, $C_4H_5O_2^+$ and TMAI but does not contain a peak corresponding to neutral loss TMA, typical of acylcarnitines. The most abundant fragment in this spectrum is the peak at m/z 185.0808, which appears to be the result of two successive neutral losses, i.e., TMA and ACYS. This is consistent with the initial loss of TMA followed by the rapid loss of ACYS. For acetylcysteine conjugates, key fragments include P − 129 (i.e., P − (ACYS − H_2S)), P − C_2H_2O (acetyl group), P − CH_2O_2, and fragment at m/z 130 (ACYS − H_2S + H^+).[37,38] Figure 2 shows multiple ACYS related fragments, providing evidence of the presence of ACYS and a tentatively assigned structure of this conjugated acylcarnitine.

Criteria used to determine conjugated acylcarnitines were similar to those for acylcarnitines, with three exceptions: (1) Fragment $C_4H_5O_2^+$ may not be among the most abundant peaks. (2) P − conjugator, P − TMA − conjugator, and N/S-containing acyl fragments should be among the most abundant peaks. (3) P − TMA or TMAI or $C_7H_{14}NO_2^+$ may be missing.

In-Source Artifacts Associated with Conjugated Acylcarnitines. Misidentification of artifacts as natural metabolites is of general concern in the analysis of LC−MS/

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Anal. Chem. 2020, 92, 6521−6528

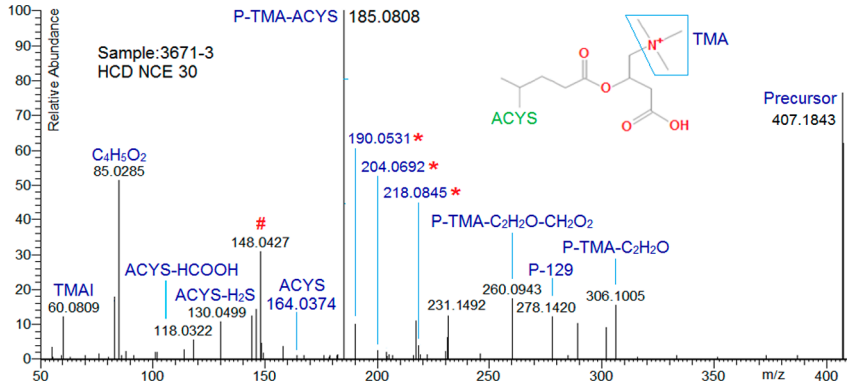

Figure 2. An HCD NCE 30 spectrum of acetylcysteinylated acylcarnitine with a tentatively assigned structure. Here, ACYS is acetylcysteine, P is precursor, and P − 129 is P − (ACYS − H_2S). # Denotes an ion ACYS − acetyl group + a part of the acyl moiety. * Denotes fragments arising from ACYS + a part of the acyl moiety, i.e., 190.0531 corresponds to ACYS + C_2H_2, 204.0692 to ACYS + C_3H_4, and 218.0845 to ACYS + C_4H_6.

Table 3. Eleven CACs and Their in-Source Artifacts, Including Formula and *m/z* Values for the CACs, Coeluting Artifact Formula, Difference in Formula (Form Diff) between Each CAC and Its Artifact AC, and Their Retention Time (RT/s)[a]

	CAC		artifact AC			identified AC	
no.	formula	*m/z*	formula	Form Diff	RT/s	RT/s	Ab ratio
1	$C_{13}H_{26}NO_5S^+$	308.1526	$C_{12}H_{22}NO_4^+$	CH_4OS	204	368−455	1.16
2	$C_{19}H_{38}NO_8S^+$	440.2313	$C_{19}H_{38}NO_5^+$	SO_3	1207	1225−1243	1.92
3	$C_{15}H_{29}N_2O_6S^+$	365.1741	$C_{12}H_{22}NO_4^+$	$C_3H_7NO_2S$*	131	368−455	0.04
4	$C_{18}H_{35}N_2O_6S^+$	407.2210	$C_{15}H_{28}NO_4^+$	$C_3H_7NO_2S$*	673	1006−1190	0.10
5	$C_{18}H_{35}N_2O_6S^+$	407.2210	$C_{15}H_{28}NO_4^+$	$C_3H_7NO_2S$*	708	1006−1190	0.12
6	$C_{20}H_{35}N_2O_6S^+$	431.2210	$C_{17}H_{28}NO_4^+$	$C_3H_7NO_2S$*	791	1170−1208	0.14
7	$C_{20}H_{37}N_2O_6S^+$	433.2367	$C_{17}H_{30}NO_4^+$	$C_3H_7NO_2S$*	859	1191−1238	0.20
8	$C_{20}H_{37}N_2O_6S^+$	433.2367	$C_{17}H_{30}NO_4^+$	$C_3H_7NO_2S$*	960	1191−1238	0.30
9	$C_{20}H_{39}N_2O_6S^+$	435.2523	$C_{17}H_{32}NO_4^+$	$C_3H_7NO_2S$*	1130	1226−1260	0.12
10	$C_{20}H_{39}N_2O_6S^+$	435.2523	$C_{17}H_{32}NO_4^+$	$C_3H_7NO_2S$*	1156	1226−1260	0.15
11	$C_{24}H_{45}N_2O_7S^+$	505.2942	$C_{21}H_{38}NO_5^+$	$C_3H_7NO_2S$*	1146	1249−1301	0.51

[a]Also shown are RT ranges of identified acylcarnitine isomers (RT/s), and the abundance (Ab) ratio of artifact AC to CAC. *$C_3H_7NO_2S$ is for the formula of cysteine.

MS data. It is expected that ESI processes can produce the in-source ions TMAI, $C_4H_5O_2^+$, and P − TMA, because these fragments are present in the low-energy spectra. While metabolite analysis should correctly identify them, these in-source ions are not artifact acylcarnitines. For confidently filtering artifact acylcarnitines, a systematic inspection was made by examining each low energy acylcarnitine tandem MS (MS2) spectrum (NCEs 10 and 15) to see (a) if the spectrum contains fragments (relative abundance (RA) > 0.01) that have the same *m/z* as that of an acylcarnitine ion, or (b) if such fragments are found, are there corresponding acylcarnitine MS1 peaks coeluting at the same retention time as that of the spectrum? This inspection did not find any such spectra.

While there are no in-source acylcarnitines generated from acylcarnitines with higher masses, this inspection did detect 11 acylcarnitines, which were coeluting with corresponding conjugated acylcarnitines (CAC) and could be neutral loss products of CACs, see Table 3. Retention times of these ACs were significantly earlier than those of identified AC ions with the same formula (Table 3). Consequently, the 11 ACs are considered to be artifacts and not included in the library.

This study found few in-source acylcarnitine adducts. For example, no observable adducts in the LC−MS peaks of the three most abundant acylcarnitines were found, except for a sodiated AC with relative abundance less than 0.001 parent (see Figures S6−S8 in the Supporting Information pdf file).

Consensus Spectral Library for Acylcarnitines and Conjugated Acylcarnitines. For each acylcarnitine and conjugated acylcarnitine, replicate spectra with the same precursor *m/z* and collision energy as well as similar RT (within 7 s of each other) were used to generate a consensus spectrum. Spectra of the same *m/z* and similar retention times but different spectral patterns were separated using the method described in section 5.1 of the Supporting Information pdf file. When generating a consensus spectrum from replicate spectra, fragments with replicates present in fewer than one-third of the number of replicates were excluded. Each library entry is accompanied by ion formula, *m/z*, RT, acquired NCE, number of replicate spectra, and annotated spectral fragments. This library contains 5 328 mass spectra, derived from 586 acylcarnitines having 157 different masses and 125 conjugated acylcarnitines having 85 different masses (Table 4). A list of unique masses of these acylcarnitine ions is provided in Table

Table 4. Unique Masses, Ions, And Spectra of Acylcarnitines and Conjugated Acylcarnitines in the Library

no.	metabolites	masses	ions	spectra
1	carnitine	1	1	9
2	acylcarnitines	157	586	4 332
3	conjugated acylcarnitines	85	125	987
sum		243	713	5 328

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Anal. Chem. 2020, 92, 6521−6528

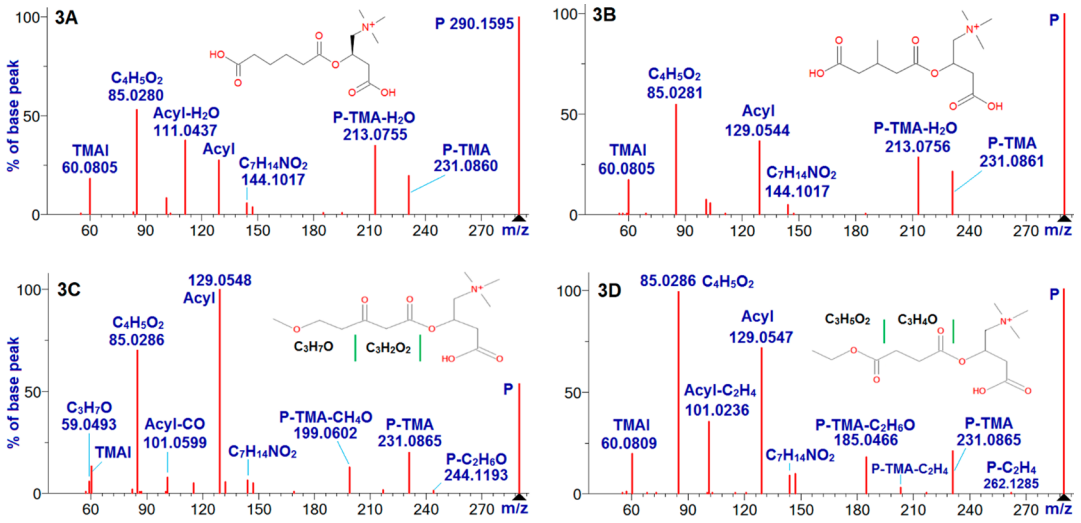

Figure 3. HCD spectra of four acylcarnitine isomers A, B, C, and D (m/z, 290.1598; ion formula, $C_{13}H_{24}NO_6^+$). (A) Spectrum of adipoyl-L-carnitine at 20 V in NIST17 Tandem MS Library. (B) Spectrum of 3-methylglutary-L-carnitine at 20 V in NIST17 Tandem MS Library. (C) Spectrum of isomer C at NCE 25. (D) Spectrum of isomer D at NCE 25. In the spectra, the code P means precursor.

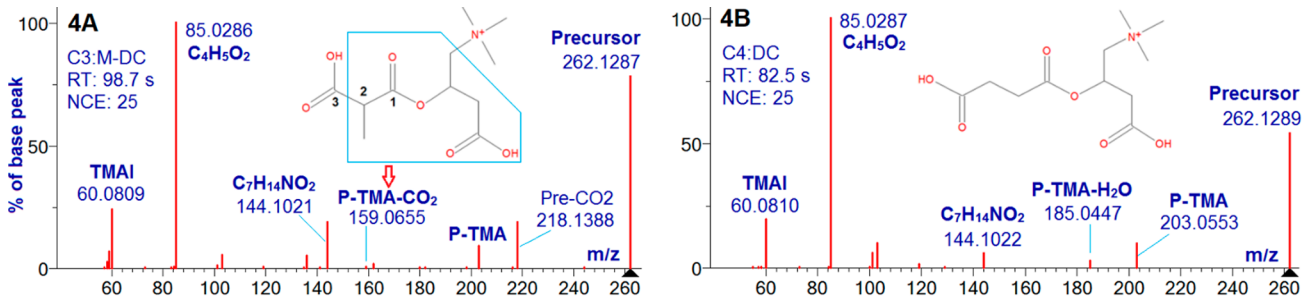

Figure 4. Spectra of isomers (m/z = 262.1285) of 2-methylmalonyl carnitine (C3:M-DC) and succinyl carnitine (C4:DC) under HCD NCE 25. (A) Spectrum of C3:M-DC sampled at RT 98.7 s. The fragments at m/z 218.1388 and 159.0655 indicate that it is 2-methylmalonyl carnitine. (B) Spectrum of C4:DC sampled at RT = 82.5 s.

ES3 in the Supporting Information Excel file; lists of chemical formulas, retention times, and relative abundances of acylcarnitines and conjugated acylcarnitines are presented in Tables ES4 and ES5 in the Supporting Information Excel file, respectively. For more information about the library spectra, see section 5.2 (Description of the acylcarnitine spectra) in the Supporting Information pdf file. Spectral data processed in this study is provided to researchers in two ways: (1) a NIST library, (2) an ASCII text file, both are downloadable from https://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:lib:acylcarnitine.

Examples of Isomer Analysis. While mass spectrometry is the most sensitive method for identifying compounds, it is often unable to establish structures of isomers. However, fragmentation details and the energy dependence of these spectra may provide clues for differentiating isomers having similar spectra. We provide two examples of using fragmentation details for establishing structures. The first involves four isomers, among them two can be identified by matching spectra in the NIST17 Tandem MS Library, while structures of the other two are not known but can be inferred from their fragment ions. The second example demonstrates that methylmalonyl carnitine and its unbranched isomer succinyl carnitine can be distinguished based on their product ions.

Example 1. The first example involves four acylcarnitine isomers (m/z = 290.1598, ion formula is $C_{13}H_{24}NO_6^+$). These isomers eluted around 280, 300, 360, and 392 s and are denoted as isomers A, B, C, and D, respectively. A spectral search against the NIST17 Library indicated that the isomer A is adipoyl-L-carnitine (Figure 3A) and isomer B is 3-methylglutaryl carnitine (Figure 3B).

Spectra of isomers C and D (Figure 3C, D) do not have neutral loss of H_2O or CH_2O_2, indicating that the acyls of the two late eluting isomers are not carboxylated. Isomer C has a fragment at m/z 59.0491 ($C_3H_7O^+$, i.e., acyl − $C_3H_2O_2$), which increased with increased collision energies and became the most abundant one at NCE 80 (data not shown). Isomers A and B exhibit low abundance for peak $C_3H_7O^+$, while isomer D does not have this ion. Furthermore, isomer C also exhibits another unique fragment at m/z 199.0601, which is a result of neutral loss of (TMA + CH_4O) from the precursor. These unique fragments and the delayed RT of isomer C imply that its acyl may have a methoxy and a carbonyl group separated by two CH_2. A putative structure of isomer C is presented in Figure 3C.

Isomer D exhibits four unique feature fragments at m/z 73.0284 ($C_3H_5O_2^+$, i.e., acyl − C_3H_4O), 101.0233 ($C_4H_5O_3^+$, acyl − C_2H_4), m/z 185.0444 ($C_8H_9O_5^+$, precursor − TMA − C_2H_6O), and m/z 203.055 ($C_8H_{11}O_6^+$, precursor − TMA − C_2H_4). The intensity of the fragment $C_3H_5O_2^+$ at m/z 73.0287

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increased with collision energy, and relative abundance reached to 0.36 at NCE 80. These feature fragments suggest that the isomer D could be an ester. A putative structure of isomer D is provided in Figure 3D.

Example 2. The second example concerns the differentiation of 2-methylmalonyl carnitine (C3:M-DC, Figure 4A) from its unbranched isomer succinyl carnitine (C4:DC, Figure 4B). Figure 4A shows that because 2-methylmalonyl carnitine has feature fragments at m/z 159.0652 ($C_7H_{11}O_4^+$) and m/z 218.1388 (neutral loss peak of CO_2), it can be confidently distinguished from isomer succinyl carnitine. The feature fragment at m/z 159.0652 relates to a phenomenon, i.e., spectra of acylcarnitines with a hydroxy in the third position of their acyl groups have a fragment at m/z 145.0495 ($C_6H_9O_4^+$)[10] or m/z 159.0652 if there is a methylation at the second position.[11] Figure 4A indicates that the hydroxy on a 2-COOH group can also facilitate this rearrangement. Actually, the neutral loss peak of CO_2 is a better indicator of 2-methylmalonyl carnitine, because it is more abundant than that of the fragment at m/z 159.0652. This is also observed in malonyl carnitine spectra, see Figure S11 in the Supporting Information pdf file.

Illustration of Library Use. Figure 5 shows results for a LC−MS/MS run of a cancer patient urine sample,[29] generated

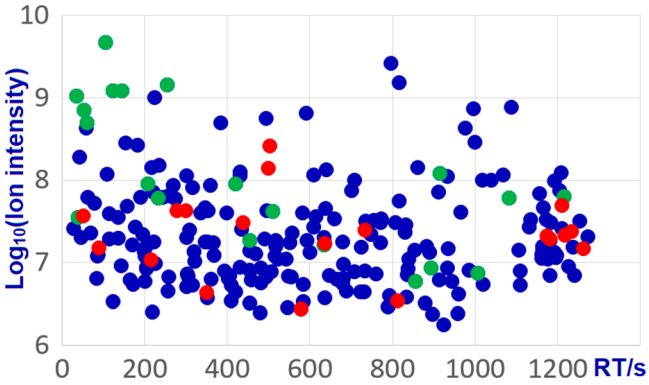

Figure 5. Acylcarnitines identified by matching the library developed in this study to spectra of a single urine analysis,[29] 19 are conjugated acylcarnitines (red) and 223 are acylcarnitines (blue), among the 223 hits, 20 were also identified by searching the NIST 17 library (green).

by an orbitrap Fusion Lumos mass spectrometer. It employs our acylcarnitine spectral library and NIST17 Library using the MSPepSearch software.[31] The acylcarnitine library identified 248 ions with a score 500 or higher and deviation less than 0.001 m/z. After removing redundant identifications, a total of 242 unique acylcarnitines were confirmed, including 223 acylcarnitines and 19 conjugated acylcarnitines. Against the NIST17 Tandem MS Library, 20 acylcarnitines were matched. A list of the 242 identifications and their ion m/z, formulas, retention times, match scores, and abundances can be found in Table ES6 in the Supporting Information Excel file. For researchers to have a better understanding of these identifications, a supplementary text file is provided in the Supporting Information. It contains 242 raw spectra, from which the 242 identifications were made.

Criteria described in Detection of Acylcarnitines are also appropriate to confirm identifications of acylcarnitines from library search results. Identifications with high scores suggest an exact match of the library compound, while low scores suggest that the identified ions may be isomers of the library compounds or the candidate spectra are of low quality due to low ion abundances or contaminants. By aligning retention times of metabolites with our data, identification confidence can be increased further. The compounds in Tables ES2, ES4, and ES5 in the Supporting Information Excel file contain such retention times.

Summary. With the pure compound spectra in the NIST17 Tandem MS Library and spectra found in the analysis of urine by the hybrid search method, we developed rules for identifying and annotating acylcarnitines as well as for excluding artifacts. Using these rules, 586 unique acylcarnitine ions were identified. In the course of this analysis, 125 novel acylcarnitine species were also identified. They appeared to primarily result from the conjugation of acylcarnitines with hydrophilic compounds, such as cysteine, taurine, glucuronic acid, and SO_3. Significantly, these conjugated acylcarnitines can have comparable abundances to their nonconjugated acylcarnitines. Based on these studies, a spectral library of acylcarnitines and conjugated acylcarnitines was created and has been made available from https://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:lib:acylcarnitine. This resource is a searchable spectral library containing thousands of mass spectra, each of which is associated with a molecular formula, precursor ion m/z, retention time, collision energy, and annotated fragment ions. This library is intended as an aid in identifying acylcarnitines in biological samples as well as for studying structure-fragmentation-retention relationships. Moreover, the methods reported appear capable of being extended to identify other classes of metabolites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c00129.

Materials and experiments, complementary description of the rules to detect acylcarnitines, annotation steps of MS2 fragments, major neutral losses, descriptions of spectra in the acylcarnitine library, brief discussions on collision energy and retention time, and artifact analyses (PDF)

List of 27 acylcarnitines in the NIST17 Tandem MS Library, 92 chemicals used to analyze retention time consistency of different runs, 157 acylcarnitine masses, carnitine, and 586 acylcarnitine ions, 125 conjugated acylcarnitines, and 242 identifications reported in the application section (XLSX)

242 raw spectra from which 242 identifications were made [Examples of Isomer Analysis (Illustration of library use)] (TXT)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Fritz, I. B.; Yue, K. T. N. *J. Lipid Res.* **1963**, *4*, 279−288.
(2) Bremer, J. *Physiol. Rev.* **1983**, *63*, 1420−1480.
(3) Rebouche, C. J.; Paulson, D. J. *Annu. Rev. Nutr.* **1986**, *6*, 41−66.
(4) Bieber, L. L. *Annu. Rev. Biochem.* **1988**, *57*, 261−283.
(5) Kelly, B. M.; Rose, M. E.; Wycherley, D.; Preece, S. W. *Org. Mass Spectrom.* **1992**, *27*, 924−926.
(6) Indiveri, C.; Iacobazzi, V.; Tonazzi, A.; Giangregorio, N.; Infantino, V.; Convertini, P.; Console, L.; Palmieri, F. *Mol. Aspects Med.* **2011**, *32*, 223−233.
(7) Millington, D. S.; Roe, C. R.; Maltby, D. A. *Biomed. Mass Spectrom.* **1984**, *11*, 236−241.
(8) Gaskell, S. J.; Guenat, C.; Millington, D. S.; Maltby, D. A.; Roe, C. R. *Anal. Chem.* **1986**, *58*, 2801−2805.
(9) Maeda, Y.; Ito, T.; Ohmi, H.; Yokoi, K.; Nakajima, Y.; Ueta, A.; Kurono, Y.; Togari, H.; Sugiyama, N. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2008**, *870*, 154−159.
(10) Su, X.; Han, X.; Mancuso, D. J.; Abendschein, D. R.; Gross, R. W. *Biochemistry* **2005**, *44*, 5234−5245.
(11) Peng, M.; Fang, X.; Huang, Y.; Cai, Y.; Liang, C.; Lin, R.; Liu, L. *J. Chromatogr. A* **2013**, *1319*, 97−106.
(12) Möder, M.; Kiessling, A.; Löster, H.; Brüggemann, L. *Anal. Bioanal. Chem.* **2003**, *375*, 200−210.
(13) Millington, D. S.; Kodo, N.; Norwood, N. L.; Roe, C. R. *J. Inherited Metab. Dis.* **1990**, *13*, 321−324.
(14) Rashed, M. S.; Ozand, P. T.; Bucknall, M. P.; Little, D. *Pediatr. Res.* **1995**, *38*, 324−331.
(15) Rizzo, C.; Boenzi, S.; Wanders, R. J.; Duran, M.; Caruso, U.; Dionisi-Vici, C. *Pediatr. Res.* **2003**, *53*, 1013−1018.
(16) Abdenur, J. E.; Chamoles, N. A.; Guinle, A. E.; Schenone, A. B.; Fuertes, A. N. J. *J. Inherited Metab. Dis.* **1998**, *21*, 624−630.
(17) Minkler, P. E.; Stoll, M. S. K.; Ingalls, S. T.; Hoppel, C. L. *Mol. Genet. Metab.* **2017**, *120*, 363−369.
(18) Soeters, M. R.; Serlie, M. J.; Sauerwein, H. P.; Duran, M.; Ruiter, J. P.; Kulik, W.; Ackermans, M. T.; Minkler, P. E.; Hoppel, C. L.; Wanders, R. J.; Houten, S. M. *Metab., Clin. Exp.* **2012**, *61*, 966−973.
(19) Minkler, P. E.; Stoll, M. S.; Ingalls, S. T.; Yang, S.; Kerner, J.; Hoppel, C. L. *Clin. Chem.* **2008**, *54*, 1451−1462.
(20) Maeda, Y.; Ito, T.; Suzuki, A.; Kurono, Y.; Ueta, A.; Yokoi, K.; Sumi, S.; Togari, H.; Sugiyama, N. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 799−806.
(21) Minkler, P. E.; Stoll, M. S.; Ingalls, S. T.; Kerner, J.; Hoppel, C. L. *Mol. Genet. Metab.* **2015**, *116*, 231−241.
(22) van der Hooft, J. J.; Ridder, L.; Barrett, M. P.; Burgess, K. E. *Front. Bioeng. Biotechnol.* **2015**, *3*, 26.
(23) Zuniga, A.; Li, L. *Anal. Chim. Acta* **2011**, *689*, 77−84.
(24) Yu, D.; Zhou, L.; Xuan, Q.; Wang, L.; Zhao, X.; Lu, X.; Xu, G. *Anal. Chem.* **2018**, *90*, 5712−5718.
(25) https://chemdata.nist.gov/.
(26) Burke, M. C.; Mirokhin, Y. A.; Tchekhovskoi, D. V.; Markey, S. P.; Heidbrink Thompson, J.; Larkin, C.; Stein, S. E. *J. Proteome Res.* **2017**, *16*, 1924−1935.
(27) Moorthy, A. S.; Wallace, W. E.; Kearsley, A. J.; Tchekhovskoi, D. V.; Stein, S. E. *Anal. Chem.* **2017**, *89*, 13261−13268.
(28) Cooper, B. T.; Yan, X.; Simón-Manso, Y.; Tchekhovskoi, D. V.; Mirokhin, Y. A.; Stein, S. E. *Anal. Chem.* **2019**, *91*, 13924−13932.
(29) Simón-Manso, Y.; Marupaka, R.; Yan, X.; Liang, Y.; Telu, K. H.; Mirokhin, Y. A.; Stein, S. E. *Anal. Chem.* **2019**, *91*, 12021−12029.
(30) Telu, K. H.; Yan, X.; Wallace, W. E.; Stein, S. E.; Simón-Manso, Y. *Rapid Commun. Mass Spectrom.* **2016**, *30* (5), 581−593.
(31) https://chemdata.nist.gov/dokuwiki/doku.php?id=peptidew:mspepsearch.
(32) Dong, Q.; Liang, Y.; Yan, X.; Markey, S. P.; Mirokhin, Y. A.; Tchekhovskoi, D. V.; Bukhari, T. H.; Stein, S. E. *mAbs.* **2018**, *10*, 354−369.
(33) Dong, Q.; Yan, X.; Liang, Y.; Stein, S. E. *J. Proteome Res.* **2016**, *15*, 1472−1486.
(34) Testa, B.; Kramer, S. D. *Chem. Biodiversity* **2008**, *5*, 2171−2336.
(35) Parkinson, A.; Ogilvie, B. W. Biotransformation of xenobiotics. In *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 7th ed.; Klaassen, C. D., Eds.; McGraw-Hill: New York, 2008; Chapter 6, p 161.
(36) Kind, T.; Fiehn, O. *Bioanal. Rev.* **2010**, *2*, 23−60.
(37) Levsen, K.; Schiebel, H.-M.; Behnke, B.; Dotzer, R.; Dreher, W.; Elend, M.; Thiele, H. *J. Chromatogr., A* **2005**, *1067*, 55−72.
(38) Xu, C.; Li, C. Y.; Kong, A. N. *Arch. Pharmacal Res.* **2005**, *28*, 249−268.
(39) Ionescu, C.; Caira, M. R. *Drug Metabolism*; Springer Netherlands, 2005.
(40) Demarque, D. P.; Crotti, A. E. M.; Vessecchi, R.; Lopes, J. L. C.; Lopes, N. P. *Nat. Prod. Rep.* **2016**, *33*, 432−455.

Supporting Information

Mass Spectral Library of Acylcarnitines Derived from Human Urine

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Contents

Abbreviation: AB, abundance; ACYS, acetylcysteine; CYS, cysteine, ESI, electrospray ionization; HCD, higher-energy C-trap dissociation; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MS1, full MS scan; MS2, tandem MS scan; NCE, normalized collision energy; NIST, National Institute of Standards and Technology; P, precursor; RA, relative abundance; RT, retention time; s, second; TIC, total ion chromatogram; TMA, trimethylamine (C_3H_9N); TMAI, positive ion of trimethylamine ($C_3H_{10}N^+$).

1. Materials and Experiment

1.1 Materials

HPLC grade methanol, Honeywell, Burdick & Jackson; HPLC grade acetonitrile, EMD Millipore Corporation, Darmstadt, Germany; HPLC grade water, Honeywell, Burdick & Jackson; LCMS grade formic acid, Fluka; creatinine-(N-methyl-d_3), purity 98%, Sigma Aldrich.

Table S1. The used urine samples in this study are six NIST standard reference materials (SRM); each one was collected from multiple individuals.

No	SRM	Sample description
1	3667	Normal human urine collected from male and female donors. It is a kind of average urine and can be used as the reference (control) for the analysis.
2	3671-1	Normal human urine collected from nonsmokers without environmental exposure to tobacco smoke.
3	3671-2	Normal human urine collected from nonsmokers with exposure to "secondhand" smoke.
4	3671-3	Normal human urine collected from smokers who smoke at least one pack of cigarettes per day.
5	3672	Normal human urine collected from smokers who smoked more than one pack of cigarettes per day (like SRM 3671-3, may be with more polycyclic aromatic hydrocarbons and nicotine metabolites).
6	3673	Normal human urine collected from nonsmokers who were not exposed to "secondhand" cigarette smoke (like SRM 3671-1).

1.2 Sample preparation

Urine samples (SRMs) were withdrawn from -80 °C and kept for three hours at room temperature to thaw. Each urine sample (300 µL) was collected in an Eppendorf and 300 µL of methanol was added to the sample. The samples were centrifuged (Beckman Coulter Centrifuge Microfuge 22R) for 20 min at 10000g and room temperature. The supernatant was dried completely (to solid) for 90 minutes under a gentle nitrogen stream at room temperature. Next, the dried material was reconstituted with 200 µL water solution of internal standard creatinine-d3 (320 ng / µL). The final 200 µL solution was processed from 300 µL urine sample. Therefore, 1 µL final solution contains 1.5 µL original urine sample. 3 µL solution was used for each run, that should contain 4.5 µL original urine sample and 960 ng internal standard. The samples were maintained at 4 °C in the auto-sampler and analyzed by LC-MS.

1.3 LC-MS analysis

Reverse phase separations were accomplished with the use of a Dionex Ultimate 3000 system (Dionex, USA) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). A Waters ACQUITY UPLC CSH-C18 Column, 130 Å (2.1x100 mm, 1.7 µm) was used at 500 to 600 bars. A gradient [(time (min), %B)]: (0, 1) (1, 1) (18, 20) (24, 90) (27, 90) (28, 1) (30, 1) was completed at 30 min. The injection volume was set to 3 µL and flow rate was 0.4 mL / min.

LC-ESI mass spectra were recorded by orbitrap Fusion Lumos (Thermo Scientific, USA). The electrospray interface was set in positive-ionization mode with ion transfer temperature 300 ^{o}C, a vaporizer temperature of 300 ^{o}C, positive spray voltage 3500 V, S-Len RF level 50, sheath gas (Arb) 45, AUX gas (Arb) 13, sweep gas (Arb) 1 to obtain maximum abundance of the standard sample in full-scan spectra (80–1200 Da). The resolutions for full MS scan was set at 120 000, and for MS/MS scan was 30 000.

2. Complementary description of the rules to detect acylcarnitines

For detecting normal acylcarnitines (defined by elements in their formulas: N = 1, S = 0, O < 9, Cl = 0), a set of rules was made to filter irrelevant or low-quality spectra as well as contaminants in AC candidate spectra. These rules include:

2.1 Exclude spectra (A)

- Charge > 1.
- Mass < 162. Hence, (R)-Aminocarnitine (*m/z*: 161) was excluded in this study.
- Mass > 700 (in the ion mode $M+H^+$, there is no AC with mass > 600, except for conjugated AC).
- Number of MS2 fragments > 400.

2.2 Exclude spectra (B)

- If the RA (relative abundance) of CNT85 < 0.05, except for spectra from low energy or conjugated AC.
- If the sum of RA values of CNT60, CNT144, precursor – TMA, and precursor – TMA – H_2O are less than 0.02, except for conjugated AC.

Note: For this part only, each RA is calculated by comparing to the largest peak that does not include precursor (P), P – TMA, and those with *m/z* values larger than precursor – 14.

Note: Under low collision energy, fragment P – TMA can be more abundant than fragment CNT85.

2.3 Remove contaminants from MS2 spectra

- Common PEG peaks: 45.0335 + n x 44.0262 (n = 1 to 5).
- Known abnormal MS2 peaks in the region 148.25 – 148.75 *m/z*.
- Abnormal peaks, such as *m/z* = 95.6, 97.8, which are impossible for charge 1 acylcarnitine fragments.

- Contaminants that cannot be explained based on precursor values. For example, the precursor *m/z* value is 312.25, it is impossible to have a fragment at *m/z* 290.05 or 290.45.
- The peaks within precursor – 12.

2.4 Match validated AC *m/z* data

We have compiled a list of 157 unique normal AC *m/z* values and their formulas. Most of the 157 acylcarnitines were manually confirmed by this study, while a small portion of them were from spectra in NIST17 Tandem MS Library as well as published papers and databases. Each candidate ion *m/z* was searched against the 157-AC list. If the candidate *m/z* was in the list, it had a greater possibility to be a true AC. If not, this candidate spectrum was manually examined to confirm its identity.

3. Annotation of MS2 fragments

3.1 Annotate fragments that involve the carnitine moiety. See the following table.

Table S2. The *m/z* (z = 1) values, formulas and codes of the carnitine moiety related fragments.

No	*m/z*	Ion formula	Code*	Notes
1	56.0495	$C_3H_6N^+$	CNT56	
2	57.0335	$C_3H_5O^+$	CNT57	Existence under higher collision energy
3	58.0651	$C_3H_8N^+$	CNT58	Existence under higher collision energy
4	59.0729	$C_3H_9N^+$	CNT59	
5	60.0808	$C_3H_{10}N^+$	CNT60 / TMAI	Feature fragment of acylcarnitine
6	61.0284	$C_2H_5O_2^+$	CNT61	
7	68.0495	$C_4H_6N^+$	CNT68	
8	70.0651	$C_4H_8N^+$	CNT70	**
9	84.0808	$C_5H_{10}N^+$	CNT84	Existence under higher collision energy
10	85.0284	$C_4H_5O_2^+$	CNT85	Feature fragment of acylcarnitine
11	86.0964	$C_5H_{12}N^+$	CNT86	**
12	88.0757	$C_4H_{10}NO^+$	CNT88	**
13	98.0600	$C_5H_8NO^+$	CNT98	**
14	100.0757	$C_5H_{10}NO^+$	$C_5H_{10}NO$	**, ***
15	102.0913	$C_5H_{12}NO^+$	CNT102	
16	103.0390	$C_4H_7O_3^+$	CNT103	
17	112.0757	$C_6H_{10}NO^+$	CNT112	**
18	129.0784	$C_6H_{11}NO_2^+$	CNT129	
19	130.0863	$C_6H_{12}NO_2^+$	CNT130	**
20	144.1019	$C_7H_{14}NO_2^+$	CNT144	Feature fragment of acylcarnitine

****** Not found from acylcarnitine HCD spectra in NIST17 Tandem MS Library. They could be the results of rearrangement or interactions between the two substructures, carnitine and acyl.

******* In N-containing conjugated acylcarnitines, this fragment may not be fully from carnitine moiety, because in N-cotaining AC spectra this fragment can be the most abundant one, while in non-conjugated AC spectra, its RA generally is less than 0.03.

3.2 Annotate precursor and major fragments, including acyl (= precursor – 161.1052), precursor – TMA, and precursor – $C_7H_{13}NO_2$ (which is carnitine – H_2O).

3.3 Annotate fragments formed by neutral loss from acyl, acyl – H_2O, and acyl – $2H_2O$, with constraints in the numbers of O and N (= 0. This means that a normal AC's acyl moiety cannot have nitrogen atom.).

3.4 Annotate fragments formed by general neutral loss from precursor, with constraints in the numbers of O and N (<=1) atoms.

3.5 Annotate fragments formed by neutral loss from precursor – TMA, precursor – TMA – H_2O, precursor - TMA – $2H_2O$, and precursor – TMA – $3H_2O$, with constraints in the numbers of O and N (= 0) atoms.

3.6 Annotate fragments formed by neutral loss from precursor – CH_2O_2 when fragment *m/z* is larger than acyl *m/z*, with constraints in the numbers of O and N (<= 1) atoms.

3.7 Calculate and assign formulas for un-annotated fragments with RA > 0.02. Because these fragments cannot be explained by a known neutral loss, they may result from unknown neutral losses, or contaminants. Hence, caution should be taken when using these peaks to confirm identity.

4. Neutral losses

4.1 Some carnitine moiety related neutral losses

The other three important carnitine moiety-related fragments are $C_3H_5O^+$ (*m/z* = 57.0335), $C_3H_8N^+$ (*m/z* = 58.0651), and $C_5H_{10}N^+$ (*m/z* = 84.0808). These three fragments are absent in low energy spectra. Their abundances increase with inceasing collision energies, indicating a stability at higher collision energies. Among the three fragments, $C_3H_8N^+$ is most abundant; in fact, it is a feature fragment in FAB spectra, with the structure $(CH_3)_2N^+{=}CH_2$.

In addition to these major acylcarnitine fragments discussed above, there are several minor ions, including $C_3H_9N^+$ (*m/z* = 59.0729), $C_5H_{10}N^+$ (*m/z* = 84.0808), $C_4H_7O_3^+$ (*m/z* = 103.0390), and $C_6H_{11}NO_2^+$ (*m/z* = 129.0784). They are generally in low abundance when collision energy is low, and some can reach to higher abudances at high collision energies.

4.2 Top neutral losses

The top 10 neutral losses from acyl moiety of AC spectra are presented by Table S3. This table indicates that the most significant neutral loss from acyl is H_2O. The numbers of loss of H_2O in the table can be much higher if we include these that are possibly in a combination of H_2O and H_2O, or H_2O and other group(s), such as CH_2O_2 (CO + H_2O), CH_4O_3 (CH_2O_2 + H_2O, or CO + 2H_2O). The major origins of the losses of H_2O should be the groups OH and COOH. However, loss of H_2O could also occur from a rearranged carbonyl CO. For example, the fragment acyl – H_2O can be detected, even the acyl moiety has only one carbonyl O.

The second and third top neutral losses from the acyl moieties are CH_2O_2 and CO (Table S3). CH_2O_2 is an indicator of carboxylic acid group, but its specificity is not high. Table S3 shows that at lower collision energies, CO is greater than that of CH_2O_2, while at higher energies, CH_2O_2 is greater, suggesting that some CH_2O_2 could be a combination of two losses: CO and H_2O. In acyl structure, one O atom always locates at the carbonyl group that connects to carnitine. Therefore, three O atoms are required for an acyl to have a carboxylic acid group. For an acyl moiety of two-O atoms, the loss of CH_2O_2 is possibly from a combination of two losses. Figure S1 shows the relationship between collision energy and neutral loss of CH_2O_2 of an acylcarnitine, whose acyl moiety has two O atoms.

Table S3. The top 10 neutral losses from acyl moiety and detected numbers at different collision energies. Note that some losses here could be a combination of two or more groups. For example, CH_4O_3 is most possibly due to a combination of CO and 2H_2O or CH_2O and H_2O.

No	NCE 15		NCE 20		NCE 30		NCE 40		NCE 50		NCE 60		NCE 80	
1	H_2O	214	H_2O	296	H_2O	350	H_2O	328	H_2O	264	$C_2H_4O_2$	206	$C_2H_4O_2$	144
2	CO	135	CO	214	CH_2O_2	270	CH_2O_2	249	CH_2O_2	223	CH_2O_2	192	$C_4H_8O_2$	136
3	CH_2O_2	114	CH_2O_2	207	CO	262	$C_2H_4O_2$	244	$C_2H_4O_2$	219	H_2O	172	$C_5H_{10}O_2$	127
4	C_2H_2O	100	2H_2O	168	C_2H_2O	242	CO	237	CO	198	C_2H_2O	164	$C_3H_8O_2$	122
5	$C_2H_4O_2$	99	C_2H_2O	163	$C_2H_4O_2$	233	C_2H_2O	231	C_2H_2O	187	CO	161	CH_2O_2	121
6	2H_2O	89	$C_2H_4O_2$	163	2H_2O	219	2H_2O	210	2H_2O	181	$C_2H_6O_2$	158	$C_2H_6O_2$	117
7	CH_4O_3	37	CH_4O_3	79	$C_2H_6O_2$	162	$C_2H_6O_2$	162	$C_2H_6O_2$	149	$C_4H_8O_2$	151	$C_4H_{10}O_2$	113
8	$C_5H_{10}O_2$	27	$C_2H_6O_2$	69	$C_4H_8O_2$	133	$C_4H_8O_2$	146	$C_4H_8O_2$	141	2H_2O	149	$C_3H_6O_2$	108

9	C_2H_4O	24	$C_4H_8O_2$	65	$C_5H_{10}O_2$	123	$C_3H_6O_2$	136	$C_5H_{10}O_2$	132	$C_3H_6O_2$	133	$C_6H_{12}O_2$	97
10	$C_2H_6O_3$	21	$C_5H_{10}O_2$	60	CH_4O_3	114	$C_5H_{10}O_2$	131	$C_3H_6O_2$	127	$C_5H_{10}O_2$	133	$C_2H_8O_2$	94

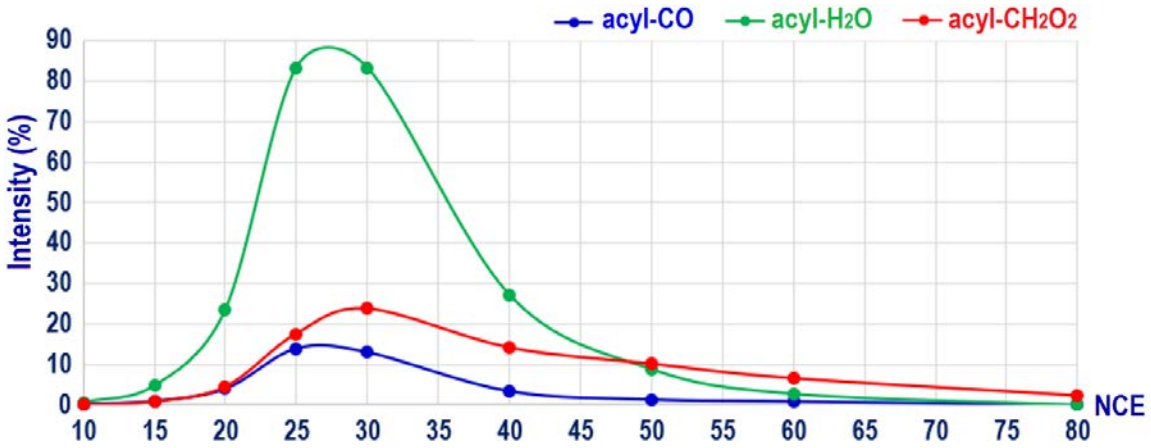


Figure S1. Abundances and collision energies (NCE) of fragments acyl-CO, acyl-H_2O and acyl-CH_2O_2 of an acylcarnitine (ion formula: $C_{17}H_{28}NO_5^+$, *m/z*: 326.1962, RT: 723 s).

The next two top neutral losses from acyl are C_2H_2O and $C_2H_4O_2$. Fragment acyl – C_2H_2O could be generated when the second bond (counted from the carbonyl group that connects to carnitine, see Figure S2) is cleaved. Figure S2 shows that the cleavage on the bond *b* produced an abundant fragment $C_3H_7O^+$ (*m/z* = 59.0488), i.e. acyl – C_2H_2O. Because all ACs have the same connection between carnitine and acyls, the fragment acyl – C_2H_2O is common in AC spectra. Generally, abundances of this fragment increase with increased collision energies, Figure S3 shows abundances of fragments $C_4H_5O_2^+$, acyl – C_2H_2O, and $C_3H_8N^+$ under different collision energies. For (2R)-3-hydroxyisovaleroylcarnitine, it is interesting to note that after 61 V (see Figure S3) the fragment $C_4H_5O_2^+$ is no longer the most abundant one, the fragment acyl – C_2H_2O becomes the most abundant one.

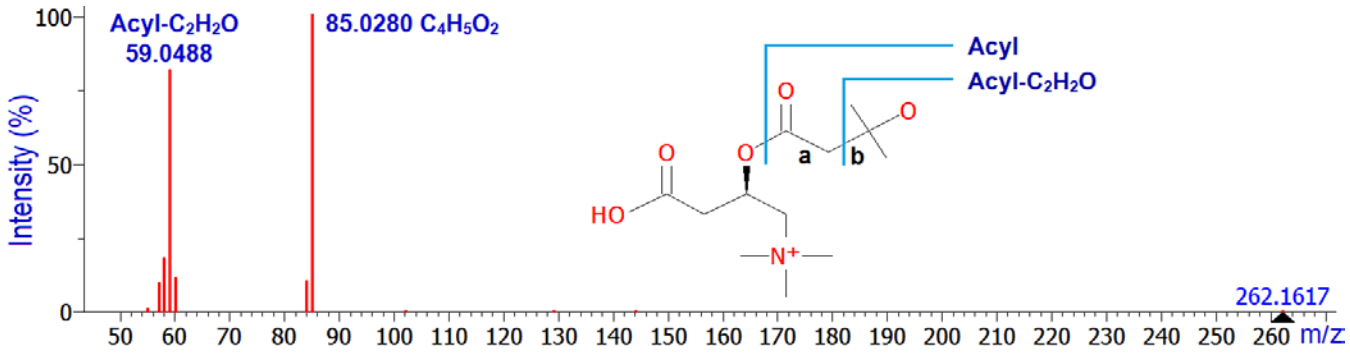


Figure S2. NIST library spectrum of (2R)-3-hydroxyisovaleroylcarnitine (ion formula: $C_{12}H_{24}NO_5^+$, *m/z*: 262.1649, collision energy: 57 V).

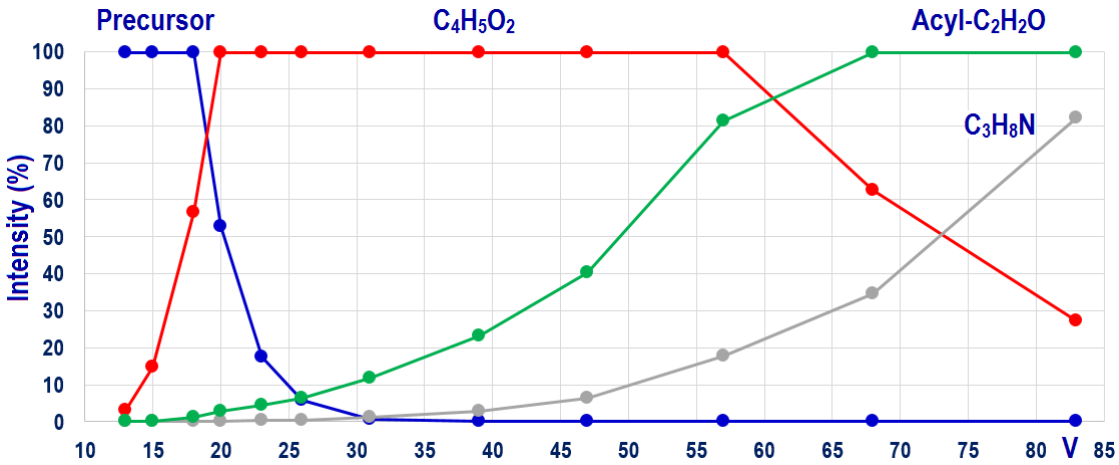


Figure S3. Abundances and collision energies (V) of MS2 fragments $C_4H_5O_2^+$, $C_3H_8N^+$, acyl – C_2H_2O, and precursor in spectra of (2R)-3-hydroxyisovaleroylcarnitine (ion formula: $C_{12}H_{24}NO_5^+$, *m/z*: 262.1649, from NIST17 Tandem MS Library).

The presence of a COOH group in an acyl indicates there are at least three O atoms in the acyl moiety. But there are a number of ACs that have the fragment acyl - $C_2H_4O_2$, and they only have two O atoms in their acyl moieties, suggesting that the loss of $C_2H_4O_2$, could be from a combination of two groups, such as $C_2H_2O + H_2O$. It is worth noting that for acylcarnitines with 2 or more O atoms in their acyl moieties, the neutral loss peak of TMA + H_2O or + $2H_2O$ can be more abundant than the fragment from loss of TMA. For acylcarnitines that have large masses (> 400 Da), the abundances of their neutral loss peak of TMA or TMA + H_2O can be very low and even may not be detectable.

5. Descriptions of spectra of the acylcarnitine library built in this study

5.1 Grouping the same ion spectra for processing consensus ones

It is critical to select relevant and quality spectra to process each consensus spectrum. Even if the spectra collected from the ion with the same *m/z* and collision energy, and similar RTs, some of these spectra may be from different ions. Hence, an analysis is necessary to filter irrelevant (or low-quality spectra). In this work, an algorithm was used to for this purpose. This algorithm first calculates the differences of the most abundant 10 fragment pairs in two spectra (only including fragments with relative abundances larger than 0.05). Then, sums up these differences to obtain the difference value of the two spectra. This algorithm is presented below:

$$SD = \sum(W[i] \,.\, FD[i]) \qquad (i = 1 \text{ to } 10) \qquad (1)$$

$$W[i] = \sqrt{RA[L, i]} \qquad (2)$$

$$FD[i] = ((RA[L, i] + B) / (RA[S, i] + B)) - C \qquad (3)$$

Here, the SD denotes the difference value of two spectra. W[i] is the weight of the fragment pair i. The FD[i] is the difference of the same *m/z* fragment pair i in two spectra, RA is relative abundance of the fragment. L is the fragment that has the larger RA value among two fragments of each pair, the S is the smaller fragment. The B is used to avoid a very small or 0 RA value that can result in an infinity large or unreasonable ratio value. In this study, 0.02 was used for the B value. The C is a correction for the abundant difference that can be caused by the instability of experiment. Because low abundant fragments can have larger variability, different C values were used and presented in Table S4. When FD[i] was less than 0 in the equation 3, 0 was used for the FD[i].

Table S4. The use C data in the spectral difference analysis

RA[large, i]	C
> 0.5	1.20
> 0.1 <= 0.5	1.40
> 0.05 <= 0.1	1.60

Generally, spectra with an RT difference less than 7 seconds and SD less than 4 were assumed to arise from the same isomer. However, if the RT difference between two spectra was much less than 7 seconds, a larger SD, up to 12 was accepted and the two spectra were treated as from the same isomer. For cases that the RT difference between two isomers is less than 7 seconds, manual analyses were made to examine if there are two ion clusters in MS1 data. Only those that were confirmed by MS1 ion clusters were employed to generate consensus spectra.

5.2 Descriptions of the acylcarnitine spectra

The codes used to describe each spectrum in the acylcarnitine library are presented in Table S5, and to annotate spectral fragments in Table S6. A simple classification of the acylcarnitines in the library is presented in Table S7.

Table S5. Codes used in describing acylcarnitines.

Codes	Description
AC or X-conjugated-AC	X indicates a conjugator, such as cysteine, glucuronide and N-containing.
(Ion formula, without code)	Positive and singly charged ion.
ionMZ	Theoretical *m/z* of singly charged ion. The deviation of observed from theoretical data is generally less than three ppm.
RT	Retention time in second.
HCDnce	Fragmented by HCD mode under normalized collision energy.

Parent	Equal to ionMZ
isomerNO	The number of unique isomers that have the same *m/z*.
Nreps	The number of replicate spectra used to process the corresponding consensus spectrum.
RT_Standard_Dev	Retention time standard deviation (in second) of spectra used for processing each consensus spectrum.

Table S6. Codes used in annotating acylcarnitine spectral fragments. CNT denotes carnitine or carnitine fragment.

Codes	Description
Acetyl	Acetyl group C_2H_2O.
Acyl	Acyl moiety of acylcarnitine.
ACYS	Acetylcysteine.
ACYS129	Mass of acetylcysteine – H_2S.
C_3H_9N / TMA	Neutral loss group of carnitine moiety.
CNT	Carnitine
CNT56	Carnitine moiety fragment $C_3H_6N^+$
CNT57	Carnitine moiety fragment $C_3H_5O^+$
CNT58	Carnitine moiety fragment $C_3H_8N^+$
CNT59	Carnitine moiety fragment $C_3H_9N^+$
CNT60	Carnitine moiety fragment $C_3H_{10}N^+$
CNT61	Carnitine moiety fragment $C_2H_5O_2^+$
CNT68	Carnitine moiety fragment $C_4H_6N^+$
CNT70	Carnitine moiety fragment $C_4H_8N^+$
CNT84	Carnitine moiety fragment $C_5H_{10}N^+$
CNT85	Carnitine moiety fragment $C_4H_5O_2^+$
CNT86	Carnitine moiety fragment $C_5H_{12}N^+$
CNT88	Carnitine moiety fragment $C_4H_{10}NO^+$
CNT98	Carnitine moiety fragment $C_5H_8NO^+$
CNT100	Carnitine moiety fragment $C_5H_{10}NO^+$
CNT102	Carnitine moiety fragment $C_5H_{12}NO^+$
CNT103	Carnitine moiety fragment $C_4H_7O_3^+$
CNT112	Carnitine moiety fragment $C_6H_{10}NO^+$
CNT129	Carnitine moiety fragment $C_6H_{11}NO_2^+$
CNT130	Carnitine moiety fragment $C_6H_{12}NO_2^+$
CNT143	Mass of carnitine – H_2O
CNT144	Carnitine moiety fragment $C_7H_{14}NO_2^+$
CYS	Cysteine
CYS87	Mass of cysteine – H_2S.
CYS88	Fragment (cysteine – H_2S).

HexA	GlcA or GalA or ManA
P	Precursor
ThioCYS	Thiocysteine

Table S7. Chemical formulas and numbers of isomers of acylcarnitines in the library. The column *Short Name* provides an abbreviation of the acyl moiety. The letter n is the carbon number in acyl moiety; OH represents hydroxyl group; the number directly after Cn: is the number of double bonds; the O atoms denoted with 2, 3 and 4 may involve multiple groups, such as COOH, -O-, C(=O), and -OH. The column *Number* shows the number of detected acylcarnitines, including these with different C numbers and isomers of the same formula.

No.	Chemical Formula	Short Name	Number	Note
1	$C_nH_{2n}NO_4^+$	Cn:0	23	
2	$C_nH_{2(n-1)}NO_4^+$	Cn:1	43	
3	$C_nH_{2(n-2)}NO_4^+$	Cn:2	24	
4	$C_nH_{2(n-3)}NO_4^+$	Cn:3	6	
5	$C_nH_{2(n-4)}NO_4^+$	Cn:4	17	
6	$C_nH_{2(n-5)}NO_4^+$	Cn:5	4	
7	$C_nH_{2n}NO_5^+$	Cn:0-OH	46	There is a possibility that the O is in the -O- format.
8	$C_nH_{2n}NO_6^+$	Cn:0-2OH	8	
9	$C_nH_{2(n-1)}NO_5^+$	Cn:1-OH	96	There is a possibility that the O is part of other groups, such as -C(=O), and -O-.
10	$C_nH_{2(n-2)}NO_5^+$	Cn:2-OH	51	
11	$C_nH_{2(n-3)}NO_5^+$	Cn:3-OH	30	
12	$C_nH_{2(n-4)}NO_5^+$	Cn:4-OH	16	
13	$C_nH_{2(n-5)}NO_5^+$	Cn:5-OH	3	
14	$C_nH_{2(n-1)}NO_6^+$	Cn:0-2O	57	In addition to the -COOH group, the two O atoms may be in ester, ether and carbonyl groups, or a double bond with a hydroxy group.
15	$C_nH_{2(n-2)}NO_6^+$	Cn:1-2O	60	
16	$C_nH_{2(n-3)}NO_6^+$	Cn:2-2O	25	
17	$C_nH_{2(n-4)}NO_6^+$	Cn:3-2O	21	
18	$C_nH_{2(n-5)}NO_6^+$	Cn:4-2O	7	
19	$C_nH_{2(n-6)}NO_6^+$	Cn:5-2O	5	
20	$C_nH_{2(n-1)}NO_7^+$	Cn:1-3O	15	These O atoms could be in the groups -COOH, -COO-, C(=O), -O-, and OH.
21	$C_nH_{2(n-2)}NO_7^+$	Cn:2-3O	17	
22	$C_nH_{2(n-3)}NO_7^+$	Cn:3-3O	2	
23	$C_nH_{2(n-4)}NO_7^+$	Cn:4-3O	6	
24	$C_nH_{2(n-5)}NO_7^+$	Cn:5-3O	1	
25	$C_nH_{2(n-2)}NO_8^+$	Cn:0-4O	3	
Sum			586	

An example of a library spectrum displayed by MS-Search program is provided by Figure S4.

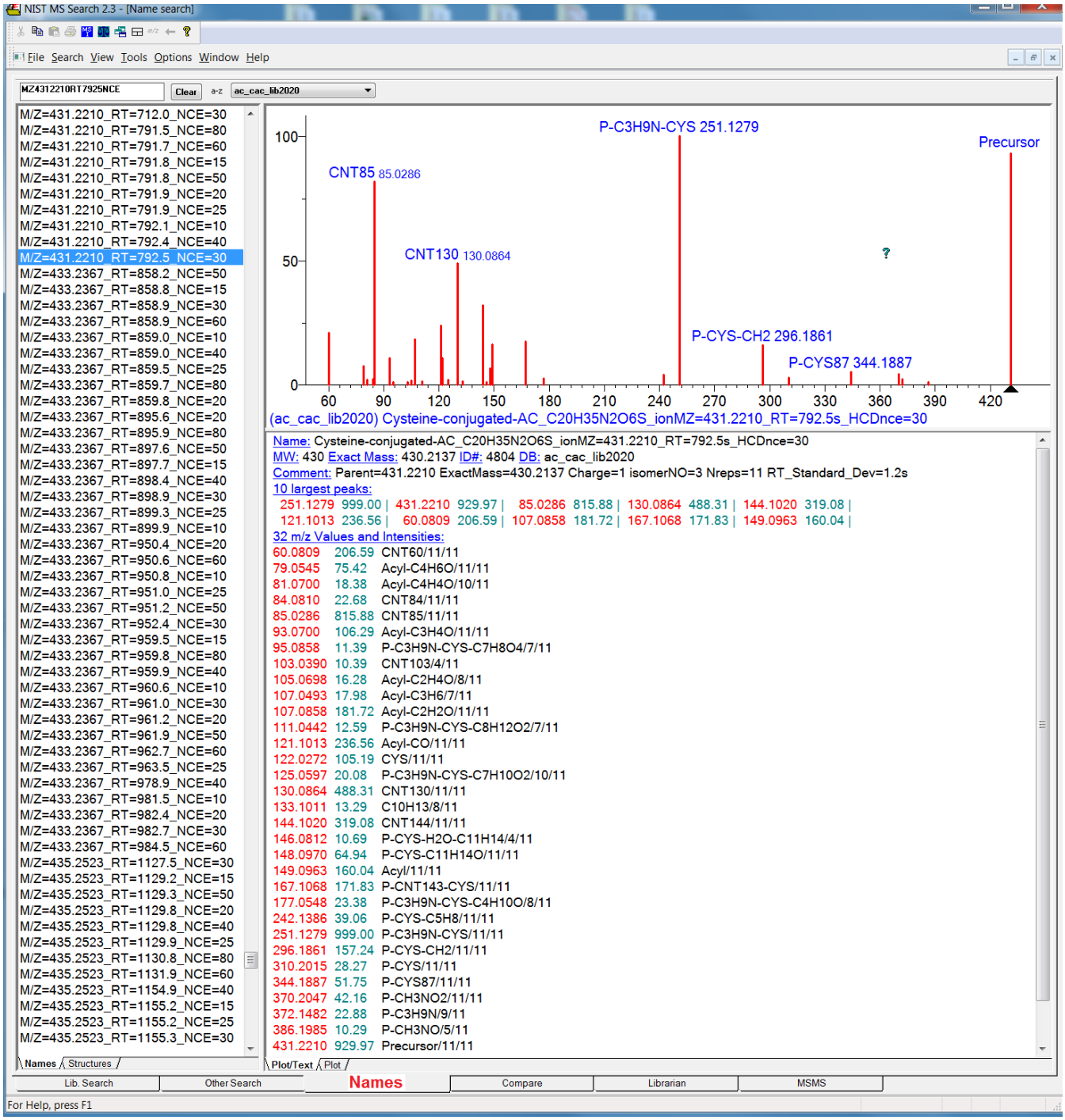


Figure S4. A cysteine-conjugated acylcarnitine spectrum is displayed by the MS-Search program. This program also provides descriptions of the parent ion, top 10 most abundant fragments, and a full list of the ion fragments and annotations. Each fragment was annotated with information of identity, the number of spectra used to process the fragment *m/z* and abundance data, and the number of total spectra of this ion at the given *m/z*, retention time and collision energy. For example, the note “11/11” in the annotation of a fragment means that this consensus spectrum was processed with 11 spectra (the second 11), and

the fragment was detected in all the 11 spectra (the first 11). Note: CNT in the figure means that the fragment is from carnitine moiety, CYS is cysteine.

6. Brief discussions on collision energy and retention time

6.1 Collision energies and fragment abundances of mass spectra

This part provides more data about collision energies and fragment abundances for the Example 1 of part 4.7 in the paper. Isomer C has a fragment at *m/z* 59.0491 ($C_3H_7O^+$, i.e., acyl – $C_3H_2O_2$), which increased with collision energies, and became the most abundant one at NCE 80 (see Figure S5A). Isomers A and B exhibit low abundance for peak $C_3H_7O^+$, while isomer D simply does not have it. This figure also shows that under NCE 20, the abundances of the fragments in the isomers A, B and C are similar, only under NCE 30 or higher, the fragment in isomer C is significant larger than these in isomers A and B, and under NCD 60 or higher, the abundance differences of the fragment in isomers A and B become obvious. This suggests that certain isomers can be distinguished by fragment differences of their spectra acquired under a set of collision energies. Figure S5B displays another unique fragment at *m/z* 199.0601 ($C_9H_{11}O_5^+$, i.e., P – TMA – CH_4O) in isomer C, which is absent in isomers A, B and D. These unique fragments and the delayed retention time of isomer C imply that the acyl moiety of isomer C may have a methoxy and a carbonyl group separated by two CH_2.

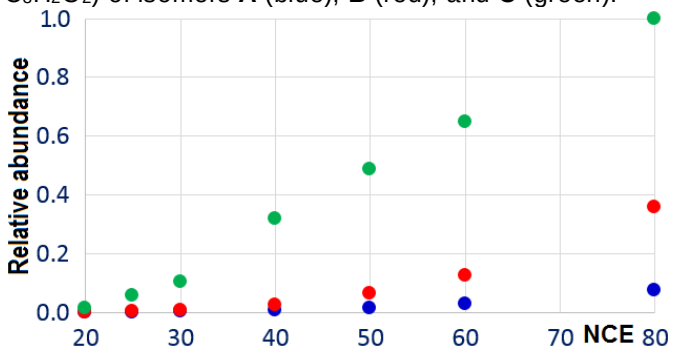
Isomer D exhibits four unique feature fragments at *m/z* 73.0284 ($C_3H_5O_2^+$, i.e., acyl – C_3H_4O), 101.0233 ($C_4H_5O_3^+$, acyl – C_2H_4), 185.0444 ($C_8H_9O_5^+$, P – TMA – C_2H_6O), and 203.055 ($C_8H_{11}O_6^+$, P – TMA – C_2H_4). The relationships between collision energies and abundances of these fragments are shown in Figures S5C, S5D, S5E and S5F. These feature fragments suggest that the isomer D could be an ester. The neutral loss C_2H_4 can be explained by McLafferty rearrangement.

6.2 Retention time

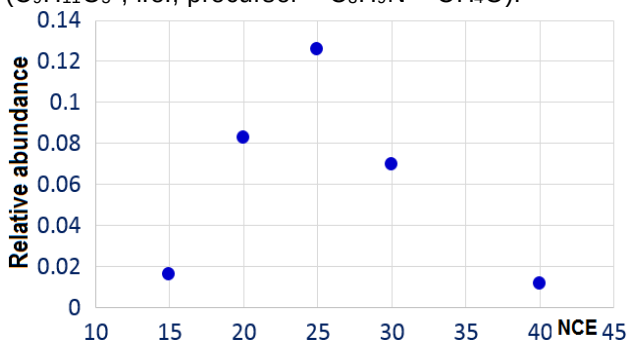
Good consistency of retention times between a known and an unknown can greatly increase the possibility that the two compounds are the same. With retention time data, some simple analyses can avoid many obvious errors. For example, if reversed-phase chromatography is used for LC separation, for the same component isomers, these with -COOH group can elute significantly earlier than these with -COO- (ester) group. Hence, we provided retention times for the acylcarnitines in the AC library, and users may use these retention times to make alignments with their data.

Figure S5. The graphs display the relative abundances of feature fragments of isomers under different normallized collision energies (NCE).

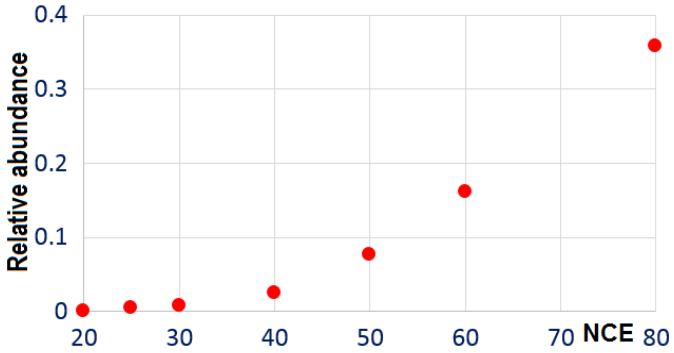
S5A. Fragment at *m/z* 59.0491 ($C_3H_7O^+$, i.e., acyl – $C_3H_2O_2$) of isomers **A** (blue), **B** (red), and **C** (green).



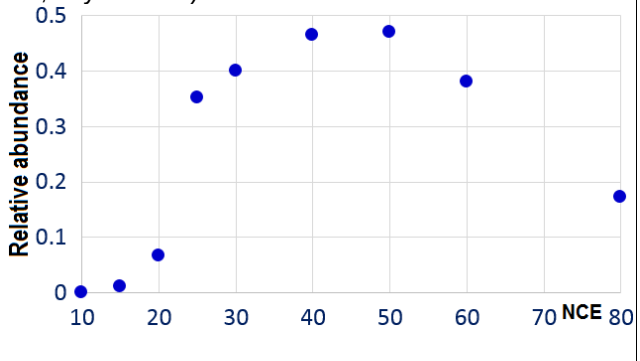
S5B. Isomer **C** fragment at *m/z* 199.0601 ($C_9H_{11}O_5^+$, i.e., precursor – C_3H_9N – CH_4O).



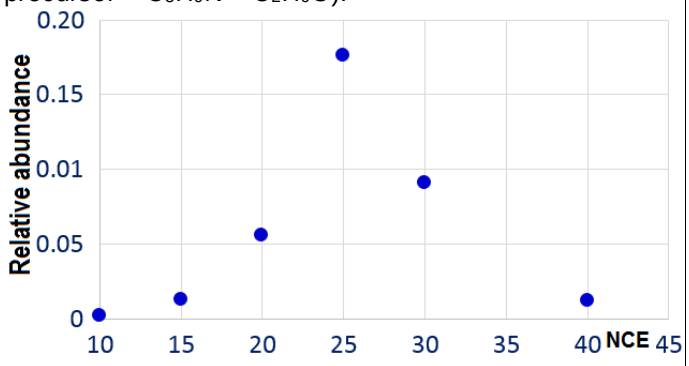
S5C. Isomer **D** fragment at *m/z* 73.0284 ($C_3H_5O_2^+$, i.e., acyl – C_3H_4O).



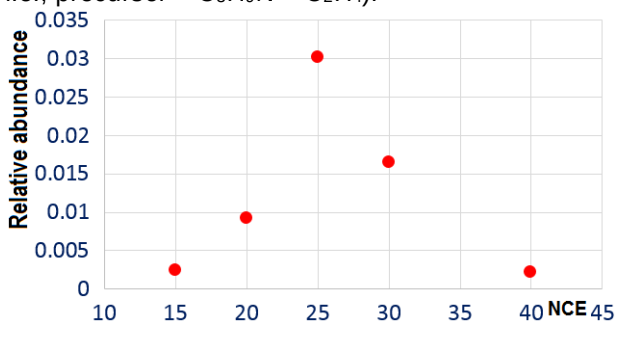
S5D. Isomer **D** fragment at *m/z* 101.0233 ($C_4H_5O_3^+$, i.e., acyl – C_2H_4).



S5E. Isomer **D** fragment at *m/z* 185.0444 ($C_8H_9O_5^+$, i.e., precursor – C_3H_9N – C_2H_6O).



S5F. Isomer **D** fragment at *m/z* 203.055 ($C_8H_{11}O_6^+$, i.e., precursor – C_3H_9N – C_2H_4).



7. Artifact analyses

7.1 Adduct analysis

In-source ions formed by adduction of targeted compounds and metal ions or small molecules are common, practically in the LC-MS based proteomic data. However, the adductions of acylcarnitines with metal ions or small molecules are very few in this study. For example, there are no any directly observable adducts in the LC-MS peaks of the three most abundant acylcarnitines detected in this study, see Figures S6-S8. When blowing up the peaks in these figures, only tiny Na-adducted ions may be found, and their abundance ratios (to their parents) are less than 0.1%. Hence, adducts are not an issue for acylcarnitines in this study.

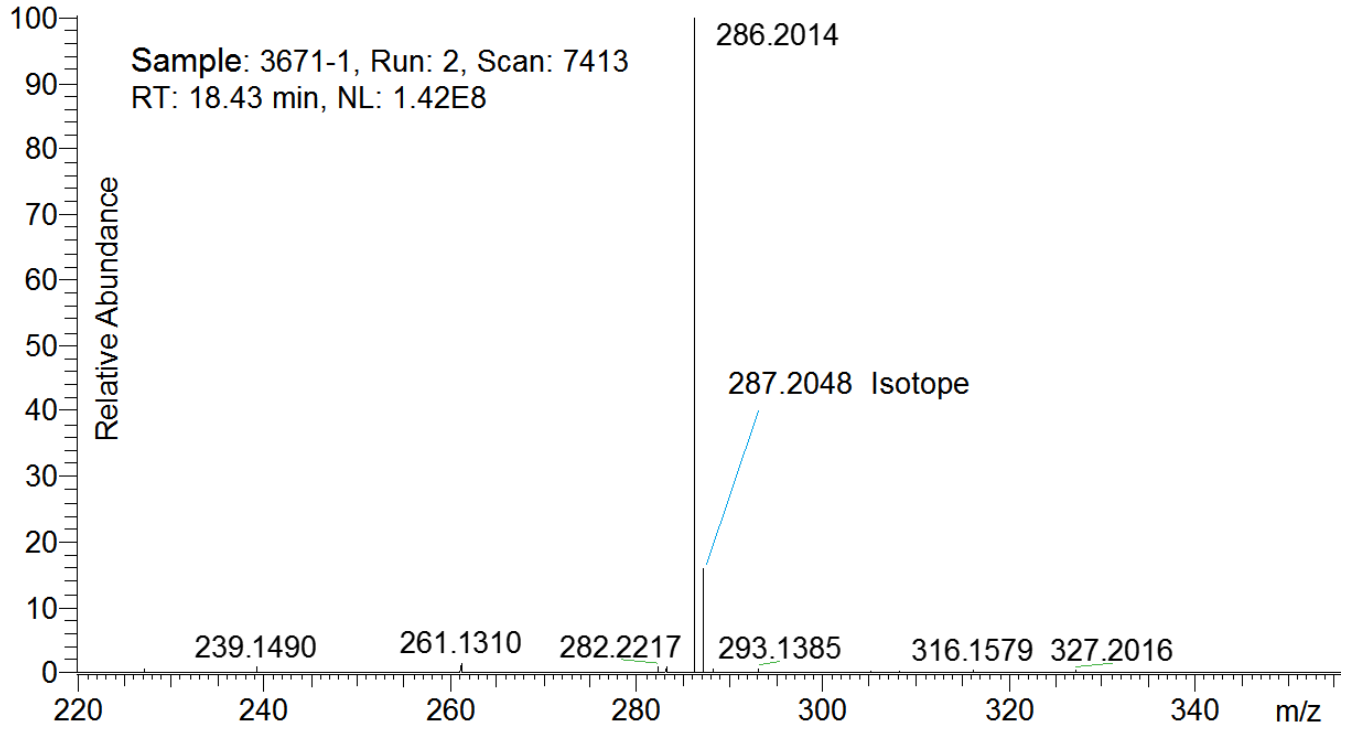


Figure S6. LC-MS peaks around the acylcarnitine at *m/z* 286.2013 and RT 18.41 min.

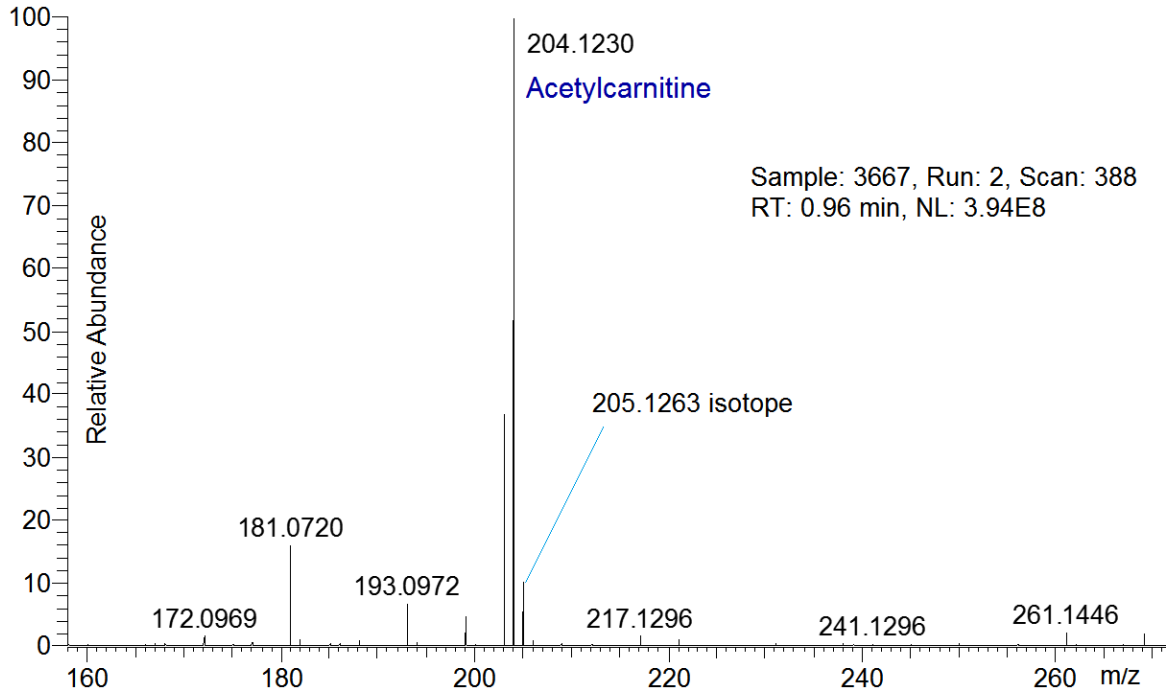


Figure S7. LC-MS peaks around the acylcarnitine at *m/z* 204.1230 and RT 0.96 min.

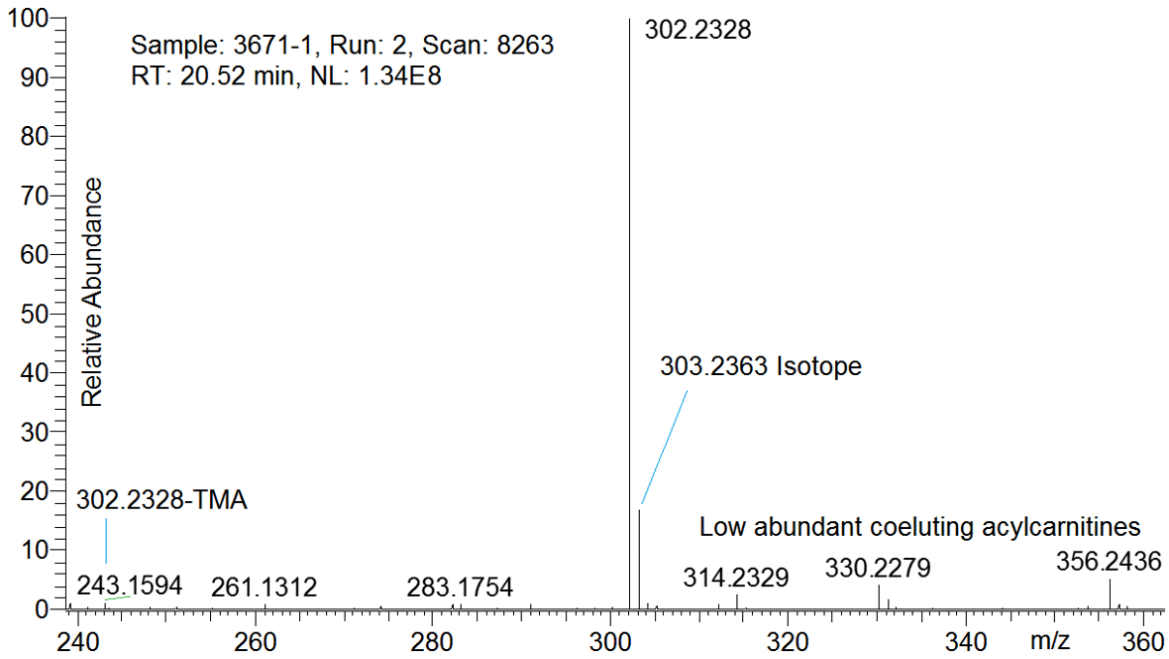


Figure S8. LC-MS peaks around the acylcarnitine at *m/z* 302.2326 and RT 20.52 min.

7.2 Confusing cases

Figure 9 shows a spectrum, which contains three acylcarnitine feature peaks, and the feature peak $C_4H_5O_2^+$ is the most abundant one. However, this spectrum is not acylcarnitine, but an unknown that was heavily contaminated by a co-eluting acylcarnitine, because it does not have the neutral loss peak of TMA, which should be 285.1548 (= 344.2283 – 59.0735).

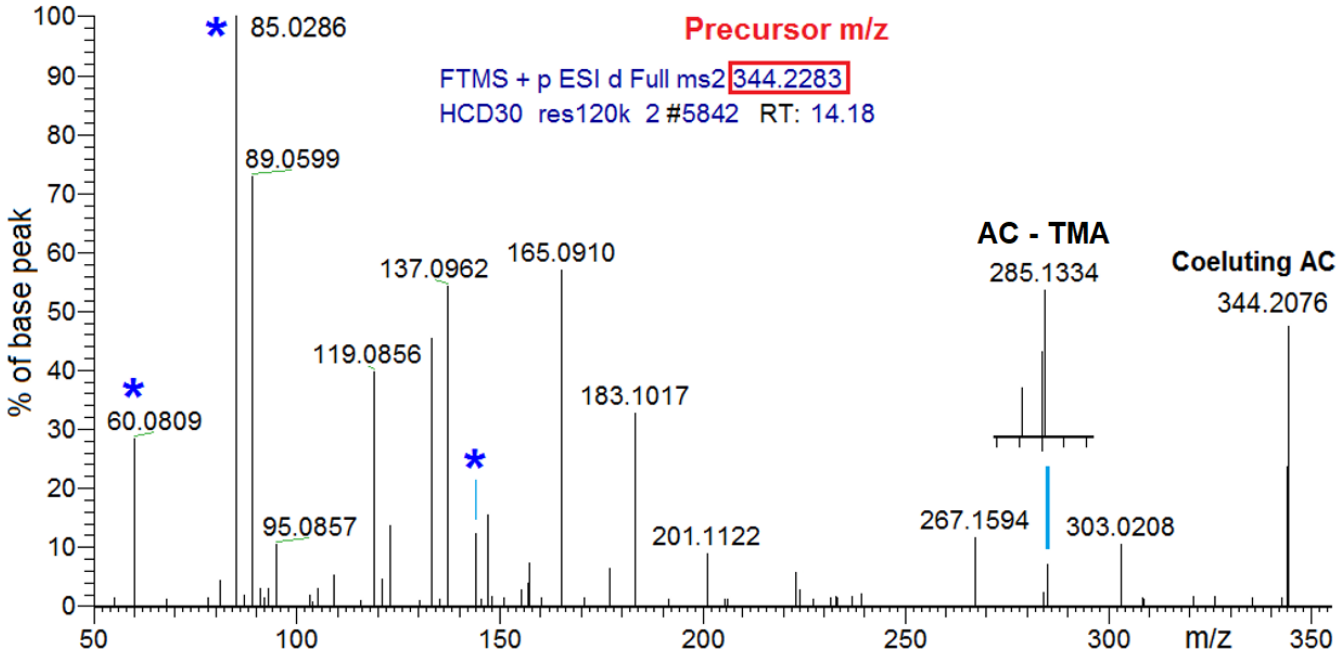


Figure S9. The figure shows a spectrum with precursor at *m/z* 344.2283. which was contaminated by a coeluting acylcarnitine (*m/z*: 344.2076). Thus, this non-acylcarnitine spectrum contains acylcarnitine feature fragments (with blue stars).

While the neutral loss peak of TMA can be used to filter false acylcarnitines, its presence is still not sufficient to guarantee a true acylcarnitine identification, because non-acylcarnitines can also have the group TMA. For example, the spectrum in Figure S10 has relatively abundant ions TMAI and P – TMA. But it may still not be an acylcarnitine spectrum because its characteristic fragment CNT85 ($C_4H_5O_2^+$) is too small, and it does not have CNT144 (= carnitine – H_2O).

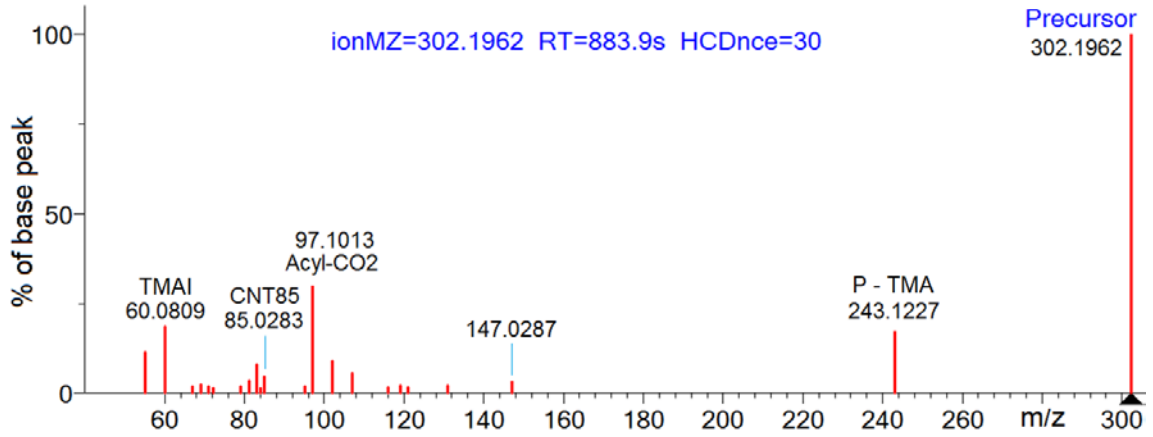


Figure S10. This spectrum has feature fragments TMAI, CNT85, precursor – TMA, and a right *m/z* as an acylcarnitine. However, this spectrum does not have CNT144 and its CNT85 is too small. Hence, there is a large possibility that it is not an acylcarnitine spectrum.

8. Others

Note: Figure S11 is mentioned in the section 4.7 (Examples of isomer analysis) in the paper.

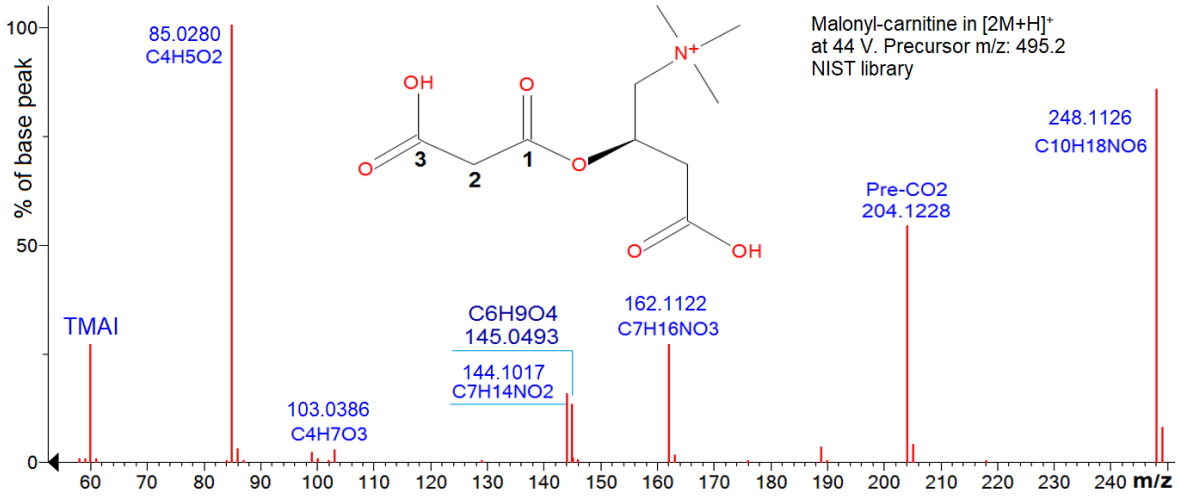


Figure S11. HCD spectrum of malonyl-carnitine in $[2M+H]^+$ ion mode, at 44 V (NIST17 Tandem MS Library).