


1 New Methodology for Known Metabolite Identification in 2 Metabonomics/Metabolomics: Topological Metabolite Identification 3 Carbon Efficiency (tMICE)

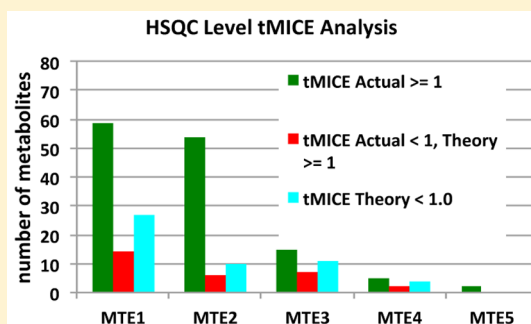
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6  Supporting Information

7 **ABSTRACT:** A new, simple-to-implement and quantitative approach to
8 assessing the confidence in NMR-based identification of known meta-
9 bolites is introduced. The approach is based on a topological analysis of
10 metabolite identification information available from NMR spectroscopy
11 studies and is a development of the metabolite identification carbon effi-
12 ciency (MICE) method. New topological metabolite identification indices
13 are introduced, analyzed, and proposed for general use, including topo-
14 logical metabolite identification carbon efficiency (tMICE). Because known
15 metabolite identification is one of the key bottlenecks in either NMR-
16 spectroscopy- or mass-spectrometry-based metabonomics/metabolomics
17 studies, and given the fact that there is no current consensus on how to
18 assess metabolite identification confidence, it is hoped that these new
19 approaches and the topological indices will find utility.

20 **KEYWORDS:** metabonomics, metabolomics, metabolic profiling, NMR spectroscopy, metabolite topology,
21 topological metabolite identification carbon efficiency (tMICE),
22 topological metabolite identification nitrogen and carbon efficiency (tMINCE)



1. INTRODUCTION

23 Metabolic profiling of animal and human biofluids and tissues
24 is emerging as a key technology in biology and especially in
25 medicine,¹ where it can be used in either a diagnostic or prog-
26 nostic mode. These metabonomics^{2,3} or metabolomics⁴ studies
27 are typically executed with NMR-spectroscopy- or mass-
28 spectrometry-based technologies for metabolite identification
29 in biofluids, cell extracts, or tissue samples. There are many steps
30 in a metabonomics experiment, and most of these steps have
31 well-described protocols for NMR-⁵⁻⁹ or mass-spectrometry
32 (MS)-based¹⁰⁻¹⁴ approaches and well-accepted statistical pro-
33 cedures¹⁵⁻²⁰ for their analysis, with the significant exception of
34 known metabolite identification. This remains a problematic
35 step for both NMR-spectroscopy-²¹⁻²⁵ or mass-spectrometry
36 MS-based^{10,26,27} metabonomics. The problem is essentially one
37 of complexity and diversity. In contrast with the 4 different bases
38 in the nucleotides of DNA and the 20 natural amino acids in
39 protein structures, there are thousands of structurally diverse,
40 metabolites in biofluids,²³ and their identification is not straight-
41 forward by NMR or MS.

42 New approaches to NMR- and MS-based known metabo-
43 lite identification are emerging,^{25,28} including new database and
44 chemical treatment approaches. The new database approaches
45 include: (i) a new isomer-specific database, ¹H(¹³C)-TOCCA-
46 TA, for the identification of metabolites from TOCSY and
47 natural abundance HSQC-TOCSY spectra,²⁹ (ii) a new, unified
48 and isomer-specific, database interrogation method that provides

improved HSQC-based metabolite identification performance 49
(COLMAR),³⁰ and (iii) SpinCouple,³¹ a new database for the 50
analysis of the J-resolved spectra of metabolite mixtures, which 51
contains a much larger number of spectra of metabolite stan- 52
dards than the similar Birmingham Metabolite Library (BML).³² 53
The isomer-specific features of ¹H(¹³C)-TOCCATA and COLMAR 54
are useful because they overcome the problem of failure to 55
identify a metabolite if only the peaks from the high abundance 56
isomer are observed, leading to low scores in overall metabolite 57
matching algorithms because the low abundance isomer peaks 58
are not scored. In addition, COLMAR contains useful confidence 59
terms including a peak matching ratio and a peak uniqueness 60
statistic that decrease false-positives. To provide an orthogonal 61
method for metabolite confirmation, Bingol and Brüscheiler³³ 62
developed a method called NMR/MS Translator that confirms 63
metabolite identifications from the HSQC COLMAR database 64
by predicting the MS adduct ions that should be observed from 65
the metabolites. 66

Metabolite identification is actually a problem in two distinct 67
categories: first, the de novo structure elucidation of novel meta- 68
bolites identified for the first time, and second, the structure 69
confirmation of known metabolites that have been identified 70
and characterized previously. To elucidate the structure of novel 71
metabolites, it is generally accepted that the rigorous processes 72

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73 used in the field of natural product structure elucidation are
74 required.³⁴ This would typically involve the isolation of the novel
75 metabolite from the biological matrix and its purification ahead of
76 full molecular structure elucidation by a panoply of spectroscopic
77 techniques. See [Supplementary Table S1](#) for a list of the struc-
78 tural features that can be revealed by these different technologies.

79 By contrast, the structure confirmation of the identities of
80 known metabolites is generally accepted to require a less rigorous
81 process because the structure has already been elucidated
82 and spectroscopic data on the pure metabolite may be available.
83 However, there is little consensus on what the identification
84 process for known metabolites should be. To address this
85 problem, the Metabolomics Standards Initiative (MSI)³⁵ set
86 up a Chemical Analysis Working Group (CAWG) in 2007.³⁶
87 This group developed a four-level classification scheme for the
88 identification of known metabolites: (1) Identified Compounds,
89 (2) Putatively Annotated Compounds, (3) Putatively Charac-
90 terized Compound Classes, and (4) Unknown Compounds.

91 To reach Level 1, Identified Compounds, the following require-
92 ments were stated: “A minimum of two independent and
93 orthogonal data relative to an *authentic* compound analyzed
94 under *identical* experimental conditions are proposed as
95 necessary to validate non-novel metabolite identifications
96 (e.g. retention time/index and mass spectrum, retention time
97 and NMR spectrum, accurate mass and tandem MS, accurate
98 mass and isotope pattern, and full ¹H or ¹³C NMR, 2-D NMR
99 spectra). The use of literature values reported for authentic
100 samples by other laboratories is generally believed to be
101 insufficient to validate a confident and rigorous identification.
102 The use of literature or external laboratory data results in level
103 2 identifications.³⁶ Thus, the CAWG stipulated that any known
104 metabolite identification made by reference to an authentic
105 standard in a database such as the Human Metabolome Database
106 (HMDB),³⁷ the BioMagResBank (BMRB),³⁸ and the Birming-
107 ham Metabolite Library (BML)³² or to a literature report should
108 be downgraded to Level 2, Putatively Annotated Compounds,
109 whereas, if that same metabolite had been identified by reference
110 to an authentic standard, actually in the laboratory of the investi-
111 gator, it would be classed as Level 1, Identified Compound.

112 The original CAWG recommendations have not been widely
113 adopted,³⁹ and recently new outline proposals have emerged^{40–43}
114 for improvements to the original four-level metabolite identi-
115 fication classification system. The proposals were either to
116 increase the refinement of the four-level system or to introduce
117 some sort of scoring method for the acquisition of certain sorts of
118 data such as a 2D NMR. However, with the exception of some
119 proposals for accurate mass and retention time fits,⁴² no pro-
120 posals were made as to how a match of the experimental data
121 with the standard data should be assessed. In addition, a call to
122 the community was made⁴¹ for engagement with this problem.
123 In response to this call, new quantitative proposals for Level
124 2 metabolite annotation confidence using LC–MS methods
125 recently emerged from the group of Daly.⁴⁴ In addition, our
126 group proposed some new, quantitative approaches to known
127 metabolite identification confidence for NMR-based metabo-
128 nomics studies including metabolite identification carbon effi-
129 ciency (MICE),⁴⁵ drawing on an approach from the drug discovery
130 field known as ligand efficiency.⁴⁶ The MICE methodology
131 simply counts the number of pieces of metabolite identification
132 information (MII) obtained for a metabolite in NMR-based
133 metabolite identification (proton chemical shifts, signal multi-
134 plicities, coupling constants (²J_{H,H} and ³J_{H,H}), COSY connectiv-
135 ities, presence of second-order spin system, HSQC cross-peaks)

and divides this sum total by the number of carbon atoms in the
metabolite. The MICE metric is thus a measure of the amount of
identification information obtained relative to the size of the
metabolite, as judged by the number of carbon atoms it pos-
sesses. The following guidelines were proposed for a metabolite
to be considered confidently identified:

- (1) MICE value ≥ 1.0 .
- (2) Experimental data are a good fit to either authentic refer-
ence standard data or literature or database values (differences
between the experimental and the expected chemical shifts
within ± 0.03 ppm for ¹H and ± 0.5 ppm for ¹³C and homonuclear
coupling constants within ± 0.2 Hz).
- (3) Experimental data provide good “coverage” across all parts
of the molecular structure of the metabolite.
- (4) Signal-to-noise ratio and resolution (actual and digital) in
the spectra should be sufficient to measure the signal features
with confidence.
- (5) Care should be applied when assigning signals in crowded
spectral regions.
- (6) HSQC data are important in metabolite identification, as
they provide an excellent orthogonal data source via the ¹³C NMR
chemical shift, which is much more sensitive to environment than
the proton chemical shift.
- (7) HMBC data should be used wherever possible to
corroborate identifications that are uncertain or those that are
critical for the biological interpretation of the experiment, that is,
those metabolites that may be biomarkers.

This current work now reports on a new topological approach
to NMR-based, known metabolite identification, which further
develops the concepts introduced in the original MICE work.⁴⁵
The MICE and related analyses were novel, simple, and
quantitative but had the disadvantage that a judgment had to
be made of how well the MII measured represented the entire
molecular structure (point 3 in the list above). This new work
overcomes that issue, again, in a simple quantitative fashion by
measuring the MII obtained by NMR spectroscopic methods
for each separate molecular topology element (MTE) of the
metabolite structure.

Molecular frameworks and topologies can be computed in a
number of different ways. In the original molecular framework
analysis by Bemis and Murcko,⁴⁷ molecules without rings were
classed as not having a framework. This causes issues for the
analysis of endogenous metabolites, where large numbers
have no rings. For example, in a recent comparative analysis of
the topologies of human metabolites relative to drugs, natural
products, and other molecules, only one-third of the 6237 human
metabolites identified in the HMDB were classed as having a
framework; that is, the majority of the human metabolites had
a structure that comprised one or more chains of atoms.⁴⁸
This methodology is therefore unsatisfactory: Any topological
analysis of metabolites must address the particular structural
features that they possess. To avoid this issue, the analysis
reported here pragmatically classifies the metabolites under study
as possessing one or more MTEs of just two types, rings and
chains, each containing protonated and nonprotonated carbon
atoms and slow-exchanging amide protons, as these are the
elements that give rise to signals in ¹H NMR spectroscopy-based
metabonomics experiments.

Metabolites may possess a single MTE, being a chain or ring,
or may possess multiple MTEs separated by, for example, hetero-
atoms without slow-exchanging hydrogens, by quaternary carbons,
or by methine (CH) carbon branching points in the structure.
Simplicity, manageability, and applicability to NMR-based metabolite

199 profiling have been taken into account, as well as the possibility
200 for automation, in the design of the entire analysis.

201 Topological metabolite identification carbon efficiency
202 (tMICE) is shown to provide a simple, robust, and quantitative
203 measure of the confidence of NMR-based identification of known
204 metabolites in complex biological matrices.

2. MATERIAL AND METHODS

2.1. Biological Samples, Metabolites, and NMR Spectroscopy

205 The 100 metabolites included in this study were recently
206 identified from ethically approved studies of the proton NMR
207 spectra of the urine of male, wildtype, and flavin monooxygenase
208 5 knockout C57BL/6 mice⁴⁹ and of Italian Type 2 diabetic
209 patients (Supplementary Table S2). This metabolite set is based
210 on that recently reported, and the sample preparation details
211 and NMR spectroscopy data acquisition parameters are also as
212 previously reported.⁴⁵ The original set of 75 metabolites was
213 augmented by additional metabolites identified in the past
214 12 months. The methods of identification of the metabolites
215 were as previously reported.⁴⁵

2.2. Analysis of Metabolite Features

217 The following information was abstracted for each metabolite
218 and is provided in Supplementary Table S2:

- 219 (1) metabolite class
- 220 (2) metabolite common name
- 221 (3) metabolite IUPAC name
- 222 (4) HMDB code
- 223 (5) number of hydrogen atoms
- 224 (6) number of carbon atoms
- 225 (7) number of oxygen atoms
- 226 (8) number of nitrogen atoms
- 227 (9) number of sulfur atoms
- 228 (10) total number of heavy atoms (non-hydrogen atoms)
- 229 (11) nominal molecular mass in Daltons
- 230 (12) a flag for molecular symmetry: 1 = some element of
231 symmetry is present in metabolite; 0 = no symmetry element in
232 metabolite
- 233 (13) a flag for chirality: 1 = ≥ 1 chiral centers; 0 = 0 chiral
234 centers in metabolite

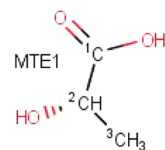
2.3. Definition of Molecular Topology Elements and Atom Numbering

236 MarvinSketch was used for drawing, displaying, and character-
237 izing chemical structures, and topology elements, Marvin 6.1.1,
238 2013, ChemAxon (<http://www.chemaxon.com>).

239 MTEs were analyzed by hand for all of the metabolites in the
240 study, and a full list of the metabolites, their assigned MTEs, and
241 their MII is provided in Supplementary Table S3. The following
242 methodology and logic was applied:

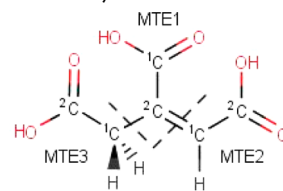
- 243 (1) MTEs are defined to be of two basic types: rings of atoms
244 (code 0) and chains of atoms (code 1).
- 245 (2) For simplicity and practicality of application, ring
246 structures are treated as singular topology elements.
- 247 (3) The first molecular topology element, MTE1, will be the
248 highest priority chain or ring according to IUPAC⁵⁰ functional
249 group and Cahn–Ingold–Prelog⁵¹ (CIP) priorities, the latter
250 determining priorities based on the atomic number of the
251 substituent ($S > O > N > C > H$, etc.) and on bond multiplicities
252 with, for example, $C=O$ treated as $C(O)-O$; that is, a carbon
253 with a double bond to oxygen is treated as if it has two single
254 bonds to two separate oxygen atoms.

(4) For molecules containing chains of atoms: (a) Breaks
255 between topology elements will occur *after* quaternary carbons
256 or heteroatoms without nonexchanging hydrogens, as these
257 atoms interrupt the proton-to-proton connectivity information
258 available from experiments such as 2D ¹H COSY NMR.⁵² (b) If
259 the start point of any MTE is a quaternary carbon it will not
260 break; for example, there is not a break after the starting
261 carboxylic acid carbon (C1) in (S)-lactic acid. The single, chain-
262 type MTE1 goes from the C1 carboxylic acid carbon through C2
263 to chain termination at the methyl group, C3. 264



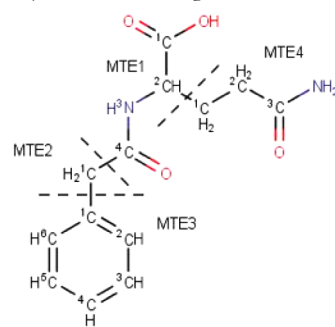
(S)-lactic acid

(c) The numbering of any subsequent MTE will start at the first
265 atom after the break point: The breaking quaternary carbon or
266 heteroatom is included in the preceding MTE. (d) The number of
267 branches after a quaternary carbon will generally equal the number
268 of atoms bonded to that quaternary carbon minus 1, and the pri-
269 ority for the next MTE will be given again by CIP rules. For example,
270 the start point for MTE1 in *cis*-aconitic acid is the carboxylic acid car-
271 bon located on the central sp^2 carbon. This carboxylic acid carbon is
272 followed by an sp^2 quaternary carbon with two branches, which go
273 first to the $=CH-COOH$ (MTE2) and then second to the $-CH_2-$
274 $COOH$ (MTE3) groups by CIP priority rules. The boundaries
275 between MTEs are shown by dashed lines in the structures. 276



cis-aconitic acid

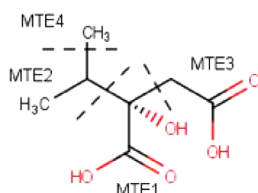
(e) If there is a branching point that is a methine carbon (CH),
277 the MTE will move across the branch point according to CIP
278 priority rules and will continue until chain termination or arrival
279 at a break point. If the chain terminates, the next MTE will then
280 be decided by CIP rules. On the contrary, if one or more new
281 chains continue after a break point, then a structural path
282 continuation rule applies and the subsequent MTE will carry
283 on the same path at that point (see 5 below). For example,
284 phenylacetylglutamine branches at the $-CH-$ (C2) of MTE1
285 ($HOOC-CH-NH-C=O$) and continues by priority via N3 to
286 the amide carbonyl carbon break point (C4). 287



phenylacetylglutamine

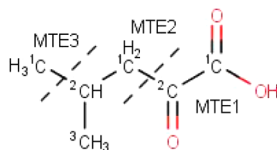
The single methylene unit then becomes MTE2 as it continues the
288 chain (rule 5 below) and coincidentally, it is higher priority (due to
289

its phenyl substituent) than the alternative methylene group at the branching CH: The phenyl moiety becomes MTE3, and MTE4 is the three carbon amide chain at the methine branching point in MTE1. Another differing example occurs in (2S)-isopropylmalic acid, which branches at the $-\text{CH}-$ (C1) of MTE2 ($-\text{CH}-\text{CH}_3$), so the $-\text{CH}_2-\text{COOH}$ becomes MTE3 by CIP priority and then the methyl group at the branching point becomes MTE4, as this is a chain termination rather than a chain continuation example.



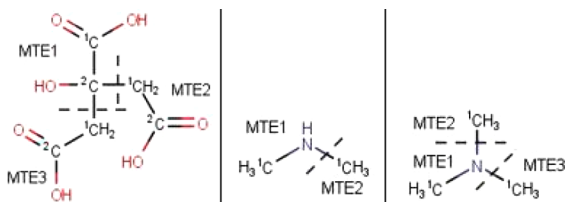
(2S)-isopropylmalic acid

(f) For metabolites with equivalent groups after a methine branching point (CH), the break will be randomly to one side or the other; for example, ketoleucine has two equivalent methyl groups: C3 in MTE2 and C1 in MTE3.



ketoleucine

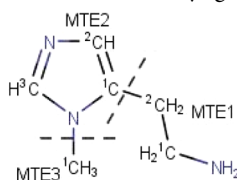
(g) For equivalent carbons allocated in different MTEs, all are registered and analyzed, for example, in ketoleucine above and in citric acid, dimethylamine, and trimethylamine, as it is critical that the topological analysis reflects the totality of the metabolite structure, including the possession of equivalent features.



MTE analysis for citric acid, dimethylamine and trimethylamine

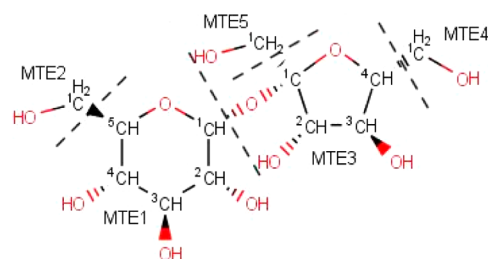
(5) Structural path continuation rule: In addition to the above rules regarding branching at CH and quaternary carbons, if after a break point there is a choice between continuing along that structural path or moving back to another structural element, continuing along the structural path will take priority (even if this breaks CIP priorities) to pragmatically align the MTE order with the metabolite structure as closely as possible. This rule applies to all metabolites, no matter of what structural type.

(6) For metabolites with a combination of rings and chains, the first molecular topology element, MTE1, will be also defined by IUPAC functional group priorities. (a) For example, MTE1 in 3-methylhistamine is the aminoethyl chain, MTE2 is the imidazole ring, and MTE3 is the methyl group.



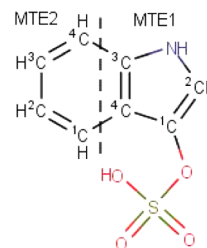
3-methylhistamine

(b) Ring priorities are decided by the number of heteroatoms of any type and if the same number, by ring size: A complex example is sucrose, whose six-membered ring (oxane) is MTE1; then, MTE2 is given by the continuation path rule to the hydroxymethyl at C5. MTE3 then corresponds to the five-membered ring (oxolane), MTE4 to the hydroxymethyl at C4, again by continuation path, and MTE5 is the remaining hydroxymethyl at C1.



sucrose

(7) For fused rings, carbons at the fusion site will be attached to the ring of greatest priority (see rule 6b), and they only will be counted once; for example, the first MTE in indoxyl-3-sulfate is the pyrrole ring (highest priority). MTE2 then corresponds to the remaining four aromatic CHs of the phenyl ring, excluding the carbons at the fusion site. The sulfate group has no observable, slow-exchanging protons and is not counted.



indoxyl-3-sulphate

2.4. Determination of the Metabolite Identification Information for each Molecular Topology Element

Once the molecular topology elements (MTEs) were defined, the experimental metabolite identification (ID) information for each metabolite was extracted from analyses of the NMR spectra of the mouse and diabetic patient urine. In parallel, the theoretical information that could possibly be obtained was calculated. Supplementary Table S2 summarizes both the theoretical and experimental information that was measured for all MTEs in all 100 metabolites.

The MII for each MTE consists of the following information, and for points 4 to 8 both actual experimental and then theoretical values are calculated:

- (1) Type of MTE, coded 1 for chains and 0 for rings.
- (2) Total number of carbon atoms in the MTE.
- (3) Total number of nonexchanging NH groups in the MTE.
- (4) MTE metabolite ID information at HSQC level,⁴⁵ which equals the sum total of the number of bits of the following information, where present and observed: (a) proton chemical shifts for each protonated carbon and nonexchanging amide NH; (b) multiplicity for each of these signals; and (c) coupling constants ($^2J_{\text{HH}}$ and $^3J_{\text{HH}}$) for each signal (note that this is different from the original MICE methodology where $^nJ_{\text{HH}}$ values were only counted once: this was seen as too conservative as each is an independent measurement). Longer-range couplings were not counted as the methodology was designed to be implemented for NMR spectra with nominal resolution: (d) presence

of second-order spin system (flag = 1 if there are additional lines present in the spectrum, not anticipated by a first-order spectral analysis, otherwise flag = 0); (e) *intra*-MTE COSY links via $^2J_{\text{HH}}$ and $^3J_{\text{HH}}$ between hydrogens (only counted once); (f) *inter*-MTE COSY links via $^3J_{\text{HH}}$ between hydrogens (only counted once and associated with the first MTE connected); and (g) HSQC cross-peaks for each protonated carbon atom (counted twice if measured separately at each of two nonequivalent hydrogens of a methylene group).

(5) HSQC-level tMICE values that equal the sum total of the information in each MTE (see point 4 above) divided by the total number of carbon atoms.

(6) HMBC connectivity information is additionally provided for metabolites, which enables the gathering of more information about information-poor MTEs. This is especially useful in symmetrical metabolites where there can be a paucity of information. The HMBC experiment can be enabling here by ^{13}C isotopomeric raising of the degeneracy within and between symmetrical MTEs. For example, even though chemically and magnetically equivalent by symmetry, HMBC cross-peaks can be observed between the methyl groups in trimethylamine due to isotopomeric raising of the degeneracy of the methyl groups, as the HMBC signals are observed via $^3J_{\text{C,H}}$ from the species $\text{H}_3^{13}\text{C}-\text{N}-^{12}\text{CH}_3$.

(7) HMBC connectivities were counted in two different ways: (1) HMBC connectivities inside an MTE were counted once only and (2) HMBC connectivities between MTEs were counted twice, at both the carbon-13 and proton that are connected. This is in contrast with the original MICE analysis where HMBC connectivities were counted only once. Inside an MTE, the HMBC connectivity is often between hydrogens and carbon-13 nuclei whose chemical shifts are known from HSQC-level analyses. Thus, the only new piece of information is the connection between two known signals: Therefore, we count one bit. However, *inter*-MTE HMBC data often provide correlations to, or through, quaternary carbons, and when it does so, it provides new information in the form of: (1) the ^{13}C NMR chemical shifts of the quaternary carbons or (2) connections between two isolated MTEs. Therefore, we counted two bits of information for *inter*-MTE HMBC connectivities, and the differential counting of *intra*- and *inter*-MTE HMBC data seems appropriate.

(8) HMBC-level tMICE+ values that correspond to the number of bits of MII at HSQC level plus the total number of HMBC links divided by the total number of carbon atoms (see Table 3 (Glossary)).

(9) A value calculated by dividing the actual experimental tMICE values by the corresponding theoretical values.

(10) As for the original MICE analysis,⁴⁵ experimental MII was classed as fitting and therefore counted when differences between the experimental and the equivalent authentic reference or literature or database chemical shifts were within ± 0.03 ppm for ^1H and ± 0.5 ppm for ^{13}C and when homonuclear coupling constants were within ± 0.2 Hz.

(11) For metabolites that are symmetrical within an MTE, identification information at the HSQC level is introduced only once; for example, succinic acid has two methylene carbon atoms that are equivalent due to symmetry, but the information is only recorded once, just as it is observed.

(12) Long-range H–H COSY information is excluded, as the analysis is intended to be applicable to 2D NMR information acquired with nominal resolution and sensitivity.

2.5. Determination of the Overall Metabolite Identification Information for Each Metabolite

Having computed the metabolite ID information available experimentally and theoretically for each MTE separately in each metabolite (Section 2.3), the following information was analyzed for each metabolite as a whole:

- (1) total number of carbon atoms in the metabolite
- (2) total number of nonexchanging NH groups in the metabolite
- (3) actual and theoretical total number of MII bits at HSQC level for all MTEs
- (4) actual and theoretical total number of *inter*-MTE COSY links in the metabolite
- (5) actual and theoretical total number of HMBC links in the metabolite
- (6) actual and theoretical MICE values for each metabolite calculated by summing the total bits of information at HSQC level for all MTEs of a metabolite (point 3 above) and then dividing by the total number of carbon atoms (point 1 above)
- (7) actual and theoretical MICE+ values for each metabolite were calculated by summing the total bits of information at HMBC level for all MTEs of a metabolite (point 3 plus 5 above) and then dividing by the total number of carbon atoms (point 1 above)

2.6. Precision of Measurements of ^1H NMR Chemical Shifts in a Variety of Metabolites in Buffered Mouse Urine and Comparison of Experimental Shifts with Those of Authentic Reference Standards in the HMDB

The chemical shifts of a variety of aliphatic, olefinic, and aromatic protons in a variety of acidic, basic, and neutral metabolites were measured to determine the variability of chemical shifts in these differing environments in mouse urine from 34 individual C57BL/6 mice and their FMO5 KO counterparts⁴⁹ at weeks 15, 30, 45, and 60. The results are summarized in Table 1 and compared with the corresponding values obtained from the HMDB.³⁷

2.7. Statistical Analyses of the Data

All statistical analyses were conducted in Microsoft Excel for Macintosh version 14.5.5. All errors are standard deviations. All significance testing used the two-tailed, unpaired Student's *t* test with a confidence threshold of >95% ($p < 0.05$).

3. RESULTS

3.1. Molecular Properties of the 100 Metabolites in the Set

The 100 metabolites were in 11 classes: carboxylic acids (10), hydroxycarboxylic acids (6), dicarboxylic acids (9), tricarboxylic acids (4), small alcohols (5), ketones (2), sugars and sugar acids (7), amines (14), amides and amino acids (32), nucleosides and nucleotides (10), and others (1) for a total of 100 metabolites. See the Supplementary Tables S2 and S3 for more information. The atomic composition and molecular weight properties of the 100 metabolites are shown in Table 2.

Table 2 gives basic statistics for the number of hydrogen, carbon, oxygen, nitrogen, and sulfur atoms present in the metabolite set, including the number of metabolites possessing these atoms (first row) and the statistics for the entire set in subsequent rows, plus basic statistics on the metabolites' nominal molecular mass. While all 100 metabolites studied contained hydrogen and carbon, 95 contained oxygen, and 56 contained nitrogen, only 4 of the sets contained one sulfur atom each. Further analysis (Supplementary Table S2) shows that 16 of the 100 metabolites

Table 1. Average Experimental Chemical Shifts ($n = 34$), Standard Deviations, Maximum and Minimum Values, Range, Corresponding HMDB Values, and Differences between the HMDB and Average Experimental Values for 10 Representative Hydrogen Environments in a Variety of Acidic, Basic, and Neutral Metabolites^a

	β -D-fucose CH ₃	cis-aconitic acid CH	TMA	MA	TMAO	N-butyrylglycine CH ₂ at 1.62	3-indoxyl sulfate, 7.7	citrate, 2.56	cinnamoylglycine, 6.7	hippurate ortho
average experimental shift	1.254	5.721	2.879	2.611	3.276	1.620	7.704	2.558	6.723	7.837
standard deviation	0.0010	0.0172	0.0028	0.0024	0.0028	0.0007	0.0030	0.0053	0.0060	0.0017
maximum value	1.256	5.752	2.885	2.616	3.282	1.622	7.710	2.570	6.734	7.840
minimum value	1.252	5.696	2.875	2.607	3.272	1.619	7.698	2.549	6.706	7.832
range in values	0.004	0.056	0.010	0.009	0.010	0.003	0.012	0.021	0.028	0.008
HMDB shift	1.25	5.693	2.893	2.59	3.253	1.613	7.712	2.527	6.706	7.820
difference HMDB vs experimental	0.004	0.028	0.014	0.021	0.023	0.007	0.008	0.031	0.017	0.017

^aTMA, trimethylamine; MA, methylamine; TMAO, trimethylamine *N*-oxide. The chemical shift of the second-order ortho protons of hippuric acid was estimated at the mid-point of the complex signal. The shifts of cinnamoylglycine were obtained from an authentic Sigma reference standard, as no data were available in the HMDB entry for this metabolite.

Table 2. Number of Metabolites Containing Hydrogen, Carbon, Oxygen, Nitrogen and Sulphur Atoms and Statistical Information on the Elemental Composition^a

	H	C	O	N	S	total heavy atoms	nominal mass in Da
metabolite count	100	100	95	56	4		
maximum	17	13	8	5	1	21	297
minimum	2	1	0	0	0	2	31
mean	8.69	5.45	3.09	0.94	0.04	9.52	138.0
median	8	5	3	1	0	9	132.0
standard deviation	3.01	2.44	1.81	1.11	0.20	3.87	54.5

^aAll data for the set of 100 metabolites in this study.

478 possessed slow-exchanging NH groups that could potentially
479 give rise to additional signals and connectivities in their ¹H NMR
480 spectra.

481 The average molecular weight of the set is 138.0 ± 54.5 Da
482 (standard deviation), and the distribution is shown in Figure 1.

3.2. Molecular Topology Elements in the Metabolites

483 Topological analysis of the set of 100 metabolites showed that
484 they possess between 1 and 5 MTEs with the distribution shown
485 in Figure 2. 67% of the metabolites possess 1 or 2 MTEs, 21%
486 possess 3, 10% possess 4, and only 2% possess 5 MTEs.

487 The 100 metabolites contained a total of 216 MTEs, of which
488 the vast majority (168, 77%) were chains. The distribution of
489 MTE types for MTEs 1 to 5 is shown in Figure 3.

490 An overview of the distribution of the topologies and their
491 types for all 100 metabolites is given in Figure 4, in the form of a
492 grid. The overwhelming preponderance of chain MTEs is clear
493 from Figures 3 and 4.

3.3. Calculation of Topological Metabolite Identification Efficiency Values

495 The number of bits of MII in each MTE was measured by default
496 at the HSQC level. When this number is divided by the number
497 of carbons in the MTE, we arrive at the tMICE value for that
498 MTE, by analogy to the previously described MICE index.⁴⁵

499 The outcome of the topological analysis for MTE1 and MTE2
500 for the first 16 metabolites, the carboxylic acids, and hydro-
501 xycarboxylic acids, is shown in Figure 5. Each metabolite is asso-
502 ciated with a symmetry flag and a chirality flag that is each set to
503 either 0 (no symmetry or no chirality) or 1 (some element of

504 symmetry or chirality, respectively). Then, each MTE is analyzed
505 separately in terms of the number of bits of MII at the HSQC
506 level and the number of inter-MTE COSY links. The inter-MTE
507 COSY information is, of course, included in the HSQC-level MII
508 but is split out separately to give a view of how much information
509 there is at this level to link MTEs together in the same metabolite.
510 The analysis is conducted from both information derived experi-
511 mentally and that which is potentially observable theoretically.
512 Naturally, not all of the MII that is theoretically available is
513 actually observable due to issues of low metabolite concen-
514 trations or crowding in the real spectra. The tMICE values can be
515 simply calculated by dividing the total number of metabolite
516 identification bits at the HSQC level in the MTE by the number
517 of carbon atoms in that same MTE.

3.4. Analysis of tMICE Values for All 100 Metabolites at HSQC Level

518
519 We next analyzed the tMICE values for each of the 217 MTEs in
520 the 100 metabolites using the three-part triage shown below
521 (Figure 6). In the subset of metabolites illustrated in Figure 5,
522 it can be observed that there are three kinds of situation with
523 respect to the tMICE values for the MTEs in each metabolite:

524 (1) It is theoretically possible to obtain sufficient MII for the
525 MTE to generate a tMICE of ≥ 1.0 (a proposed cutoff threshold
526 value for confident identification of the MTE), and this is
527 achieved experimentally (green).

528 (2) It is theoretically possible to obtain a tMICE of ≥ 1.0 for
529 the MTE but this is NOT achieved experimentally (red).

530 (3) It is theoretically impossible to obtain a tMICE of ≥ 1.0
531 (cyan in Figure 6).

532 135 MTEs (62%) have actual tMICE values ≥ 1.0 (green);
533 29 MTEs (13%) have actual tMICE values < 1 but theoretical
534 tMICE values ≥ 1.0 (red); and 52 MTEs (24%) have theoretical
535 tMICE values < 1.0 (cyan).

3.5. tMICE+ Values for all 100 Metabolites at HMBC Level

536 In the tMICE+ analysis at HMBC level, 168 MTEs (77%) have
537 actual tMICE+ values ≥ 1.0 ; 40 MTEs (19%) have actual tMICE
538 + values < 1 but theoretical tMICE values ≥ 1.0 ; and only 8 MTEs
539 (4%) have theoretical tMICE+ values < 1.0 .

540 In moving from the HSQC-level tMICE analysis to the HMBC-
541 level tMICE+ analysis, 27 MTEs changed from cyan (theoretical
542 tMICE < 1) to green (actual tMICE+ ≥ 1.0); 6 MTEs changed from
543 red (actual tMICE values < 1 but theoretical tMICE values ≥ 1.0) to
544 green (actual tMICE+ ≥ 1.0), and 17 MTE changed from cyan

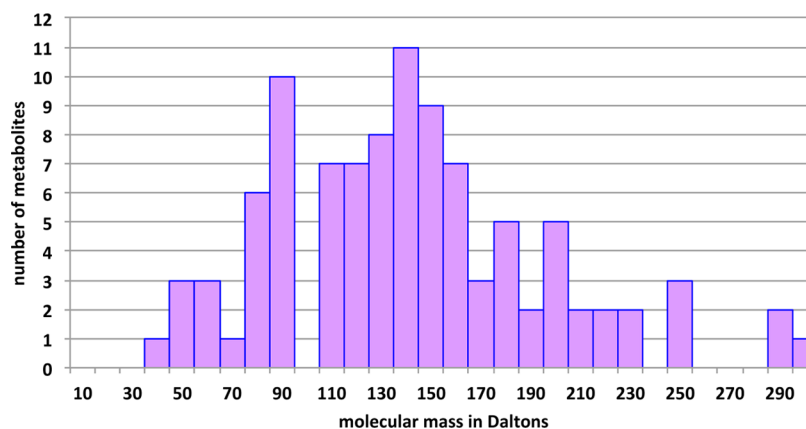


Figure 1. Histogram of the distribution of molecular weights of the 100 metabolites in the set.

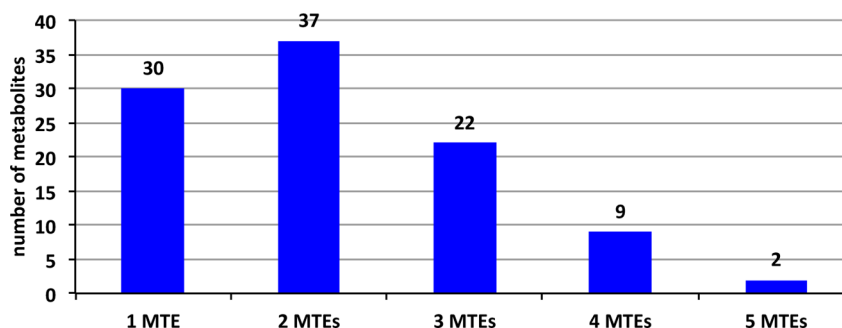


Figure 2. Chart of the number of metabolites with 1, 2, 3, 4, or 5 MTEs in the 100 metabolites studied.

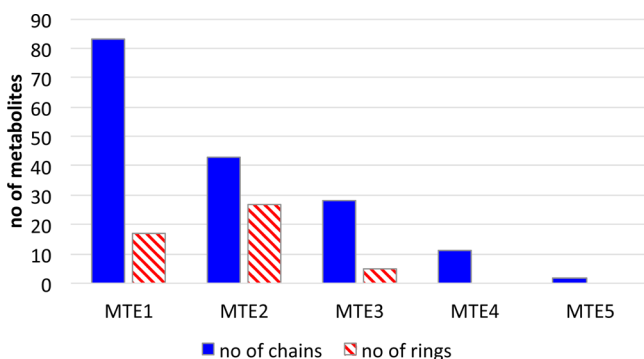


Figure 3. Chart of the distribution of chains or rings across MTEs 1 to 5 in the 100 metabolites in the current cohort.

(theoretical $tMICE < 1$) to red (actual $tMICE+ < 1$ but theoretical $tMICE+ \geq 1.0$). See also Figure 7.

$tMICE+$ values for metabolites with some degree of symmetry (actual 2.18 ± 1.93 and theoretical 3.49 ± 2.62 , $n = 79$) were lower than those for unsymmetrical metabolites (actual 2.68 ± 2.43 , $p = 0.101$ and theoretical 7.20 ± 5.50 , $n = 137$, $p = 2.30 \times 10^{-10}$) but were only statistically significantly lower for the theoretical $tMICE+$ values. Similarly, $tMICE+$ values for achiral metabolites (actual 2.37 ± 1.90 and theoretical 3.93 ± 2.31 , $n = 140$) were lower than those for chiral metabolites (actual 2.73 ± 2.82 , $p = 0.318$ and theoretical 9.35 ± 6.51 , $n = 76$, $p = 4.82 \times 10^{-10}$), although again only the theoretical $tMICE+$ differences were statistically significant.

4. DISCUSSION

Confidence in known metabolite assignment is one of the key issues facing metabolomics/metabonomics at present. Many

current studies do not report in detail their MS- or NMR-based spectroscopic analyses, let alone any assessment of the confidence ratings for the identification of important metabolites.

The Metabolomics Standards Initiative (MSI)³⁵ recognized this as an important issue as far back as 2007 and proposed a set of guidelines,³⁶ but few studies currently refer to these,³⁹ and improvements/amendments to the guidelines have been suggested recently.^{28,41–45}

There are three key issues with the current MSI guidelines for metabolite identification in our view: (i) they are qualitative and not quantitative, referring to having data such as 2D NMR spectra; (ii) there is no guidance on how good a fit of the experimental data to reference data should be; and (iii) there is an assertion that data in the literature or in databases such as the HMDB are “generally believed insufficient to validate a confident and rigorous identification”.³⁶ The work reported here addresses these issues. First, both the recent MICE and the new $tMICE$ methods presented here are quantitative in basis but simple and easy to calculate. Second, clear guidance is given on the goodness of fit required for experimental NMR data to be seen as a good match for literature or reference standard data. Third, the assumption that data generated from a reference standard of a metabolite in the investigator’s laboratory will be significantly different from that recorded on a different sample of that standard in the literature or in a database such as the HMDB is shown to be generally incorrect for NMR-based metabolomics (Table 1). This may be an issue and a concern for MS-based experiments such as UPLC–MS or LC–MS, where, for example, metabolite retention time could be influenced by a number of factors including the exact column type, history and age, and mass spectral intensities could be influenced by sample- and spectrometer-specific ion suppression and enhancement effects, in addition to differential

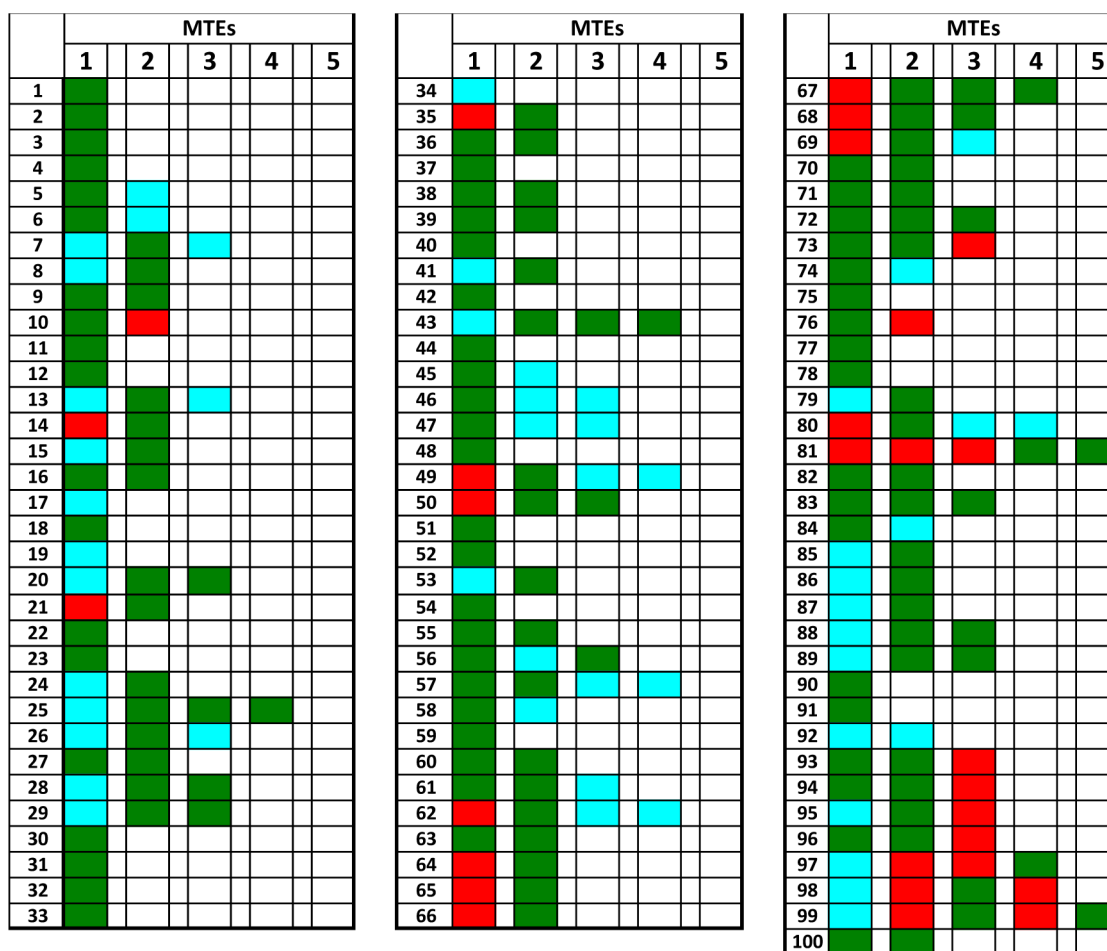


Figure 6. Analysis of the tMICE value for each MTE in each of the 100 metabolites at the HSQC level. Green: actual tMICE value ≥ 1.0 ; red: tMICE actual < 1.0 but tMICE theoretical ≥ 1.0 ; cyan: tMICE theoretical < 1.0 .

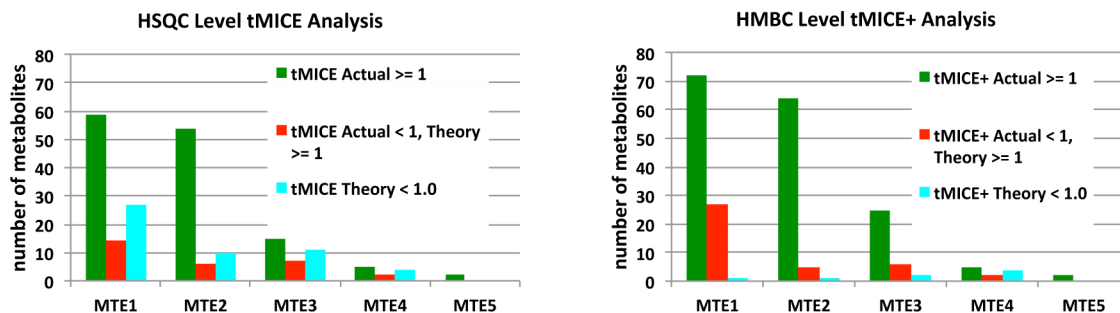


Figure 7. Left: Histogram of the distribution of HSQC-level tMICE values in MTEs: Green: actual tMICE value ≥ 1.0 ; red: tMICE actual < 1.0 but tMICE theoretical ≥ 1.0 ; cyan: tMICE theoretical < 1.0 . Right: Corresponding HMBC-level tMICE+ analyses with analogous color coding.

640 varied from a low of 0.004 ppm (methyl protons in β -D-fucose)
 641 to a high of 0.031 ppm for the high-frequency methylene proton
 642 in citric acid, with an average difference of 0.017 ± 0.009 ppm
 643 (standard deviation). Our proposal that comparisons of experi-
 644 mental data can be confidently made with reference standard
 645 data from databases such as the HMDB rather than generat-
 646 ing new NMR data on an authentic reference standard on the
 647 same spectrometer seems valid. In addition, the guideline of
 648 matching proton chemical shifts between experimental data
 649 and database or literature values of ± 0.03 ppm also seems
 650 reasonable.

651 The group of 100 metabolites studied here is typical of
 652 those identified by NMR spectroscopy in metabolomics studies.

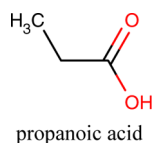
653 These 100 metabolites have a molecular weight distribution as
 654 follows: 138.0 ± 54.5 Da in this set versus 126.7 ± 46.6 Da for the
 655 set of 75 subjected to MICE analysis.⁴⁵ Correspondingly, the
 656 number of carbon atoms in the metabolites in this set is 5.5 ± 2.4
 657 versus 4.9 ± 2.2 atoms previously (all figures means \pm standard
 658 deviations). These changes are due to the fact that further work
 659 had identified metabolites that were less obvious in the year since
 660 the previous analysis was completed. These less obvious meta-
 661 bolites generally had larger and more complex structures with
 662 increased molecular weight.

663 The topological approach to metabolite identification intro-
 664 duced here is a natural approach for NMR spectroscopy. Net-
 665 works of proton-to-proton connectivity that are discovered by

666 methods such as 2D ^1H COSY NMR are frequently broken or
 667 interrupted by so-called “spectroscopically silent centers” such as
 668 heteroatoms bearing no slow-exchanging protons or quaternary
 669 carbons. The topological analysis defines these “spectroscopi-
 670 cally silent centers” as two of the four types of break between
 671 the MTEs in the structure of the metabolite, with the other two
 672 breaks between MTEs being ring junctions and branching methine
 673 carbons. Thus, in this analysis, there tends to be a natural align-
 674 ment between the topology elements in the metabolites and
 675 subnetworks of proton–proton connectivity derived from the
 676 NMR spectra.

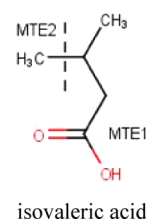
677 The basic tMICE approach measures the number of bits of
 678 MII derived from NMR spectra from each MTE at a level up to
 679 2D ^1H , ^{13}C HSQC, that is, information gathered from 1D
 680 ^1H NMR, 2D ^1H J-resolved NMR, 2D ^1H COSY NMR, and 2D
 681 ^1H , ^{13}C HSQC experiments. These bits of information include
 682 the following: (1) the number of proton chemical shifts for
 683 each protonated carbon and nonexchanging amide NH; (2) the
 684 number of signal multiplicities identified (usually from 1D
 685 ^1H NMR or 2D ^1H J-resolved NMR); (3) the number of
 686 coupling constants measured ($^2J_{\text{HH}}$ and $^3J_{\text{HH}}$) for each signal
 687 (note that this is different from the original MICE methodology
 688 where $^nJ_{\text{HH}}$ values were only counted once: this was seen as
 689 too conservative); (4) the presence of second-order spin system
 690 (flag = 1 if there are additional lines present in the spectrum, not
 691 anticipated by a first-order spectral analysis, otherwise flag = 0;
 692 the bit is only counted if it is set to 1); (5) the number of intra-
 693 MTE COSY links via $^2J_{\text{HH}}$ and $^3J_{\text{HH}}$ between hydrogens (only
 694 counted once) and the number of inter-MTE COSY links via
 695 $^3J_{\text{HH}}$ between hydrogens (only counted once and associated with
 696 the first MTE connected); and (6) the number of HSQC cross-
 697 peaks for each protonated carbon atom (counted twice if mea-
 698 sured separately at nonequivalent hydrogens of a methylene
 699 group). The sum total number of bits of information for each
 700 MTE is then divided by the number of carbon atoms in that MTE
 701 to give the tMICE value.

702 For example, simple metabolites like propanoic acid just have
 703 one chain-class MTE from $\text{C1}=\text{O}$ to C3H_3 .



704 In this case, a total of seven bits of MII were experimentally
 705 observed (see Figure 5): two ^1H chemical shifts (1.061, C3H_3
 706 and 2.190 ppm, C2H_2), two ^1H signal multiplicities (triplet
 707 and quartet), two $^3J_{\text{HH}}$ coupling constants (7.7 and 7.7 Hz),
 708 and a COSY between the signals for the methyl and methylene
 709 groups, all matching the values given for the authentic meta-
 710 bolite HMDB00237 to within ± 0.03 ppm and ± 0.2 Hz for
 711 ^1H chemical shifts and coupling constants, respectively
 712 (1.04 and 2.17 ppm, 7.7 Hz, all for a 10 mM sample at pH
 713 7.0 in H_2O referenced to DSS, accessed from HMDB on
 714 April 25, 2016).³⁷ With three carbons in the single MTE for
 715 propanoic acid, this gives it a tMICE value of $7/3 = 2.3$. Theo-
 716 retically, at an HSQC level, two ^{13}C chemical shifts could have
 717 been observed, but these were below the sensitivity of our
 718 experiment and were not observed. The theoretical tMICE
 719 value is $9/3 = 3.0$.

720 Slightly more complex metabolites like isovaleric acid were
 721 characterized as follows.



In MTE1 (four carbons) of isovaleric acid, a total of eight
 722 bits of information were experimentally obtained at an HSQC
 723 level, including two ^1H shifts, two multiplicities, two coupling
 724 constants, one ^{13}C shift, and one COSY connectivity (0.916, d,
 725 6.6 Hz, 24.8 ppm with COSY to 1.958, multiplet and 2.062, d,
 726 7.4 Hz), matching that of the authentic metabolite HMDB00718
 727 (0.90, d, 6.6 Hz, 24.7; 1.94, triplet of septet (HMDB erroneously
 728 has doublet of quartet), 7.9, 6.6 Hz and 2.045, d, 7.5 Hz (HMDB
 729 erroneously gives 0.5 Hz?), all for pH 7.0 in H_2O referenced to
 730 DSS (n.b. the raw free induction decay data files associated with
 731 this entry in the HMDB are for another unrelated metabolite)
 732 accessed on April 25, 2016).³⁷ Note that the signal for the methine
 733 proton H3 is a very weak 1-proton triplet of septets and was only
 734 observed indirectly in the COSY and is therefore not included in
 735 the chemical shift or multiplicity counts. For MTE2, the symmet-
 736 rically equivalent methyl group, no information was obtained
 737 and, at an HSQC level, no MII can be obtained because of equiv-
 738 alence. The tMICE values are thus 2.0 (8/4) and 0 for MTE1 and
 739 MTE2, respectively. The errors found in the HMDB data analysis
 740 do highlight the importance of checking database entries for the
 741 quality of the sample, the spectrum, and the data analysis.

The analysis of isovaleric acid highlights the importance of the
 743 topological approach to known metabolite identification con-
 744 fidence. While the main portion of the metabolite is well iden-
 745 tified, there is no information available on the second methyl
 746 group in MTE2 due to molecular symmetry and equivalence.
 747 The HSQC-level tMICE information was analyzed separately
 748 for different overall classes of metabolite. tMICE values for meta-
 749 bolites with some degree of symmetry (actual 1.26 ± 1.30 and
 750 theoretical 1.87 ± 1.72 , $n = 79$) were statistically significantly
 751 lower than those for unsymmetrical metabolites (actual $1.97 \pm$
 752 1.94 , $p = 0.0015$ and theoretical 3.88 ± 3.53 , $n = 137$, $p = 6.45 \times$
 753 10^{-8}). It is natural that the tMICE values of the metabolites with
 754 some element of symmetry are lower than those of metabolites
 755 without symmetry, as in the metabolites with symmetry, there
 756 will be elements of the structure for which it will not be possible
 757 to obtain independent NMR data. For example, in dimethyl-
 758 amine, the two methyl groups are chemically equivalent by
 759 symmetry and at an HSQC level it is not possible to get infor-
 760 mation separately from these MTEs. Even though both methyl
 761 groups contribute to the singlet signal at 2.720 ppm, the infor-
 762 mation is ascribed solely to MTE1, and the second methyl group
 763 in MTE2 is allocated no bits of MII. This is a conservative approach
 764 and reflects what is actually observed.

Similarly, tMICE values for achiral metabolites (actual $1.52 \pm$
 766 1.42 and theoretical 2.08 ± 1.54 , $n = 140$) were lower than those
 767 for chiral metabolites (actual 2.04 ± 2.23 , $p = 0.067$ and theo-
 768 retical 5.11 ± 4.24 , $n = 76$, $p = 4.14 \times 10^{-8}$), although only the
 769 theoretical tMICE differences were statistically significant; the
 770 actual tMICE differences are just insignificant. This effect is
 771 due to the chiral center causing nonequivalence of the geminal
 772 protons on the methylene carbons in chiral metabolites. In chiral
 773 metabolites, each geminal proton typically resonates at a distinct
 774 frequency, thus doubling the amount of information available
 775 from, and to, these groups.

777 The key factor in the identification of known metabolites is
778 whether sufficient information has been collected to be confident
779 in the identification. The tMICE approach enables this analysis
780 to be done quantitatively for each separate topology element in
781 the metabolite, but the question is still, how much information is
782 enough? Following on from the original MICE work where
783 an overall value of MICE ≥ 1.0 was determined to be generally
784 sufficient to confidently identify a metabolite,⁴⁵ we chose to have
785 a tMICE value of ≥ 1.0 also as the cutoff between confidently and
786 not confidently identified MTEs.

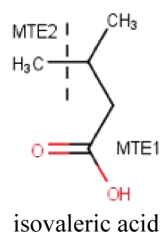
787 We then triaged the 100 metabolites into those where all of
788 their MTEs were confidently identified (tMICE ≥ 1.0 , green), as
789 opposed to those metabolites that contained MTEs where
790 the experimental tMICE values were < 1.0 when the theoretical
791 tMICE values were ≥ 1.0 (red) or those metabolites that con-
792 tained MTEs where the theoretical tMICE value was < 1.0 (cyan
793 in Figures 6 and 7).

794 At a metabolite level, as opposed to an MTE level, only 42
795 metabolites had all of their MTEs green (tMICE ≥ 1.0 , confident
796 assignment); 24 had at least one red MTE (actual tMICE < 1.0 ,
797 whereas theoretical ≥ 1.0), and a further 34 had at least one cyan
798 MTE (theoretical tMICE < 1.0) with no red MTEs. Thus, 58 of
799 the 100 metabolites had at least 1 MTE with insufficient infor-
800 mation for confident assignment (Figure 7, all analyses for HSQC-
801 level data).

802 Given that nearly a quarter of MTEs had theoretical tMICE
803 values of < 1.0 (cyan in Figure 6) and that nearly 60% of the
804 metabolites as a whole had at least 1 MTE without sufficient
805 information for confident assignment, the analysis was repeated
806 at the HMBC level to generate the corresponding theoretical
807 and experimental tMICE+ values for each MTE for comparison.
808 The only difference between the HSQC-level tMICE value and
809 the HMBC-level tMICE+ value for the same MTE is that the
810 number of HMBC connectivities observed for that MTE is added
811 to the HSQC-level MII before dividing by the number of carbon
812 atoms in the MTE. 2D ^1H , ^{13}C HMBC data are very important in
813 metabolite identification as it allows additional carbon-13 to
814 hydrogen connectivities to be determined over two or three
815 bonds via $^2\text{J}_{\text{CH}}$ and $^3\text{J}_{\text{CH}}$. HMBC data allows: (1) connectivities to
816 quaternary carbons to be observed, which are otherwise generally
817 invisible, and (2) via $^3\text{J}_{\text{CH}}$, connectivities through heteroatoms
818 and quaternary carbons can be observed. These new HMBC data
819 enable: (1) the acquisition of MII for MTEs which have signifi-
820 cant numbers of quaternary carbons, (2) the linking of MTEs
821 to one another, which are separated by “spectroscopically silent
822 centers”, and (3) the acquisition of information on symmetrically
823 equivalent MTEs or parts of an MTE due to isotopomeric
824 breaking of MTE symmetry.

825 Similar differences to those observed for tMICE values were
826 seen between the tMICE+ values of both symmetric and non-
827 symmetric metabolites and between achiral and chiral metabo-
828 lites (see Results).

829 It is important to note that for metabolites possessing some
830 degree of symmetry, such as isovaleric acid,



831 while no information could be obtained directly on the presence
832 of the second methyl group in MTE2, due to molecular
833 symmetry in HSQC-level experiments, in a 2D ^1H , ^{13}C HMBC
834 experiment, connectivities can be seen between one methyl
835 group and the other via the isotopomer $\text{H}_3^{13}\text{C}-\text{CH}-^{12}\text{CH}_3$,
836 with observation at the $-^{12}\text{CH}_3$ group. The presence of the
837 H_3^{13}C -isotope in one methyl group only (statistically very
838 unlikely to observe ^{13}C isotopes in both methyl groups) means
839 that the two methyl groups are no longer symmetrically equiv-
840 alent and a true, cross-methyl HMBC connectivity is seen. Thus,
841 this is a third important reason for the use of HMBC data in
842 metabolite identification experiments.

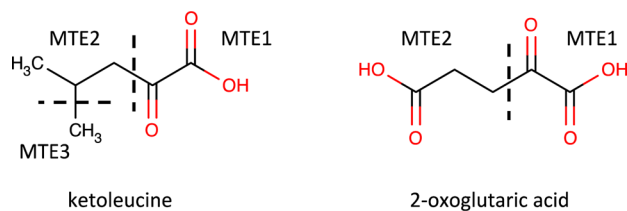
843 The difference between the tMICE analysis at HSQC level and
844 the tMICE+ analysis at HMBC level is illustrated in Figure 7.

845 In the HSQC-level tMICE analysis, 135 MTEs (62%) have
846 actual tMICE values ≥ 1.0 (green); 29 MTEs (13%) have actual
847 tMICE values < 1.0 but theoretical tMICE values ≥ 1.0 (red);
848 and 52 MTEs (24%) have theoretical tMICE values < 1.0 (cyan).
849 By contrast, in the tMICE+ analysis at HMBC level, 168 MTEs
850 (77%) have actual tMICE+ values ≥ 1.0 ; 40 MTEs (19%) have
851 actual tMICE+ values < 1.0 but theoretical tMICE values ≥ 1.0 ;
852 and only 8 MTEs (4%) have theoretical tMICE+ values < 1.0 .

853 At an HMBC level, 61 metabolites now have all MTEs with
854 sufficient identification information for confident assignment
855 (green, cf. 42 in tMICE level), but 34 still have at least one MTE
856 with an actual tMICE+ < 1.0 , while the theoretical tMICE+ ≥ 1.0
857 (red, cf. 24 in tMICE analysis) and only 5 now have at least
858 1 MTE with a theoretical tMICE+ of < 1.0 (cyan, cf. 34 in tMICE
859 analysis), with no red MTEs.

860 It is important to address the issue of MTEs that do have
861 tMICE+ < 1.0 . In cases where the actual tMICE+ value is < 1.0
862 but the theoretical value is ≥ 1 , it is possible to look for the
863 missing information in other samples for confirmation. In cases
864 where the theoretical tMICE+ is < 1.0 , this is not possible and
865 other approaches will be needed. We illustrate these approaches
866 now with some examples from this work.

867 Ketoleucine and 2-oxoglutaric acid are examples where even
868 though the theoretical tMICE+ was > 1.0 the actual HMBC-level
869 tMICE+ values in both cases were < 1.0 , and in fact no infor-
870 mation was obtained from MTE1 at an HMBC level in either
871 case. This is because, the first MTE in both cases is a carboxylate-
872 keto group with two quaternary carbons joined together, resulting
873 in no information at the HSQC level and a difficulty in obtaining
874 HMBC-level data for samples in which metabolite concentrations
875 are low.



876 In these cases, we were fortunate to have access to female
877 C57BL/6 urine samples containing higher concentrations of the
878 metabolites (the original samples were all from male mice). For
879 ketoleucine, female C57BL/6 mice have 2.619 (d, 7.1 Hz, CH_2),
880 51.1 with COSY to 2.10 (CH), TOCSY to 0.944 (CH_3), and
881 HMBC to 24.2 and 210.8 (ketone carbonyl), thus linking MTE1
882 to MTE2 and providing information on MTE1. In addition, they
883 have 0.941 (d, 6.8 Hz, CH_3), 24.5 with COSY to 2.103, TOCSY
884 to 2.614, and HMBC to 50.7 and 24.0 (low digital resolution in

t1, so shift imprecise; cross-methyl connectivity), thus linking MTE 2 to the symmetrically equivalent group in MTE3 and confirming the structure. For 2-oxoglutaric acid, female C57BL/6 mice have 2.448 (t, 6.8 Hz), 33.4 with COSY to 3.013 (t, 6.9 Hz), 38.4 and HMBC from 2.444 to 184.2 and 207.6. The HMBC link from the methylene to the ketone carbon at 207.6 proves the linking of MTE1 to MTE2 and confirms the identity of the metabolite.

Trimethylamine and trimethylamine-*N*-oxide are examples of metabolites where, even at an HMBC level, it is impossible to obtain any identification information on the third MTE, which is a symmetrically equivalent methyl group. HMBC data provide information that there is a symmetrically equivalent methyl group from the cross-methyl HMBC peaks, but it cannot define that there are two of these. In these cases, the situation is made more difficult by the fact that there is so little information available: The signals of both these metabolites comprise just one singlet. Their identification is considered safe but not definitive on the basis of spectroscopic signal density and signal characteristics arguments, as follows. The information density in the ^1H NMR spectrum of a biofluid such as urine varies with the chemical shift and is not at a maximum in the region of the signals from trimethylamine and trimethylamine-*N*-oxide. In addition, the sharp singlet signals from these metabolites are relatively characteristic. Nevertheless, to be prudent and avoid errors, it would be recommended to confirm the identities of these metabolites by a complementary technology such as MS if they were determined to be statistically significant biomarkers in a study. From the NMR spectroscopy perspective, they are considered Putatively Annotated, Level 2 in the MSI notation. It is possible that in other cases additional information could be obtained from long-range, proton–proton coupling constants, as $^4J_{\text{HH}}$, $^5J_{\text{HH}}$

and even $^6J_{\text{HH}}$ couplings can be either observed directly or inferred from COSY experiments in biofluid NMR spectra.²⁵ In addition, information from heteronuclear couplings such as $^2J_{\text{NH}}$ due to ^{14}N isotopes in highly symmetrical environments²⁵ or $^3J_{\text{PH}}$ and $^4J_{\text{PH}}$ due to the presence of ^{31}P in nucleotides may also provide additional confirmatory information.

Other approaches to the analysis are possible. One alternative topological measure of metabolite identification confidence at the HSQC level would be the topological metabolite identification nitrogen and carbon efficiency index: tMINCE, which is equivalent to tMICE but where the number of bits of MII for an MTE, is divided by the total number of nitrogens with non-exchanging protons (NHs) plus the number of carbon atoms in the MTE. The analogous tMINCE+ index could be used for HMBC-level information. All of the information required to calculate this is provided in [Supplementary Table S2](#). In this group of 100 metabolites, only 17 MTEs and 16 metabolites had nitrogens with slow-exchanging hydrogens attached (urea has two such MTEs). The tMINCE or tMINCE+ approaches are, in principle, more conservative than the tMICE or tMICE+ approaches due to the denominator in the equation being at least as large or larger, but in practice not one single MTE would have changed classification, that is, actual or theoretical tMINCE or tMINCE+ < 1.0 when tMICE or tMICE+ \geq 1.0, respectively.

5. CONCLUSIONS AND FUTURE WORK

The new topological approach to metabolite identification confidence (tMICE) presented here is an improvement upon the original metabolite identification carbon efficiency (MICE) method, as it objectively monitors whether MII covers all relevant parts of the metabolite's structure. It is clear when reviewing this more stringent approach to metabolite identification confidence

Table 3. Glossary

no.	term	explanation
1	HSQC	2D, heteronuclear single quantum coherence spectroscopy, allowing correlations between protons and directly attached ^{13}C nuclei to be elucidated (2D ^1H , ^{13}C HSQC)
2	HMBC	2D heteronuclear multiple bond correlation spectroscopy, allowing correlations between protons and ^{13}C nuclei two to three bonds away to be elucidated (2D ^1H , ^{13}C HMBC)
3	MTE	molecular topology element: a chain or ring in a metabolite that forms part or all of the structure
4	MII	metabolite identification information
5	MICE	metabolite identification carbon efficiency ⁴⁵ <ul style="list-style-type: none"> • can be defined at a number of levels • at HSQC level, equals sum total of information bits from proton chemical shifts, multiplicities, coupling constants, second-order flag, COSY connectivities, and HSQC connectivities divided by the total number of carbon atoms in the metabolite, that is, equals MII at HSQC level divided by the number of carbon atoms in the metabolite
6	MINCE	metabolite identification nitrogen and carbon efficiency <ul style="list-style-type: none"> • can be defined at a number of levels • at HSQC level, equals sum total of information bits from proton chemical shifts, multiplicities, coupling constants, second-order flag, COSY connectivities, and HSQC connectivities, divided by the total number of carbon atoms and nonexchanging NHs in the metabolite
7	tMICE	topological MICE <ul style="list-style-type: none"> • can be defined at a number of levels • at HSQC level, equals sum total of information bits from proton chemical shifts, multiplicities, coupling constants, second-order flag, intra- and inter-MTE COSY connectivities, and HSQC connectivities, that is, the MII, divided by the total number of carbon atoms in the MTE
8	tMINCE	topological MINCE <ul style="list-style-type: none"> • can be defined at a number of levels • at HSQC level, equals sum total of information bits from proton chemical shifts, multiplicities, coupling constants, second-order flags, intra- and inter-MTE COSY connectivities, and HSQC connectivities, divided by the total number of carbon atoms and nonexchanging NHs in the MTE
9	tMICE+	topological MICE+ <ul style="list-style-type: none"> • equivalent to tMICE at HMBC level, and equals sum total of information bits from proton chemical shifts, multiplicities, coupling constants, second-order flag, intra- and inter-MTE COSY connectivities, HSQC connectivities, and HMBC connectivities, divided by the total number of carbon atoms in the molecular topology element (MTE)
10	tMINCE+	topological MINCE+ <ul style="list-style-type: none"> • equivalent measure to tMINCE at HMBC level and equal to the total amount of information divided by the total number of carbon and nonexchanging NHs in the MTE

947 that, in many cases, HMBC-level information is important for
948 improved confidence. This is especially the case for metabolites
949 that (i) possess MTEs that are bounded by, or composed largely
950 of, quaternary carbons or heteroatoms without slow-exchanging
951 hydrogens, or (ii) possess MTEs that are rendered wholly or
952 partly “HSQC-invisible” due to some form of symmetry, which
953 can be broken by observation of long-range connectivities from
954 an asymmetric ^{13}C isotopomer of the metabolite in an HMBC
955 experiment.

956 The tMICE and tMICE+ methodology systematizes NMR-
957 based known metabolite identification by (i) taking a coherent
958 topological approach, ensuring that each element of a meta-
959 bolite’s structure is considered in the analysis, (ii) using a simple
960 quantitative measure of the number of bits of MII available in
961 each MTE, and (iii) expressing that amount of MII in ratio to the
962 number of carbon atoms in each MTE: $\text{tMICE} = (\text{no. of MII bits})/\text{number of carbon atoms in the MTE}$.

964 In the future, automated topological analysis of all possible
965 metabolite structures would be advantageous, and this should be
966 readily computed, as the rules for topology definition are quite
967 deterministic, while aligned with the needs of NMR-based meta-
968 bolite identification. In addition, the automated analysis of the
969 theoretical tMICE and tMICE+ scores for each MTE for each
970 metabolite should be readily computable. Metabolite identi-
971 fication would also be aided by precise and accurate NMR pre-
972 diction programs, as it would then be possible to identify many
973 metabolites, known and unknown by comparison with computed
974 as opposed to experimental chemical shifts and other parameters,
975 as experimental values are often missing.

976 The analysis of tMICE indices is recommended for all key
977 biomarkers discovered in untargeted NMR-based metabonomics
978 studies to give a measure of confidence in any biological con-
979 clusions drawn from the identification of these biomarkers.
980 We see no reason to apply the methodology to all of the known
981 metabolites identified and would advocate an approach that is as
982 simple as possible to implement and use, consistent with rigor.

983 Known metabolite identification is a significant issue for both
984 MS- and NMR-based metabolic profiling experiments. We hope
985 that the tMICE and tMICE+ approaches and variants will find
986 utility in the field. We expect that other researchers will seek to
987 improve on these initial proposals and also develop similar
988 approaches for MS-based metabolic profiling.

989 ■ ASSOCIATED CONTENT

990 ● Supporting Information

991 The Supporting Information is available free of charge on the ACS
992 Publications website at DOI: [10.1021/acs.jproteome.6b00631](https://doi.org/10.1021/acs.jproteome.6b00631).

993 Table S1. Metabolite molecular structure information deter-
994 mined by different spectroscopic techniques. Table S2.
995 Metabolite names and properties and experimental and
996 theoretical tMICE and tMICE+ analyses. Table S3.
997 Analysis of molecular topology elements and associated
998 metabolite identification information, both experimental
999 and theoretical. (PDF)

1000 ■ AUTHOR INFORMATION

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1003 Notes

1004 The authors declare no competing financial interest.

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