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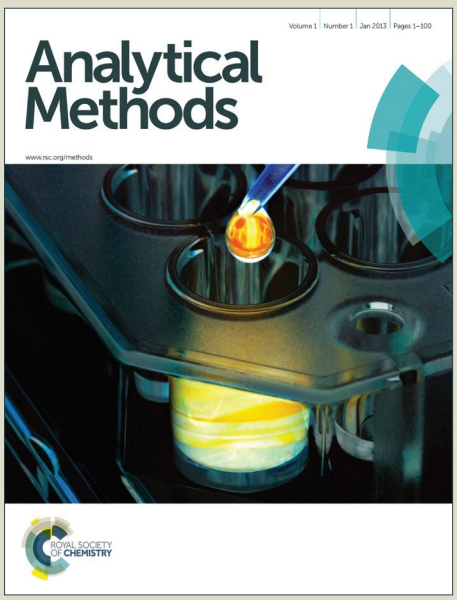

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Isotopically Coded *N*-Methoxy Amide Reagents for GC-MS Profiling of Carbonyl Compounds via Mass Spectral Tag Generation

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Abstract: Synthesis and application of isotopically labeled *N*-methoxy-*N*-(2-aminooxyethyl)-propionate (MAP), a chemoselective carbonyl derivatization reagent, is reported. To exploit the ready measurement of fragments serving as reporter ions in the *m*/*z* 32-34 range, MAP is designed to undergo electron ionization (EI)-induced fragmentation to expel labeled ethyl carbenium ions for relative quantifications in multiplexed analyses. A study of the EI-MS fragmentation behavior of a panel of MAP–carbonyl adducts revealed that the *N*-methoxy amide motif of MAP is highly predisposed to undergo carbonyl alpha cleavage to produce corresponding labeled carbenium ions in the targeted *m*/*z* range. Use of the *N*-methoxy amide functionality decreased undesired (e.g., uninformative) mass spectral fragmentations as well as provided good resistance to cleavage by amines or base relative to ester functionality. These properties should facilitate the use of MAP in multiplexed GC-MS analyses of complex mixtures containing aldehyde and ketone analytes. A representative multiplexed experiment using MAP isotopologues illustrates this approach for quantification of a carbonyl analyte in pooled sample mixtures.

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Introduction

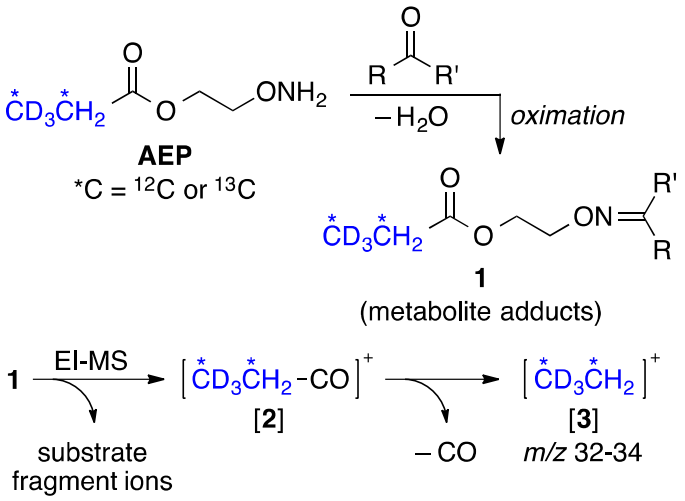
Liquid chromatography–mass spectrometry (LC-MS) is widely used for analysis of complex mixtures since analyte derivatization generally is not required.[1] Gas chromatography–mass spectrometry (GC-MS), on the other hand, often requires analyte derivatization to increase volatility and/or alter ionization character for suitable analysis.[2] The strategy of chemoselective derivatization,[3] wherein compounds selectively react based on the presence of a common functional group, allows for targeted, covalent attachment of isotopic labels to create "light" and "heavy" reagent-compound adducts.[4] Chemoselective derivatization enabled the practice of analyzing multiple samples at a time by LC-MS, or multiplexing (i.e., with a single injection).[4] Consequently, many derivatized metabolites, including carboxylic acids,[5] fatty acids,[6] steroids,[7] amino[8] and non-amino[9] acids, have since been analyzed in multiplexed experiments by LC-MS.

Whereas the use of isotopic labeling for LC-MS analyses has become prevalent in recent years, its application in GC-MS analyses is far less.[10] A recent review by Bruheim *et al.*[10] described only three cases of binary derivatization strategies for analysis by GC-MS. The reagents *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA),[11] *N*-methyl-*N*-(*t*-butyldimethyl-silyl)trifluoroacetamide (MTBSTFA)[12] and methyl chloroformate (MCF)[13] have been used in conjunction with the isotopologue reagents d_9-MSTFA,[11] d_6-MTBSTFA,[12] and d_3-MCF,[13] respectively, for simultaneous analyses of sample mixtures. To date, these limited examples of matched derivatizing agents allowed only for the GC-MS analyses of amino acids, organic acids, fatty acids and metabolites amenable to silyl derivatization. Furthermore, these approaches are binary, allowing only two samples to be analyzed at a time.

The ability to analyze three or more samples at a time has only recently been established and utilized.[14] Progress in developing multiplexed approaches, however, has largely been

confined to the LC-MS platform. For example, recent advances in multiplexed proteomic studies include the use of isobaric tags for relative and absolute quantification (iTRAQ)[15] and mass differential tags for relative and absolute quantification (mTRAQ).[16] Recently, groups have utilized iTRAQ[17] and mTRAQ,[18] and the methodology has since been expanded to analyze up to eight samples simultaneously (OxiTRAQ).[19] A limited number of papers have described LC-MS multiplexing for analysis of lower molecular weight compounds, such as metabolites from biological extracts. In one example, Torde *et al*.[20] described analyses of carboxylic acids and other fatty acids in eggs from caged versus cage-free chickens using cholamine-d_0, -d_3, or –d_9 reagents. Given the benefits of more rapid sample analysis with enhanced analyte detection illustrated by the above-cited works, there is a surprising dearth of multiplexed GC-MS analyses. Extrapolation of multiplexing strategies to this platform may provide a means for rapid and efficient metabolite or biomarker detection. We report herein our efforts to develop and optimize chemoselective derivatization reagents for this purpose.

We recently described an isotope coding strategy to enable multiplexed analysis of carbonyl compounds from multiple samples using GC-MS.[21] Our approach relies on the electron ionization-induced expulsion of isotopically labeled and quantifiable mass spectral reporter ions (Scheme 1). Specifically, treatment of a metabolite mixture with the aminooxy reagent AEP results in the chemoselective derivatization (oximation) of all aldehydes and ketones. The use of aminooxy reagents to label aldehydes and ketones is among the most efficient of chemoselective derivatizations.[22] This click chemistry approach[23] has been widely studied[24] and is more robust than analogous derivatizations that utilize amine-[25] or hydrazine-based[26] reagents. Subsequent GC-MS analysis of the AEP adducts **1** (Scheme 1) results in their ionization and accompanying

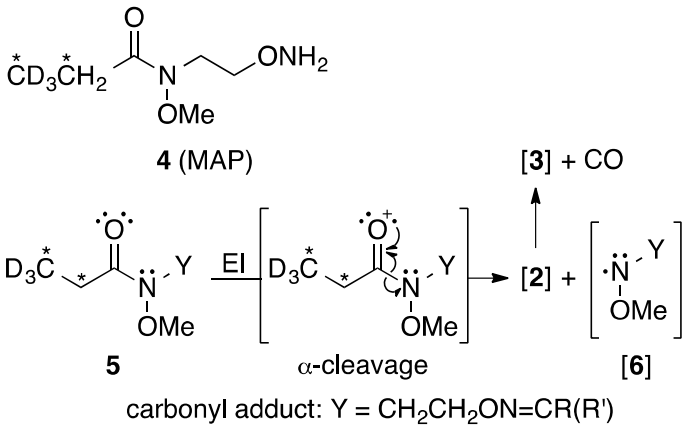


Scheme 1. Formation and EI-induced fragmentation of AEP-derived oxime ether adducts.

fragmentation. One of the principal fragmentation modes for adducts **1** is an ester α-cleavage that gives rise to acylium ion [**2**], which spontaneously loses CO to yield the labeled mass spectral tag [**3**]. The ethyl carbenium ion [**3**] is purposely substituted with deuterium so that its $^{12}C_2$-isotopologue is observed at *m/z* 32. An analysis of the mass range *m/z* 31-37 revealed sparse fragment ion population (unless specifically targeted) and low ion intensities for the few fragment ions that register within this range.[21] Thus, fragments within this mass range, a zone of minimal interference (ZMI), can be cleanly quantified using ion count measurements. In a multiplexing scenario, the $^{12}C_2$-isotopologue adducts **1** can be pooled with the adducts obtained from derivatization of a second and third metabolite mixture, using the $^{13}C_1$- and $^{13}C_2$-isotopologues of AEP, respectively, and then simultaneously analyzed by GC-MS. Importantly, for a given carbonyl analyte, the isotopic AEP-derivatives are chromatographically indistinguishable. Comparison of the unobstructed ZMI mass spectral tag ion counts at *m/z* 32, 33, and 34 then reveals the relative abundance of a given carbonyl metabolite in the three mixtures. In our initial disclosure,[21] we demonstrated the average accuracy of the method to be

95%. One concern, however, for applicability under more forcing conditions is the susceptibility of the ester linkage toward hydrolytic cleavage as well as possible transacylation reactions with nucleophiles, either by the reagent AEP or its adducts **1**. These undesired reactions could have the effect of cleaving or transferring the mass spectral tag onto metabolites not targeted by the aminooxy moiety. Also of interest in a redesign of the AEP reagent would be to enhance the α-cleavage process so as to boost mass spectral tag ([**3**]) detection in the ZMI while minimizing uninformative fragmentations that arise from EI-induced cleavage along the reagent backbone. With these goals in mind, we pursued synthesis and evaluation of an *N*-methoxy amide analog of AEP based on the rationale presented below.

Whereas amides are considerably more stable toward hydrolysis than esters, their α-cleavage fragmentations in EI-MS occur at a significantly lower rate, thus suggesting that conversion of AEP to an amide analog is likely to be counterproductive. However, we postulated that substitution of the amide nitrogen with alkoxy (e.g., amide **4**, Scheme 2) would



Scheme 2. Incorporation of an *N*-methoxy amide group to impart reagent and adduct stability and to promote EI-induced generation of the ZMI reporter ion.

maintain the stability associated with amides yet possibly promote α-cleavage (5 → **6**) due to neighboring oxygen stabilization of the resultant *N*-centered radical species.[27] Consequently, we set out to synthesize *N*-methoxy-*N*-(2-aminooxyethyl)-propionate (MAP; **5**, Y = $CH_2CH_2ONH_2$) and examine the propensity for formation of mass spectral tag [**3**] from derived oxime ether adducts (Y = $CH_2CH_2ON{=}CR(R')$). Although syntheses and applications of *N*-methoxy amides are abundant in the literature (e.g., Weinreb amides[28]), there are few reports on the EI-induced fragmentation of this functionality.

Experimental

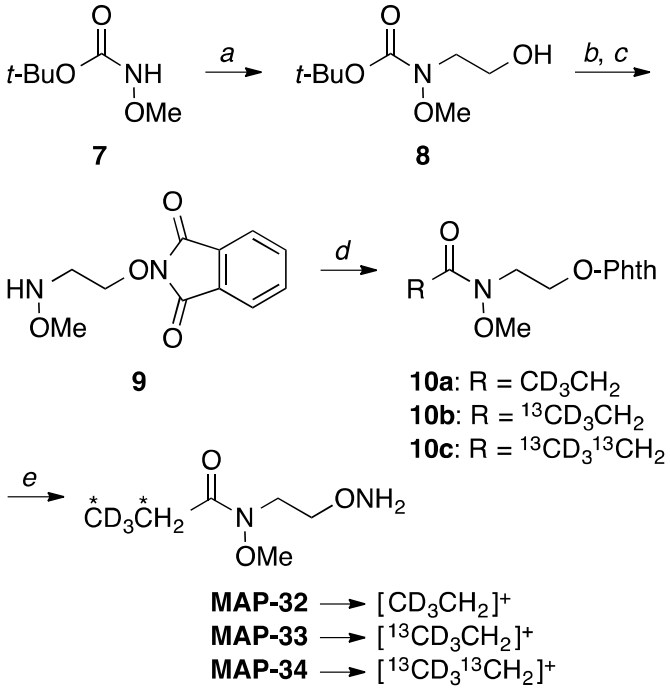
MAP Synthesis

We prepared the MAP isotopologues from Boc-protected methoxyamine[29] (**7**, Scheme 3). *N*-Alkylation using silyl-protected 2-bromoethanol[30] followed by mild acid work-up delivered amide-alcohol **8**. Alcohol to alkoxy phthalimide transformation according to the method of Grochowski and Jurczak[31] followed by Boc deprotection gave platform amine **9** in good yield. At this stage, *N*-acylations of **9** with $^{2}H_3$-labeled propionic acid and corresponding $^{13}C_1$- and $^{13}C_2$-isomers using standard carbodiimide methodology afforded entry to the propionamide isotopologues **10a-c**, respectively. Hydrazinolyses of **10a-c** furnished the labeled MAP panel. Detailed synthetic procedures and spectral characterizations are provided in the Electronic Supplementary Information (ESI).

Sample Derivatization

MAP and AEP adducts were prepared by adding an equimolar mixture of MAP-32 (2 mg, 0.01 mmol) and AEP-32 (1.4 mg, 0.01 mmol) to a slight excess of select aldehydes and ketones (0.02 mmol) in CH_2Cl_2 (1 mL) at room temperature. After stirring 48 h, the solvent and

unreacted carbonyls were removed by rotary evaporation. The isolated oxime ether adducts then were redissolved in CH_2Cl_2 (1 mL) for analysis by GC-MS.



Scheme 3. Synthesis of MAP reagents (Phth = phthaloyl, *C = ^{12}C or ^{13}C). Conditions: *a. i.* NaH (1.05 eq), DMF, 0 °C, *ii.* $Me_3SiOCH_2CH_2Br$ (1.0 eq), rt, 12 h, *iii*. H_2O, citric acid, 50%; *b*. *N*-hydroxy-phthalimide (1.15 eq), PPh_3 (1.15 eq), DIAD (1.2 eq), THF, rt, 16 h, 90%; *c*. CH_2Cl_2 : CF_3CO_2H (1:1), 0 °C, 1 h, 66%; *d*. RCO_2H (1.1 eq), DIC (1.5 eq), DMAP (cat.), CH_2Cl_2, 0 °C – rt, 16 h, 14-91%; *e*. $N_2H_4 \bullet H_2O$ (1.05 eq), EtOH, 0 °C – rt, 1 h, 20-53%.

MAP Multiplexing Study

Each MAP isotopologue was dissolved in acetone (5 mL) at room temperature. After stirring 24 h, the acetone was removed by rotary evaporation. Quantitative transformation to the corresponding oxime ether adducts was confirmed by GC using a reference sample (MAP-32–

acetone adduct, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.18 (t, J = 8.0 Hz, 2H), 3.86 (t, J = 8.0 Hz, 2H), 2.43 (br. s, 2H), 1.87 (s, 3H), 1.83 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 69.3, 61.7, 45.4, 26.1, 25.2, 21.8 ppm). The adducts then were redissolved in CH_2Cl_2 (1 mL) for the multiplexing experiment. Multiplexed samples were prepared by combining the isotopic adducts MAP-32–acetone, MAP-33–acetone, and MAP-34–acetone in the following respective proportions: 1:2:5, 2:5:1, 5:1:2. Details for analysis using GC-MS and accompanying spectra are provided in the ESI.

Stability Tests

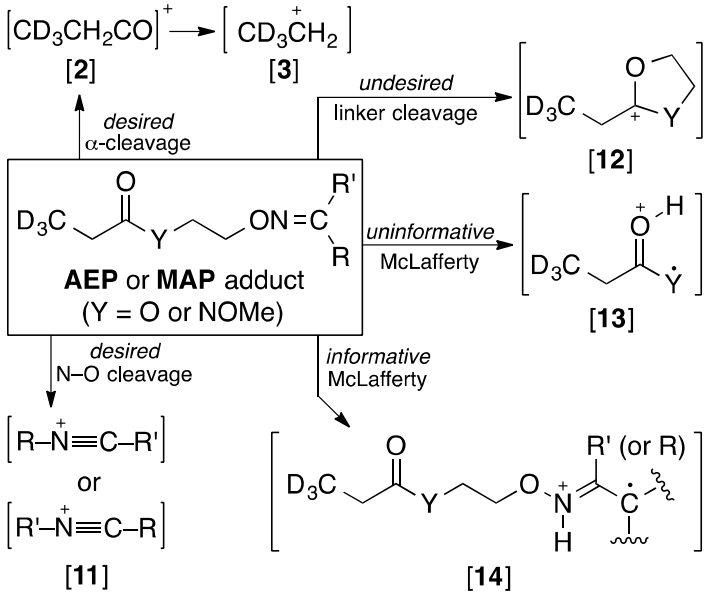
A solution of MAP-32–acetone adduct (2.0 mg, 0.01 mmol) dissolved in CH_3CN (1 mL) was added to benzyl amine (2.18 mL, 0.02 mmol). The reaction mixture was heated to 85 °C and stirred overnight. Upon cooling, the solution was transferred to a vial for analysis using GC-MS. This procedure also was conducted using *N*-methyl-benzyl amine (2.58 mL, 0.02 mmol).

In a separate experiment, MAP-32–acetone (25.0 mg, 0.12 mmol) and 1,4-dimethoxy-benzene (5.0 mg, 0.04 mmol), serving as internal standard, were added to 1× PBS buffer (1 mL, pH 8.8). The reaction mixture was heated to 65 °C for 16 h. Upon cooling, the aqueous layer was extracted with $CDCl_3$ (0.5 mL) and the organic extract was analyzed using ^{1}H-NMR.

Results and Discussion

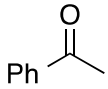
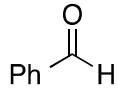
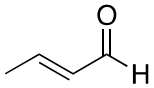
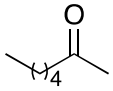
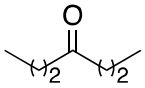
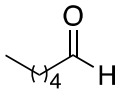
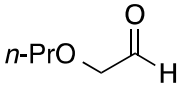
A 1:1 mixture of MAP-32 and AEP-32 was reacted in turn with select aldehydes and ketones to form the corresponding 1:1 mixture of oxime ether adducts for subsequent comparisons of EI-induced fragmentations. For structural diversity, the panel of carbonyl compounds included saturated (acetone, 2-heptanone, 4-heptanone, hexanal), unsaturated ((*E*)-2-butenal) and aromatic examples (benzaldehyde, acetophenone) as well as an α-alkoxy substituted compound (2-(*n*-propoxy)ethanol). The 1:1 mixtures of MAP- and AEP-derived adducts

obtained from reactions of the carbonyl compounds then were analyzed by GC-MS (Table 1). In addition to the formation of acylium [**2**] and mass spectral tag [**3**], the reagent-carbonyl adducts exhibited several other characteristic fragmentations (Scheme 4). Of interest to this study is the formation of the substrate-derived nitrilium ion [**11**] that accompanies oxime ether N–O cleavage.[32,33] This mode of fragmentation provides useful substrate-dependent information (i.e., nitrilium *m/z* + 1.992 provides carbonyl parent MW). In contrast, fragmentations leading to dioxolium (from AEP adducts) or oxazolium (from MAP) ions ([**12**], Scheme 4) provide no useful information in that these fragment ions are reagent-derived. The adducts also underwent classic McLafferty-type fragmentations. McLafferty fragmentations involving the reagent carbonyl (ester in AEP, amide in MAP) furnish radical cations [**13**] that yield no substrate information. When structurally possible, McLafferty fragmentations of the oxime ethers,[32] however, furnish radical cations [**14**] that provide structural information specific to the adducts. An ideal 'next-generation' AEP reagent should improve the prevalence of [**3**] for



Scheme 4. EI-MS induced fragmentations of oxime ether adducts.

Table 1. Relative abundance of fragment ions observed on EI-induced fragmentation of AEP- and MAP-derived oxime ether adducts (Scheme 4).[a]

Entry	Carbonyl	Adduct (isomer)[b]	[**2**]	[**3**]	[**11**]	[**12**]	McLafferty[c] [**13**]	McLafferty[c] [**14**]
1		AEP	83.5	21.0	100	53.1	0.2	na[d]
		MAP	100	17.5	37.7	12.5	0.1	
2		AEP (*Z*)	100	30.2	2.4	61.2	7.6	na
		MAP (*Z*)	100	20.9	17.3	1.2	0.4	
		AEP (*E*)	100	56.0	0.3	49.3	33.4	
		MAP (*E*)	100	25.5	0.1	21.3	0.8	
3		AEP (*Z*)	72.8	17.5	100	83.3	57.7	na
		MAP (*Z*)	100	14.7	32.8	16.3	3.5	
		AEP (*E*)	74.3	20.5	100	77.3	71.7	
		MAP (*E*)	100	17.3	26.2	9.9	3.6	
4		AEP (*Z*)	100	35.9	49.9	68.4	12.0	na
		MAP (*Z*)	100	18.2	21.0	14.5	0.0	
		AEP (*E*)	100	31.4	0.0	59.4	4.7	
		MAP (*E*)	100	14.9	9.9	8.9	0.1	
5		AEP (*Z*)	37.4	8.6	25.4	100	0.2	3.6
		MAP (*Z*)	100	17.8	55.8	36.8	0.2	0.1
		AEP (*E*)	35.0	8.3	3.4	100	0.2	17.2
		MAP (*E*)	100	18.7	7.7	49.5	0.2	7.5
6		AEP	51.7	17.3	1.7	100	1.7	2.1
		MAP	100	19.4	8.8	56.6	0.1	0.3
7		AEP (*Z*)	60.6	11.7	13.4	100	0.3	0.5
		MAP (*Z*)	100	14.8	4.8	8.9	0.1	0.2
		AEP (*E*)	50.4	10.5	2.5	100	0.2	8.9
		MAP (*E*)	100	13.1	2.1	6.5	0.1	0.3
8		AEP (*Z*)	51.2	11.7	8.8	100	1.9	0.3
		MAP (*Z*)	100	13.7	0.5	17.2	0.2	0.1
		AEP (*E*)	30.7	7.2	0.9	100	17.3	12.6
		MAP (*E*)	100	21.9	1.6	2.0	0.0	0.2

[a] Parent *m/z* peak assigned a value of 100; [b] oxime ether stereochemistry; [c] fragments resulting from γ-H abstraction leading to α,β-scission; [d] not applicable (no carbonyl γ-H).

quantifications and/or [**11**] and [**14**] to assist with analyte identifications while minimizing the formation of uninformative fragmentations, such as formation of [**12**] and/or [**13**]. The GC-MS data (Table 1) show that the MAP modification provides these benefits.

Examination of the MAP-adduct fragmentations reveals that α-cleavage of the *N*-methoxy amide construct to generate acylium ion [**2**] is very facile. In every case examined this fragment ion was the predominant ion measured. In agreement with this observation, formation of the mass spectral tag [**3**] from the MAP-adducts also was robust, although there was neither substantial increase nor decrease in the productions relative to the corresponding AEP-adducts. Substitution of amide functionality with methoxy, as anticipated, thus greatly enhanced EI-induced α-cleavage to furnish a mass spectral tag in the ZMI for quantification purposes. We also were gratified to observe that formation of the structurally informative nitrilium ion [**11**] from MAP-adducts was well populated and occurred to a greater extent in a few cases than the corresponding AEP-adducts (e.g., entries 5, 6). A notable difference between MAP and AEP behavior is fragmentation along the linking carbon chain to form the uninformative ions [**12**]. AEP-adducts are prone to give uninformative dioxolium ions [**12**] — these are often the predominant fragment ions (entries 5-8). In comparison, analogous formation of the oxazolium ion [**12**] from MAP-adducts was suppressed in every case examined. Incidence of McLafferty fragmentation along the linking carbon chain also was greatly reduced in MAP-adducts relative to AEP-adducts. Radical cation [**13**] was formed to a lesser extent from MAP-adducts than AEP-adducts in all cases except with 2-heptanone (entry 5). McLafferty fragmentations involving the oxime ether moieties of either AEP or MAP adducts to form [**14**] was minimal relative to the aforementioned modes of fragmentation.

In modeling a multiplexed experiment, we pooled acetone adducts of the three isotopic reagents MAP-32, MAP-33 and MAP-34 at different ratios. Three sample mixtures with respective adduct ratios of 1:2:5, 2:5:1, 5:1:2 were prepared and analyzed by GC-MS.

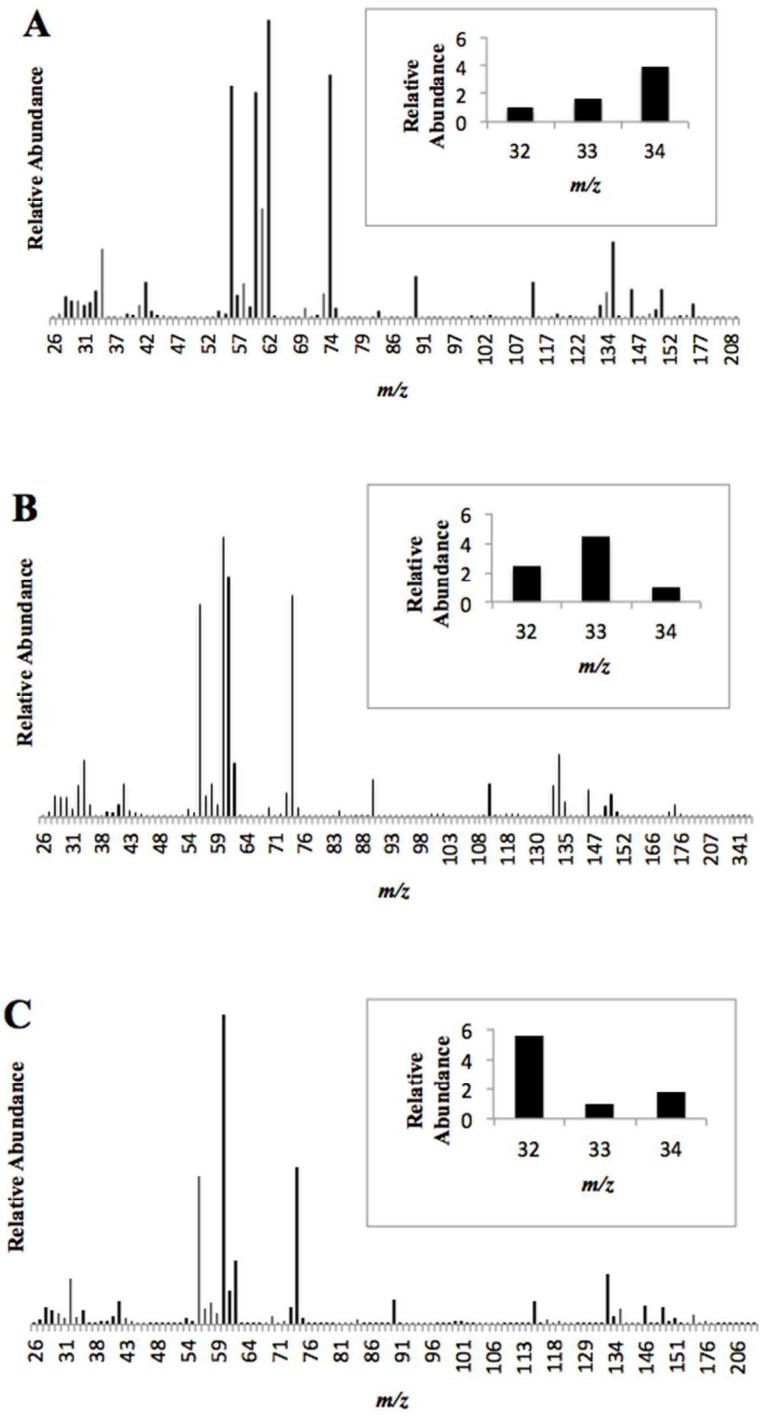


Figure 1. Multiplexed GC-MS analyses of three sample mixtures containing the isotopic MAP-32–, MAP-33– and MAP-34–acetone adducts as follows: mixture **A**, 1:2:5; mixture **B**, 2:5:1; mixture **C**, 5:1:2. Insets depict the relative abundance of mass spectral tags [**3**] in the ZMI.

Importantly, since no chromatographic separations based on differences in ^{13}C incorporation occurred, the relative quantification of adducts in each mixture is achieved by direct comparison of the isotopic carbenium ion mass spectral tags in the ZMI at *m*/*z* 32-34, as shown in Figure 1.

To test the resistance of the *N*-methoxy amide functionality of MAP toward biological nucleophiles, we reacted the MAP-32–acetone adduct, as a representative carbonyl metabolite adduct, with excess benzyl amine and *N*-methyl-benzyl amine under forcing conditions (acetonitrile, 85 °C, 16 h). No observable adduct degradation or label transfer was noted in either reaction. The MAP-32–acetone adduct also exhibited robust stability when incubated in 1X PBS buffer (pH 8.8) at 65 °C for 16 h — no amide or oxime ether cleavage was observed by ^{1}H NMR analysis.

Conclusion

Substitution of the ester moiety of the previously reported[21] AEP reagent with an *N*-methoxy amide affords MAP, a robust and chemoselective carbonyl derivatization reagent. Examination of the electron ionization-induced fragmentations of a representative carbonyl-MAP adduct panel reveals that *N*-methoxy amide functionality is highly predisposed to produce an acylium ion fragment and corresponding isotopically labeled carbenium reporter ion fragment in the *m*/*z* range 32-34. A representative multiplexed experiment using the MAP isotopologues demonstrated how relative quantification is achieved through reporter ion comparison in this range. The substitution to *N*-methoxy amide also decreased the prevalence of undesired (e.g., uninformative) fragmentations, such as oxazolium ion formation or McLafferty fragmentation associated with the reagent carbonyl. Finally, the *N*-methoxy amide functionality is suitably resistant to reaction with amines or base at elevated temperatures. These properties should

facilitate the use of the MAP reagent panel in multiplexed GC-MS analyses of complex mixtures containing aldehyde and ketone analytes.

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Notes and references

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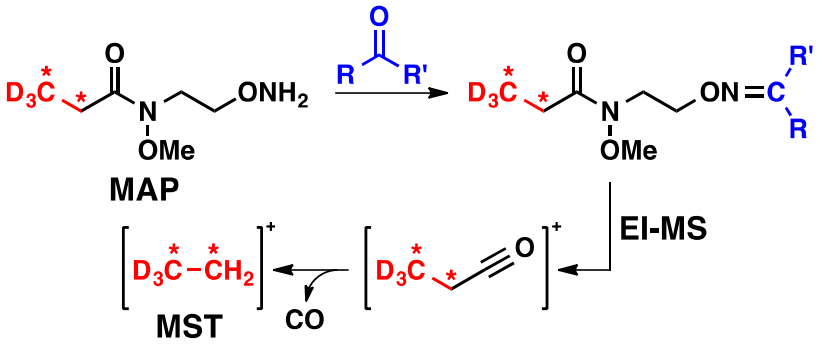
Graphical Abstract



Isotopologues of reagent MAP (* = ^{13}C) are described for chemoselective carbonyl labeling. Electron ionization–induced fragmentation of the Weinreb amide moiety gives rise to labeled ethyl carbenium ion mass spectral tags (MST) for quantifications in multiplexed GC-MS experiments.