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Electron Ionization–Induced Release of Coded Isotopic Reporter Ions in an m/z Zone of Minimal Interference for Quantifiable, Multiplexed GC-MS Analyses

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Abstract

We describe an isotope coding strategy that enables simultaneous GC-MS analysis of multiple samples for substrate identification and quantification. The method relies on direct measurement of isotopic ethyl carbenium ions serving as mass spectral tags in a zone of minimal interference (ZMI) at m/z 31–37. Sample aldehyde and ketone mixtures were reacted with isotopic 2-aminooxyethyl propionates to illustrate the method, which determined the relative abundance of the mixed compounds with an average 95% accuracy. ZMI reporter ion detection also enables chemoselective substrate profiling and absolute quantification, as demonstrated using a biologically derived sample.

Introduction

The quest to obtain chemical composition ‘snap-shots’ of complex biological systems drives much research in the fields of genomics, proteomics and metabolomics. In particular, the search for biomarkers of disease in biological samples, such as blood, urine or tissue, is a common goal among these disciplines and has led to numerous advances in analytical technologies.¹ Profiling endogenous and exogenous small molecules with molecular weights below 1500 Da² is especially challenging since such collections of ‘metabolites’ typically contain diverse chemical structures and functional groups that make analyte extraction, enrichment, separation, and analyses difficult.³ Mass spectrometry⁴ (MS) and its tandem combinations with principal separation techniques, such as liquid chromatography⁵ (LC-MS) and gas chromatography⁶ (GC-MS), currently are among the most applied analytical tools for metabolomic profiling.⁷

Stable isotope coding is a strategy that has been used to streamline LC-MS analyses in metabolomics.⁸ Analyte samples containing common functional groups are first derivatized with differentially coded isotope reagents and then the derivatized mixtures are combined and analyzed simultaneously by LC-MS. The relative concentrations of the coded analyte adducts are provided by an isotope ratio analysis of the isotopic molecular ions (M^+) obtained on electrospray ionization. Isotopic derivatization reagents have been applied in this way for the quantification of carboxylic acid,^{8a,d} amine^{8b,c} and fatty acid^{8e} metabolites. In contrast, stable isotope coding strategies for GC-MS analyses are scarce.⁹ The ionization method employed in a GC-MS system is electron ionization (EI), in which the electron

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energy (~70 eV) is so high that metabolite adducts are directly fragmented to yield mass spectra that are considerably more difficult to quantify. Given that GC-MS and comprehensive two-dimensional GCxGC-MS are the principal analytical platforms for metabolomics due to their superior chromatographic resolution and reproducibility,² and that EI enables the use of searchable mass spectra libraries,¹⁰ an isotopic coding strategy for GC-MS that addresses the aforementioned quantification challenge is very much needed.

We present here the first isotope coding strategy that features EI-induced expulsion of a reporter tag for quantification using GC-MS (Figure 1). Specifically, we have developed a panel of isotopologous aminooxyethyl propionate (AEP) reagents for chemoselective derivatization of aldehydes and ketones. The reagents are fitted with ²H- and ¹³C-isotope labels such that EI-induced fragmentation of the AEP-adducts results in identifiable reporter mass spectral tags (MSTs) for relative quantification of individual members of a multiplex set, as depicted in Figure 1. Derivatization of carbonyl substrates using the AEP isotopologues (Figure 1, step A) followed by pooling the samples (step B) generates a multiplex set for a single-injection analysis by GC-MS. If the isotope labeling does not influence GC elution of the pooled adducts, then identical substrates that have been labeled with different isotope tags will co-elute on GC separation (step C). Subsequent electron ionization of the eluted substrates induces fragmentation (step D) to yield characteristic fragment ions for substrate identification as well as release of coded isotopic tag ions for quantification. Although previously published GC-MS isotope coding approaches have used isotopically labeled derivatizing agents,⁹ none of these approaches exploit a common reporter tag that enables rapid quantification by direct mass spectral measurements. Furthermore, earlier coding strategies have relied on binary sets of reagents, and this limitation as well as noted chromatographic isotope effects leading to partial GC resolution of derivatized substrates impede high throughput. A multiplex strategy that overcomes chromatographic separation of isotopically derivatized substrates would enable multiple samples to be labeled, mixed and analyzed simultaneously to increase throughput and reduce technical variation.

Reagent Design and Synthesis

An important consideration to actuate such a coding strategy is selection of an unambiguous reporter MST, one that can be observed free from obfuscation by other fragment ions. Deconvolution approaches that align and match derived fragment ions to resolve MSTs using pre-processing software for MS identification and quantification have been shown to be error prone.¹¹ Ideally, direct ion count comparisons of MST isotopologues would be most practical. To select a mass range m/z most likely to be free from interfering fragment ions, we consulted a NIST compilation¹² of the EI mass spectra for 163,198 organic compounds (Figure 2). Tabulation of the incidence for every m/z signal in the library of compounds reveals several gaps where the frequency of a signal is minimal (Figure 2A). Whereas the gap between m/z 19–24 (see Figure 2A insert) is sparsely populated, this m/z range is too low for synthetic application. However, the next zone of minimal interference (ZMI) that is suitable for engineering an MST based on ¹³C and ²H isotopes appears at mass range m/z 31–37. Importantly, the intensities of any fragment ions that register within this ZMI are quite low as indicated by the summed ion count for each m/z (Figure 2B). Consequently, we have designed a derivatization reagent to generate isotopic MSTs in the mass range m/z 31–37 for distinct observation.

Preparation of the AEP reagent panel followed from two principal considerations: (1) the target ZMI suggested modification of an ethyl carbenium ion, C₂H₅⁺ m/z 29, to its ²H₃-analog, D₃CCH₂⁺ m/z 32; and (2) our prior experience with aminooxy reagents for chemoselective labeling¹³ of carbonyl metabolites led us to pursue oximation as a

representative mode of substrate derivatization. Finally, we selected EI-induced ester-cleavage¹⁴ as the mode for tag expulsion. Thus, electron ionization of AEP-derived oxime ether adducts **1** (Scheme 1) gives rise to acylium ion **[2]** as well as characteristic substrate-specific fragment ions. Spontaneous loss of CO from **[2]** generates the target m/z 32 carbenium ion MST **[3]**. Corresponding ¹³C-isotope insertions at the - or -, -positions of the AEP ester moiety yield isotopic MSTs of **[3]** having m/z 33 and 34, respectively.

Synthesis of the AEP reagents proceeded by carbodimide-mediated acylation of *N*-(2-hydroxyethoxy)phthalimide (**4**) with ²H₃-labeled propionic acid and corresponding ¹³C₁- and ¹³C₂-isomers to give esters **5a-c** in good yield (Scheme 2). Hydrazinolyses of **5a-c** afforded the labeled AEP panel having embedded isotope tags for observation within the ZMI at m/z 32–34.

Proof of Concept

To validate the concept that release of isotopic carbenium ions on GC-MS analysis of AEP-derived oxime ether adducts can be used to determine relative proportions of carbonyl substrates in different sample mixtures, we prepared three model carbonyl mixtures with known substrate ratios (Scheme 3). In example, mixtures A, B, and C were formulated to contain equimolar quantities of hexanal whereas the quantity of 2-heptanone in mixture B was tripled relative to 2-heptanone in mixtures A and C (Scheme 3, entries 1–2). Each mixture was derivatized with a single AEP isotopologue by simple mixing in dichloromethane at room temperature, and the resultant AEP-oxime ether mixtures A₃₂, B₃₃, and C₃₄ then were combined to yield the pooled sample that was analyzed by GC-MS.

Whereas the *Z*- and *E*-diastereomers derived from the unsymmetrical substrates gave separate GC peaks, we were gratified to find that no GC separation occurred based on carbon isotope incorporation (Scheme 3). GC separation of derivatives based on deuterium incorporation previously has been observed^{9a,15} and constitutes a major limitation for effective multiplexing. The use of carbon isotopes, however, appears to overcome the chromatographic isotope effect. MS analysis of the eluted compounds revealed each derivatized carbonyl substrate generated characteristic fragment ions that enabled its structural assignment and designation of either *E*- or *Z*-oxime ether stereochemistry.¹⁶ The isotopic MSTs at m/z 32–34 were abundant and clearly measurable in the ZMI of each spectrum. Relative ratios for the carbonyl substrates that gave diastereomeric oxime ethers were determined readily using MST measurements from either the *Z*- or *E*-isomer. Importantly, direct MST ion count comparisons gave substrate ratios that were in close agreement with the actual formulation ratios of the starting compound mixtures (Figure 3). The average accuracy of the method obtained by comparison of the MST-derived ratio to the known carbonyl substrate compositions in mixtures A–C is 95.1% ± 0.3%.

Analysis of a Biologically Derived Sample

To demonstrate additional attributes of the ZMI tag approach, we examined samples of the root extract of the turmeric plant (*Curcuma longa*) to determine compositions of ketones and aldehydes. The complexity of such an extract¹⁷ makes it ideal for challenging the technology described above. Carbonyl metabolites from plants, in particular aliphatic methyl ketones, have a variety of important natural and commercial roles, including pheromonal and insecticidal roles in plants,¹⁸ as possible sources of biofuels,¹⁹ and providing scents in essential oils as well as flavorings for cheese and other dairy products.²⁰ First, to profile only the carbonyl substrates in the extract, we sought to apply the unique ZMI reporter MSTs as a selection tool to provide an extracted ion chromatogram (in this case, the profile

of plant metabolites that had reacted with the AEP reagents). Second, we aimed to quantify the prominent methyl ketone substrates identified in the root extract.

Carbonyl Profiling

Since AEP-derivatized substrates generate reporter MSTs in such a unique m/z region of a mass spectrum, we theorized that the appearance of these isotopic tag ions might be used to identify AEP-adducts in the total ion chromatogram (TIC), thus providing a rapid means for profiling the carbonyl metabolites. By using two isotopologues of AEP at a specified ratio in the derivatization step, we aimed to minimize the extraction of false-positive peaks from the TIC. Consequently, we derivatized the turmeric extract with both AEP-33 and AEP-34 in a 1:1 ratio. Subsequent profiling of the carbonyl adducts then was achieved by generating an extracted ion chromatogram (EIC) through the selection of peaks that exhibited the reporter MSTs at m/z 33 and 34 in a 1:1 ratio in their accompanying mass spectra (Figure 4).

The GCxGC-TOF-MS total ion chromatogram of the derivatized turmeric extract (Figure 4A) clearly is populated with a multitude of compounds. By applying the ion selection tool for reporter ions at both m/z 33 and 34, an extracted ion chromatogram is obtained (Figure 4B) that profiles the carbonyl substrates (i.e., AEP-reactive). With the profile of carbonyls in hand, we next sought to identify the subset of aliphatic methyl ketones. Given that ketoxime ethers undergo characteristic EI-MS fragmentations (Figure 5),¹⁶ a targeted m/z mass spectral analysis of the EIC revealed the presence of several AEP-methyl ketone adducts (Figure 4C, Table 1). The AEP-adducts were present as both *E*- and *Z*-isomers, and assignment of stereochemistry followed from analysis of the relative abundance of McLafferty fragment ion versus nitrilium ion formation.¹⁶

Each AEP-methyl ketone adduct generated McLafferty fragment ions at m/z 177 and 178 (one from each AEP-adduct isotopologue), and these values thus functioned as a convenient searchable m/z for methyl ketone substrates. The nitrilium ion m/z is substrate specific and provides information on the constitution of the ketone aliphatic chain. These features are illustrated by the representative mass spectra of the *E*- and *Z*-2-nonanone adducts (Figure 6). The structural assignments for 2-nonanone, 2-decanone and 2-undecanone were confirmed by comparison to AEP-derivatized standards (e.g., library spectra), and the remaining suggested methyl ketones, while likely members of the same linear family, may be constitutionally isomeric at positions remote to the carbonyl. It is noteworthy that even when applying powerful separation conditions (i.e., GCxGC, 5 °C gradient) we did not observe chromatographic separation of AEP-adduct isotopologues, as confirmed by the observable 1:1 ratio of the reporter MSTs in the ZMI (Figure 6).

Quantification Using AEP-Labeled Standards

Absolute quantification of the principal methyl ketone constituents in the root extract, 2-nonanone and 2-undecanone, followed from preparation of labeled standards. The standards were derivatized using AEP-34, while the turmeric extract was treated with AEP-33. Known concentrations of the derivatized standards then were added to the derivatized extract and the resulting spiked mixtures were analyzed by GCxGC-TOF-MS. Once again, the direct comparison of the ion intensities for the two reporter MSTs in the ZMI was key and provided the measures for determining the minimal concentrations of both 2-nonanone and 2-undecanone in the turmeric extract at 178 ± 14 $\mu\text{g/mL}$ and 218 ± 24 $\mu\text{g/mL}$, respectively.²¹

Conclusions

In conclusion, we have demonstrated a new method for the quantification of substrates from multiple samples using a multiplexed, single injection GC-MS approach enabled by direct measurement of isotopic mass spectral tags. We illustrated the method using mixtures of sample aldehydes and ketones. To do so, we identified a zone of minimal interference (ZMI) at the m/z range 31–37 and engineered the expulsion of labeled ethyl carbenium ions from derivatized substrates to serve as observable reporter tags within this zone. The unobstructed detection of the ZMI reporter ions also enables rapid profiling and quantification of substrates that have been chemoselectively derivatized, as exemplified by the turmeric extract study. The results here provide a useful guideline for the development of isotopic carbenium ion- or other ZMI ion-producing reagents that can facilitate multiplexed GC-MS analyses. By simultaneously analyzing multiple samples, such an approach can significantly increase the analytical throughput, reduce the experiment costs and, importantly, improve the accuracy of compound identification and quantification. We are currently extending the technology to chemoselectively label other functional groups, such as amines and alcohols, and to analyze complex biological samples for biomarker profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representation of a multiplexed GC-MS analysis using isotope-coded reagents designed for electron-induced expulsion of reporter mass spectral tags (MSTs). The derivatization reagents provide corresponding MSTs for relative quantification of substrates from separate sample mixtures.

Figure 2.

Delineation of the zone of minimal interference; **A**: fragment ion frequency for the compound library (NIST/EPA/NIH NIST 08 Mass Spectral Library), **B**: corresponding summed ion count (peak intensity) for a given m/z .

Figure 3.

ZMI plots obtained from the eluted peaks of the pooled sample mixture of Scheme 3 showing the MST ion abundance (vertical) for the range m/z 32–34. Shown are the mean values of three separate experiments and the standard deviation from the mean. The starting A:B:C substrate ratio is given in brackets. Ion counts are normalized to 100 for the parent fragments (not shown).

Figure 4.

A. GCxGC-TOF-MS total ion chromatogram (TIC) of turmeric root extract after derivatization using AEP reagents; **B.** Extracted ion chromatogram (EIC) for peaks that exhibit MS signals at m/z 33 and 34 (same field of view as in **A** with lower threshold); **C.** Expanded field of view of peaks within the highlighted rectangle of **B** where circled peaks 1–6 are, respectively, the AEP-adducts of 2-nonanone, 2-decanone, 2-undecanone, 2-dodecanone, 2-tridecanone and 2-octadecanone.

Figure 5.
Characteristic EI-MS fragmentations of ketoxime ethers.

Figure 6.

EI-Mass spectra of AEP-2-nonanone adducts: **(A)** *Z*-isomer, **(B)** *E*-isomer. Adducts were obtained by derivatization of turmeric root extract using AEP-33 and AEP-34 in a 1:1 ratio.

Scheme 1.

EI-MS-induced fragmentation of AEP derived oxime ether adducts.

Scheme 2.

Synthesis of AEP reagents. Reagents: (a) $\text{CD}_3\text{CH}_2\text{CO}_2\text{H}$ (to **5a**) or $^{13}\text{CD}_3\text{CH}_2\text{CO}_2\text{H}$ (**5b**) or $^{13}\text{CD}_3^{13}\text{CH}_2\text{CO}_2\text{H}$ (**5c**), DIC (1.5 eq), DMAP (cat.), CH_2Cl_2 , rt; (b) MeNHNH_2 (1.1 eq), CH_2Cl_2 , 0 °C, 45 min.

Scheme 3.

Formulation, labeling and pooling of carbonyl sample mixtures for analysis. Depicted at bottom is the total ion chromatogram of the pooled sample mixture showing elution of the oxime ether adducts of the substrates indicated by name.

Table 1

AEP-methyl ketone adducts extracted from the EIC (Figure 4B). Entry numbers correspond to GCxGC retention properties (Figure 4C).

Entry	AEP-Methyl Ketone Adduct (Carbonyl Parent)	McLafferty Fragment Ions (m/z)	Nitrilium Ion (m/z)	Normalized GC Peak Area
1	2-nonanone	177, 178	140	66.0
2	2-decanone	177, 178	154	6.7
3	2-undecanone	177, 178	168	100
4	2-dodecanone	177, 178	182	9.4
5	2-tridecanone	177, 178	196	5.2
6	2-octadecanone	177, 178	266	20.4