Increasing Molecular Ion Production for Unknown Formula Elucidation with Chemical Ionization and Low Energy-Electron Ionization on Orbitrap GC/MS

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ABSTRACT

The ability to generate accurate molecular ion information is critical for true unknown identification in complex samples. In electron ionization (EI), lower electron energies can reduce fragmentation of molecules, thus increasing the signal-to-noise ratio and relative intensities of high masses, including molecular ions. But this technique is compound dependent, in particular for molecules that are very fragile or containing active leaving groups. It can be difficult to retain molecular ions even with electron energies lower than 12 eV. In these cases, traditional chemical ionization (CI) is an alternative approach to generate protonated molecules that can aid in chemical formula elucidation. In this study, a mixed reagent gas of 5% methylamine and 95% methane has been used as a CI reagent gas for trimethylsilyl (TMS) derivatized metabolites and compared with 100% methane. The study demonstrates that this gas mixture can significantly reduce fragmentation and dramatically increase [M+H]⁺and adduct ion intensities relative to fragment ions, while maintaining excellent mass accuracies of less than 1 ppm for unknown identification.

INTRODUCTION

High resolution/accurate mass (HRAM) gas chromatography-mass spectrometry (GC/MS) has become an increasingly popular tool for unknown compound identification in diverse applications and is especially important for global metabolomics analysis. Acquisition of accurate mass spectra can aid in identifying compounds where no reference spectra exist. However, to fully utilize an HRAM mass spectrometer's ability to predict an unknown compound's formula, a molecular ion must be observed. Most GC/MS systems are operated with an EI source at an electron energy of 70 eV, which can be too energetic to prevent fragmentation. Thus, there has been increasing interest in lower electron energy electron ionization on GC/MS that can reduce or eliminate low mass ions that do not contain useful structural information while simultaneously boosting higher mass ions and/or molecular ions that significantly help in qualitative analysis. However, the effectiveness of this technique can be compound specific. In cases where low energy EI proves ineffective, the conventional softer ionization CI is an alternative approach to generate protonated molecules or molecular ion adducts by using different reagent gases such as methane, ammonia, isobutane or customized gases.

Methane is the most commonly used CI reagent gas due to its high ionization efficiency. The reason for the robust ionization with methane is its low proton affinity, making it a good proton source for compounds with higher proton affinities. In contrast, ammonia has relatively higher proton affinity and so transfers a lower amount of energy to the analytes it ionizes, which is why it is considered a softer CI reagent gas. Even so, for acid labile compounds with halogenated, acetyl, hydroxyl, thiol and alkoxy leaving groups, more fragmentation and missing protonated molecules are frequently observed when using ammonia as a CI reagent gas[1].

An alternative approach is to use a mixture of reagent gasses. In this study, a 5% methylamine in methane gas mixture has been chosen and evaluated for TMS derivatized metabolites. Methylamine has a much higher proton affinity (214.1 kcal/mol) than methane (127 kcal/mol) but similar to ammonia (205 kcal/mol). It has been demonstrated as an excellent CI reagent gas to significantly reduce fragmentation for a variety of analytes including alcohols, ketones, aldehydes, acetals, and others[1]. Different TMS derivatized metabolites have been employed using variable electron voltage (VeV) EI acquisition, and CI acquisition with either 100% methane or 5% methylamine in methane for comparison on a Thermo Scientific™ Q Exactive™ GC Orbitrap™ mass spectrometer.

MATERIALS AND METHODS

Sample Preparation and Derivatization

A common two-step sample derivatization method was carried out before analysing on the GC/MS. Initially 20 µL of a 20 mg/mL methoxyamine/pyridine solution was added to the sample to enable the methoximation of any potentially labile ketone groups. Incubation of this solution at 60 °C for 60 min was followed by silylation in which 90 µL of MSTFA +1% TMCS (N-methyl-N-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane) was added. Subsequent heating at 60 °C for 60 min afforded volatility to any labile hydroxyl and amine groups by the addition of the TMS moiety. The TMCS acted as a catalyst to ensure optimal TMS addition.

GC/MS Analysis

In all experiments, a Q Exactive GC Orbitrap GC-MS/MS system was used. Sample injection into a hot split/splitless injector (250°C) was performed using a Thermo Scientific™ TriPlus RSH™ autosampler, and chromatographic separation was obtained with a Thermo Scientific™ TRACE™ 1310 GC system and a Thermo Scientific™ TraceGOLD™ TG-5SilMS 30 m × 0.25 mm I.D. × 0.25 µm column with 10 m integrated guard column. A total GC run time of 37 min per sample was used. Additional details of instrument parameters are shown in Table 1.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software, which allowed for both quantitative and qualitative sample analysis. In addition, compound identification was performed by library searching either a custom-made or commercially-available spectral library such as NIST 2017.

Table 1. Gas chromatograph and mass spectrometer analytical parameters.

TRACE 1310 GC Parameters	
Injection Volume (µL):	1.0
Liner	Single taper without glass wool
Inlet (°C):	250
Inlet Module and Mode:	Split 5:1 (EI) Splitless 2min (CI)
Carrier Gas, (mL/min)	He, 1.0
Oven Temperature Program:	
Temperature 1 (°C):	60
Hold Time (min):	1
Temperature 2 (°C):	325
Rate (°C/min)	10
Hold Time (min):	9.5

Q Exactive GC MS Parameters	
Transfer line (°C):	290
Ionization type:	EI / PCI
Ion source(°C):	250
Electron energy (eV):	70
Acquisition Mode:	Full scan
Reagent gas, (mL/min)	5% CH3NH2 in CH4, 100% CH4, 2.0
Mass range (*m/z*):	60-800 (EI) 100-1000 (CI)
Lock masses (*m/z*):	73.04680; 133.01356; 207.03235; 281.05114; 355.06990

RESULTS

Variable Electron Voltage (VeV) for Metabolomics Analysis

Variable Electron Voltage EI acquisition can be used to operate an EI source at energies lower than the conventional 70 eV, usually at 12 or 10 eV. This can reduce fragmentation or eliminate low mass ions that do not contain useful structural information, while simultaneously boosting higher mass ions and/or molecular ions that can be very helpful for chemical formula elucidation, especially for untargeted metabolomics studies. This softer EI technique is a promising and informative ionization mode that possesses the merits of both EI and CI.

Figure 1. Comparison of kynurenine, 3TMS mass spectra acquired using VeV at 70 eV and 10 eV with all mass accuracies of all intense ions within 1 ppm.

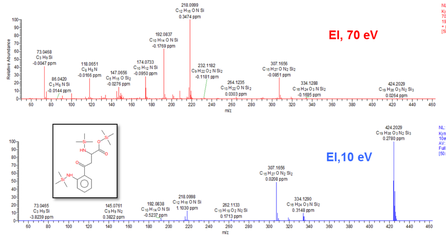

Figure 2. Comparison of cholesterol, 1TMS mass spectra acquired using VeV at 70 eV and 10 eV with all mass accuracies of all intense ions within 1 ppm.

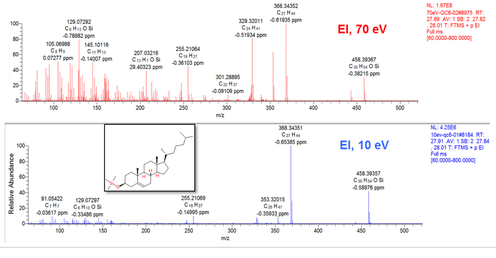

Figure 1 and 2 show examples for kynurenine, 3 TMS and cholesterol, 1 TMS spectra acquired under both 70 eV and 10 eV using the VeV technique. Acquisition at 10 eV significantly boosts higher mass intensities including the molecular ions *m/z* 424.20282 and 458.39384 for kynurenine, 3TMS and cholesterol, 1TMS , respectively. The enhanced higher mass signals can be used as more selective ions for quantitation, especially when analyzing complex matrix samples. Furthermore, the promoted molecular ions with excellent mass accuracies (<1 ppm) are essential for chemical formula elucidation to increase confidence in identification of known unknown metabolites. It is worthy to note that there is no need to change the ion source when switching between conventional 70 eV and VeV, acquisition, which significantly reduces downtime on the instrument for high throughput metabolomics laboratories.

Chemical Ionization for Metabolomics Analysis

Chemical ionization is considered a softer ionization mode since CI ion formation involves much lower energy and the CI technique is much gentler than EI. Due to lowered fragmentation, a higher abundance of molecular ion adducts (e.g. [M+H]⁺) can be generated in CI for chemical formula elucidation when molecular ion is absent or present with low intensity in EI, which can help to avoid ambiguous identification. In Figure 3, a TCA cycle metabolite, derivatized oxaloacetic acid, is shown as an example where no molecular ion was detected at both 70 eV and 12 eV in EI acquisition. VeV just reduces the abundance of lower masses but molecular ion information is still missing. In contrast, CI acquisition is readily able to generate protonated molecule [M+H]⁺ using both 100% methane and 5% methylamine in methane. However, 5% methylamine in methane dramatically increases the relative abundance of [M+H]⁺ in the spectrum and considerably decreased the fragmentation. Additionally, an [M+CH$_3$NH$_3$]⁺ adduct was also found in that CI spectrum.

Figure 3. Comparison of oxaloacetate, 1 MOX, 2TMS mass spectra acquired using VeV at 70 eV and 10 eV and CI mode with 100% methane and 5% methylamine in methane as reagent gases. Less than 1 ppm mass accuracies were maintained in both EI and CI modes.

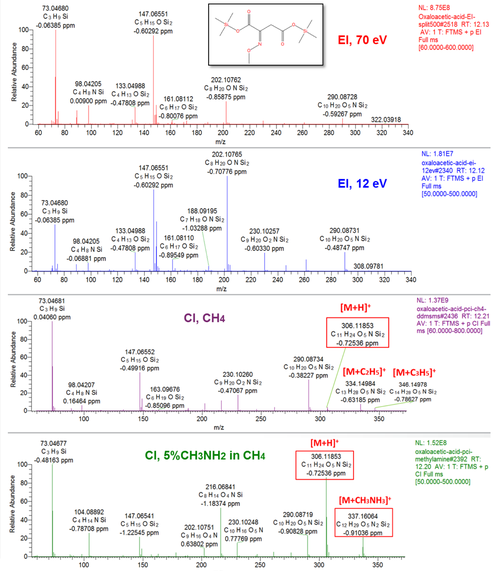

Figure 4. Comparison of niacinamide, 1TMS mass spectra acquired using VeV at 70 eV and 10 eV and CI mode with 100% methane and 5% methylamine in methane as reagent gases. Less than 1 ppm mass accuracies were maintained in both EI and CI modes.

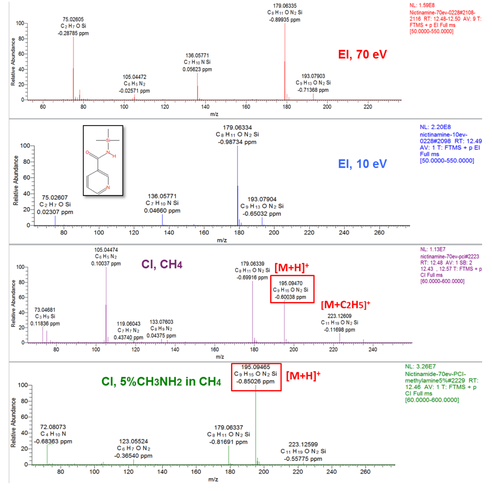

Table 1. Comparison of the ability to generate [M+H]⁺ for TMS derivatized amino acids between 100% methane and 5% methylamine in methane reagent gases.

	100% methane as reagent gas			5% methylamine as reagent gas		
Compound	**[M+H]⁺**	**TIC**	**[M+H]⁺ /TIC**	**[M+H]⁺**	**TIC**	**[M+H]⁺ /TIC**
Alanine, 2TMS	25,162,863	348,675,057	7.22%	35,862,577	56,941,071	62.98%
Glycine, 2TMS	22,462,976	1.65E+08	1.36%	54263115	3.66E+08	14.84%
Glutamic acid, 3TMS	1,129,259	26,749,984	4.22%	2,971,298	13,656,111	21.76%
Phenylalanine, 2TMS	4,512,577	55,119,398	8.19%	7,619,533	21,831,968	34.90%
Serine, 3TMS	4,592,628	62,864,365	7.31%	7,539,979	24,379,856	30.93%
Threonine, 3TMS	6,847,773	94,601,822	7.24%	13,773,807	38,231,267	36.03%
Arginine, 3TMS	164,713	7,267,225	2.27%	679,715	4,909,587	13.84%
Asparagine, 3TMS	114,814	3,554,274	3.23%	151,302	900,865	16.80%
Valine, 2TMS	13,982,635	170,065,118	8.22%	23,466,264	62,262,336	37.69%
Tyrosine, 3TMS	4,253,531	30,941,146	13.75%	5,513,548	18,708,674	29.47%
Lysine, 4TMS	2,867,069	108,050,481	2.65%	5,500,731	26,074,025	21.10%
Proline, 2TMS	3,410,313	31,821,469	10.72%	6,118,653	14,820,911	41.28%
Citric Acid, 4TMS	313,653	77,028,390	0.41%	3,657,970	34,290,219	10.67%

Another example of CI acquisition is for niacinamide, 1TMS shown in Figure 4. At 70 eV and 10 eV, a molecular ion is detected but in extremely low intensities that can't be seen in the spectrum. Also, its fragment ions are at relatively low masses, so it could be problematic when analyzing complex samples that potentially could give a false positive identification. Thus, the ability to detect molecular ion information is critical for confident identification. In CI acquisition mode, a dominant [M+H]⁺ adduct was detected using 5% methylamine in methane and dramatically eliminated fragmentation compared with 100% methane gas.

Results from further study of the ability to generate [M+H]⁺ adducts between 100% methane and 5% methylamine in methane using TMS derivatized amino acids are listed in Table 1. It is commonly accepted that relative sensitivity drops as the proton affinity of the reagent gas increases[2]. Thus, the TIC intensities generated from 5% methylamine is lower than that of 100% methane, but the absolute intensities of [M+H]⁺ ions increase on average and the relative fragmentation percentage over the total ion chromatogram, ([M+H]⁺ /TIC), is dramatically higher than that of 100% methane. The average of [M+H]⁺ /TIC with 5% methylamine (30% on average) is six times higher than that of 100% methane (5% on average).

Due to the much high proton affinity (214.1 kcal/mol) of methylamine, only analytes that have higher proton affinity than that of ionized methylamine ($CH_3NH_3^+$) can successfully take the proton from ionized methylamine to form a positively charged analyte [M+H]⁺ adduct. Furthermore, the energy difference (proton affinities) between methylamine and the target analyte is much lower than the one between methane (127 kcal/mol) and the target analyte. Hence, less excess energy will be applied to the [M+H]⁺ adduct after proton transfer, which is the main reason why methylamine causes less fragmentations and is considered a softer reagent gas than methane.

Pure (100%) methylamine has extremely low sensitivity compared with 100% methane[1]. Thus, 5% methylamine in methane could be a good combination to balance fragmentation and sensitivity, based upon source design, CI reagent gas flow rate and source chamber pressures. Moreover, methane is the gas that will be first ionized by electrons in the source chamber due to its much lower proton affinity. After this initial ionization, methane adducts (CH_5^+ $C_2H_5^+$ $C_3H_5^+$) will rapidly react with methylamine to form positively charged methylamine ions. Five-percent methylamine is enough to suppress ionized methane ions, hence much lower [M+C_nH_{2n}]⁺ adducts were detected in the spectra using 5% methylamine in Figure 3 and 4. Also, 95% methane can decrease ligand-switching reactions[1] when using amines as CI reagent gases[1].

CONCLUSIONS

Two different ionization modes EI and CI were evaluated using TMS derivaitzed metabolites.

- VeV can significantly lower the limit of detection for specific compounds in matrix for confident qualitative and quantitative analysis.
- VeV allows for enhanced sensitivity for more specific high mass ions.
- CI is an efficient approach in the generation of pseudo-molecular ions for chemical formula elucidation.
- Five-percent methylamine in methane has been proved as a promising softer CI reagent gas especially for TMS derivatized metabolites.
- Excellent mass accuracy (<1 ppm) is consistently maintained on the Orbitrap for both EI and CI, which significantly increases the confidence of unknown identification.
- VeV and CI acquisitions are both excellent tools to generate molecular ion information on the Q Exactive GC for unknown identification. It is necessary to alternate them when one approach yields poor molecular ion information for certain compounds.

REFERENCES

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TRADEMARKS/LICENSING

Thermo Fisher SCIENTIFIC