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Infrared isomer-specific fragmentation for the identification of aminobutyric acid isomers separated by differential mobility spectrometry

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Keywords: isomer separation; aminobutyric acid; ion mobility spectrometry; differential mobility spectrometry; IRMPD; specific activation

Highlights:

- First results based on the coupling of differential mobility spectrometry and infrared multiple photon dissociation spectroscopy with high resolution FT-ICR mass spectrometry are presented
- Two operation modes of the DMS-MS/MS(IRMPD) are illustrated for the structural assignment of DMS peaks associated with three structural isomers of aminobutyric acids
- Peak assignment and structural identification is performed through IRMPD spectroscopy of DMS- and mass-selected ions
- Peak assignment and structural characterization can also be based on selected DMS peak depletion at selected IR wavelengths.

Abstract

Metabolomics strongly relies on liquid chromatography hyphenated to tandem mass spectrometry (LC-MS/MS) for the separation and identification of metabolites. Retention times and fragmentation mass spectra are used as identification parameters. A complementary approach is proposed here based on alternative approaches for both the separation and identification, and is illustrated in the case of isomeric forms of aminobutyric acids ($C_4H_9NO_2$). Differential mobility spectrometry (DMS), a very efficient method for the separation of isomers of small molecules, is coupled with high resolution tandem mass spectrometry Fourier transform ion cyclotron resonance (FT-ICR). Identification of the DMS peaks associated with the various isomers is based on isomer-specific infrared multiple photon dissociation (IRMPD) in the mid-infrared range. Two operation modes of the DMS-MS/MS(IRMPD) are used. Structural identification of the various isomers is first achieved based on the comparison of IRMPD spectra of DMS- and mass-selected ions with IR absorption spectra of candidate structures derived from quantum chemical calculations. For this purpose, the DMS device is used in selective mode. Alternatively, when the DMS is using in scanning mode, identification can also be achieved based on specific depletion of DMS peaks at selected IR wavelength. These two operation modes for identification of isomers are illustrated in conjunction with the use of modifier-assisted DMS separation.

Introduction

Nowadays, the screening of biomarkers allows for an early diagnosis of many diseases,¹⁻² and can also answer the need for emergency diagnosis.³ Subtle differences of metabolite abundances in urine or plasma, for example, can be the signature of specific diseases. Small molecules of different classes such as amino acids, acylcarnitines, organic acids are common target molecules of many diseases.⁴ The large number of metabolic pathways and corresponding metabolites are at the origin of the analytical challenge.

Metabolomics profiling based on chromatographic separation coupled with tandem mass spectrometry (MS/MS) is considered to be a method of choice for the identification and quantification of metabolites.⁴⁻⁵ Due to the complexity of biological samples, chromatographic separation is routinely used for the separation and identification, but this is not without its drawbacks. Liquid chromatography (LC) requires handling large volumes of solvents most often and difficult to recycle solvents. Diagnosis relies on multiple LC-MS/MS protocols since only a set of molecules with comparable polarities can be isocratically separated under a given set of chromatographic conditions. In addition, while isobar resolution can be achieved with high resolution MS/MS, isomer separation often remains a challenge.⁶⁻⁷ In this context, ion mobility separation could provide an added value for the separation on its own, or could also be integrated to LC-MS/MS protocols.⁸⁻¹¹

Additional identification parameters of biomarkers to retention time derived from chromatography and fragmentation mass spectra, which rely on library of standards, would be needed. In this perspective, isomer-selective or even specific fragmentation based upon resonant IR photo-dissociation is particularly interesting. Comparison of gas phase IR spectra with calculated IR absorption spectra allows for structural characterization of the isomeric ions.¹² This has been demonstrated for the case of an unknown metabolite where reference compounds are not available.¹³⁻¹⁶ Infrared ion spectroscopy was used to assign the structure of an unknown drug metabolite from a collected chromatographic fraction.¹⁵⁻¹⁶ Better resolved IR ion spectra can be derived when ions are cryogenically cooled, as demonstrated by the structural characterization of metabolites of the Varsatan drug.¹⁴ These recent studies clearly show the promised of IR ion spectroscopy to differentiate isobaric and isomeric variants.

Ion mobility spectrometry (IMS) method could be an alternative and/or a complement to chromatography for the separation step of metabolites.¹⁷⁻¹⁸ An additional advantage of IMS is that it could provide an additional identification parameter to LC and MS/MS.¹⁹ In a recent identification study of pesticides in standard solutions and in the presence of different matrices,²⁰⁻²¹ it had been shown that IMS drift time or better collision cross section (CCS) could

be used as identification parameter of ions. Both dispersive and selective, relying on time and space separation, respectively, can be used in an IMS-MS/MS workflow or integrated in an LC-MS/MS workflow. Selective differential mobility spectrometry (DMS) is recognized as being more orthogonal than dispersive drift-tube IMS to MS, which is an important aspect for improving the resolving power of the analytical method.¹⁷ DMS has been successfully used for resolving various sets of molecules featuring isomers in pure solution or in uncomplicated matrices.²²⁻²⁵ It has also been shown to be effective in particular in cleaning the baseline and rising up the signal-to-noise ratio in complex matrices to improve the detection and quantification of analytes of interest.^{10, 26}

In this paper, two DMS-MS/MS(IR) workflows combining three orthogonal techniques are presented for the separation, identification, and structural characterization of isomers of aminobutyric acid (ABA). Separation of ABA isomers is an important issue for their quantification.²⁷ For instance, γ -aminobutyric acid (GABA) is known for being an inhibitor of neurotransmission in the nervous system of animals,²⁸ whereas β -aminobutyric acid (BABA) can act as a partial agonist of the glycine receptor.²⁹ α -aminobutyric acid (AABA) has been investigated as biomarker for liver toxicity and alcohol abuse.³⁰ In a recent study, LC-MS/MS performance for the separation of these derivatized species (with Marfey's reagent) has been evaluated.²⁷ In the present study, a chromatography free DMS-MS/MS(IR) approach is used. This is the first application of the coupling of DMS and isomer-selective infrared induced fragmentation with high resolution MS/MS Fourier transform ion cyclotron resonance (FT-ICR). DMS- and mass-selected ions are subjected to infrared (IR) irradiation of the CLIO Free Electron Laser³¹ allowing for infrared multiple photo-dissociation (IRMPD). Isomer-selective fragmentation of each isomer at a selected wavelength allows for the structural characterization. This is exploited for the identification of modifier-assisted DMS peaks in a workflow where the IR wavelength is fixed and while the DMS parameter is scanned (DMS(scan)-MS/MS(IR fixed)). Alternatively, structural characterization of the DMS peaks can be achieved through the comparison of IRMPD spectra of DMS- and mass-selected ions with IR absorption spectra predicted by density functional theory.

Methods

Sample preparation

The solutions of AABA, BABA, and GABA isomers were prepared by diluting from high concentration of 10^{-2} to 10^{-4} M using methanol/water (50/50, v/v) and 5% acetic acid. Similarly, the mixture solution of three isomers was formulated at the final concentration of 10^{-4} M for each of them. Protonated ABA ($[C_4H_{10}NO_2]^+$, m/z 104.071) were generated by electrospray

ionization (ESI). The sample was pumped into the ESI emitter at a flow rate of 70 $\mu\text{L}\cdot\text{h}^{-1}$ by a syringe pump.

Mass spectrometry instrumentation

All the experiments were performed on a 7 Tesla APEX-Qe (Bruker Daltonics), a hybrid Fourier transform ion cyclotron resonance (FT-ICR) tandem mass spectrometer (MS/MS). Infrared Multiple Photon Dissociation (IRMPD) experiments were carried out using the IR beam delivered by the CLIO infrared free electron laser (IR FEL).³¹ The coupling of the IR FEL with the FT-ICR has already been described in details elsewhere.³² The FT-ICR was equipped with an Apollo II ESI ion source, on which a homebuilt differential mobility spectrometry (DMS) device has been mounted. The conception of this DMS device is based on the one developed by G.L. Glish,³³ which was designed for the coupling with the ESI source of a quadrupole ion trap ESQUIRE 3000+ (Bruker Daltonics). The coupling of such DMS device and IRMPD activation with our quadrupole ion-trap has already been described.³⁴⁻³⁵

The DMS cell is integrated between the ESI emitter and the capillary transfer. Positive ions are generated by setting the ESI emitter at 4 kV, and the DMS housing to the ground. Electrosprayed ions are transported at atmospheric pressure by a carrier gas stream (N_2) between two parallel electrodes (dimensions 0.7 × 6 × 20 mm for gap height, width and length, respectively) towards the glass capillary transfer. The carrier gas flow is a part of the N_2 desolvation gas, originally implemented on the mass spectrometer, partially redirected through the electrodes gap due to the pressure difference between the ESI source and the mass spectrometer vacuum. The temperature and flow rate of the desolvation gas are set using the Bruker control software at 150 °C and 6 $\text{L}\cdot\text{min}^{-1}$, respectively. A custom built ESI chamber allows for a better control of the composition of the carrier gas flow. This ESI chamber allows for the adjustment of the position of the electrospray emitter next to the two DMS electrodes.

Upstream the ESI-DMS part, polar modifier molecules can be introduced in the desolvation gas using a NanoHPLC (LC-20AD nano, Shimadzu). A liquid flow rate of methanol set at 1.5 $\mu\text{L}\cdot\text{min}^{-1}$ was used. The gas line is also heated at ~80-100 °C to ensure homogeneous distribution of the small liquid amount in the N_2 gas before reaching the ion source. The molar percentage of the methanol vapor in the N_2 carrier gas was on the order of ~0.01%. Considering the gap between the electrodes (0.7 mm), and assuming that the carrier gas is near atmospheric pressure and a temperature around 70°C (measured temperature of the electrodes using a thermal camera), the reduced electric field value corresponding to a voltage of 1800 V can be estimated to ~120 Td. In the following, the DMS spectra correspond to

extracted ion chromatogram plotted as a function of the CV value, where a 1 V value correspond to ~ 0.7 Td.

A LabView program (v12.0.1f5, National Instrument) has been developed in order to control the radiofrequency field and the data acquisition, which is synchronized with the Bruker acquisition software under chromatogram mode. Space separation of ions through the DMS device is obtained by applying a high frequency bisinusoidal waveform, alternating phase of high and low electric field, on the DMS electrodes. The dispersion voltage (DV), which corresponds to the maximum amplitude of the radiofrequency waveform, can be adjusted in the 0-2 kV range and has been set at 1.8 kV in this study. The maximum electric field between the two DMS electrodes is thus on the order of $\sim 2.6 \cdot 10^6$ V.m⁻¹. Selection of the ions to be transmitted based on their differential ion mobility is made by applying a DC offset called compensation voltage (CV) to one electrode.

DMS-selected ions can be mass-selected in a quadrupole, and accumulated in a hexapole ion trap. Typical accumulation time is 500 ms. DMS- and mass-selected ions are then pulse-extracted toward the ICR cell, where they are irradiated by IR light for a fixed time period. The energy of the IR FEL electron accelerator was set to 42 MeV. A fairly stable power was maintained in the 900-1900 cm⁻¹ wavenumber ($\tilde{\nu}$) range. Typical irradiation time for these experiments was 250-500 ms.

DMS spectra are recorded by scanning the CV value by steps of 0.1 V. The DMS spectrum is derived from the ion chromatogram. The centroid of each peak and its full width at half maximum (FWHM) are obtained from Gaussian fits of the experimental DMS spectra. Uncertainties on the CV and FWHM values, estimated with the standard deviation over several experiments, are on the order of magnitude of the bin width of the DMS spectra, i.e. 0.1 V.

Two operation modes of the DMS-MS/MS(IRMPD) experiment are possible, a selective and scanning mode. DMS selective mode (fixed CV, scanned $\tilde{\nu}$) allows for structural identification of DMS- and mass-selected ions based on the comparison of IRMPD spectra with IR absorption spectra of candidate structures derived from quantum chemical calculations. Alternatively, when the DMS is used in scanning mode (fixed $\tilde{\nu}$, scanned CV), identification can also be achieved based on specific depletion of DMS peaks at selected IR wavelength.

Quantum chemical calculations

IRMPD spectra were compared to calculated IR absorption to provide a guide for selecting IR signature of each isomer to be used for selective fragmentation of specific isomers. Hybrid density function calculations were carried out with the Gaussian package of programs³⁴ at the

B3LYP/6-311+G** level of theory. A manual search using different starting guess structures featuring a hydrogen bonding motif were optimized using the gradient minimization algorithm of Gaussian and the B3LYP/6-311+G** level. Vibrational frequency values were determined within the harmonic approximation, and the corresponding values were scaled by 0.98.

Result and discussion

DMS peak capacity and resolution can be significantly improved by introducing dopant molecules, also called modifier gas, into the N₂ carrier gas.³⁶⁻³⁷ The term modifier-assisted DMS-MS/MS method for detection and quantification of metabolites in complex samples has been coined recently.³⁸ By seeding polar molecules in the carrier gas, differential mobility between low and high electric field change. During each cycle of the waveform applied to the DMS electrodes, clustering and declustering phenomena is assumed to occur at low and high electric field, respectively. The drawback of this modifier-assisted DMS-MS/MS method is that the peak ordering is difficult to predict, although some recent correlation between DMS peak shifts and ion-molecule binding energy³⁹ have been proposed. Identification of the modifier-assisted DMS-MS/MS peaks through MS/MS is thus important.

Separation of protonated ABA isomers with modifier-assisted DMS is investigated with methanol. The DMS spectra of an equimolar mixture of protonated AABA, BABA and GABA isomers is shown on Figure 1 with only N₂ desolvation gas flow (Figure 1a) and with methanol when injected at 1.5 $\mu\text{L}\cdot\text{min}^{-1}$ (Figure 1b). Fig 1a displays two peaks, one centered at -7.0 V, and another, broader, centered at -5.5 V. By adding methanol in the N₂ flow, the DMS spectrum exhibits three baseline separated peaks shifted towards more negative CV values and centered at -24.6, -20 and -16.9 V. This allows in particular for the resolution of the broadband observed near -5.5 V in the absence of modifier.

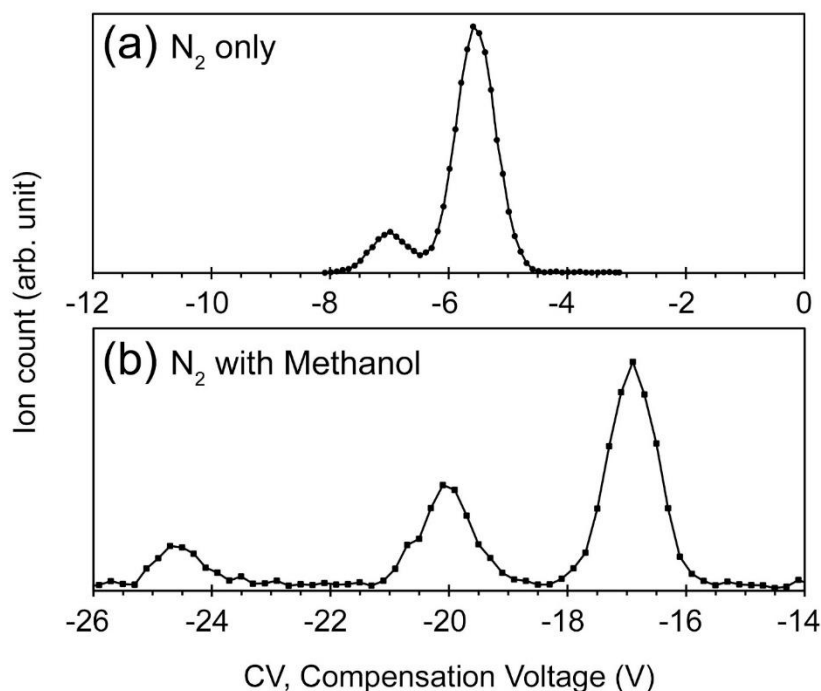


Figure 1: DMS spectra of an equimolecular mixture of AABA, BABA, and GABA. All spectra were recorded with a dispersion voltage value of 1.8 kV. The DMS spectrum recorded in the absence of modifier is shown in panel a). Modifier-assisted DMS spectrum recorded with a methanol at a $1.5 \mu\text{L}\cdot\text{min}^{-1}$ flow rate and $DV=1.8 \text{ kV}$ is given in panel b).

Identification of the DMS peaks is presented in the following using IR isomer-specific fragmentation. The three peaks observed using methanol can be assigned by recording the IRMPD spectrum of the DMS- and mass-selected m/z 104 ions. Then, a second operation mode of the DMS-MS/MS(IRMPD) is used for the identification of the peaks when no modifier is added to the carrier case. In this case, a special emphasis is made on the resolution of the broadband (Figure 1a).

Isomer-specific IR-activation is used for the identification of the three DMS peaks in Figure 1b. For this purpose, DMS-MS² (IRMPD) experiments are performed with the DMS device used in a selective mode, *i.e.* at a fixed CV value. The IRMPD spectrum of the DMS- and mass-selected m/z 104 ions is thus recorded at three fixed CV values (-24.6, -20 and -16.9 V) corresponding to the maximum transmission of protonated ABA isomers with using methanol as modifier. Identification of each DMS peak can then be done by comparing experimental IRMPD spectra with calculated IR absorption spectra of the three protonated isomers (Figure 2).

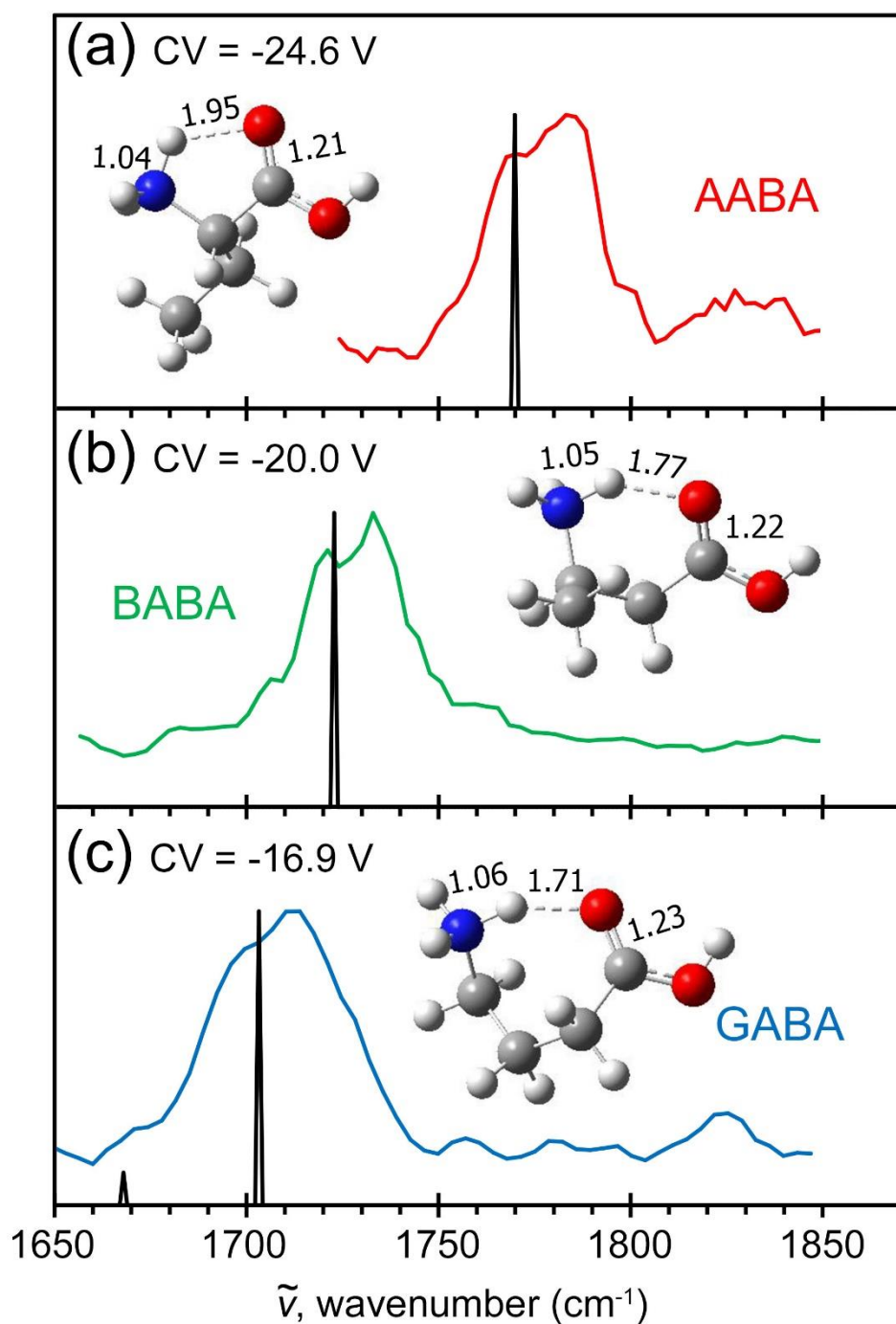


Figure 2: IR spectra of DMS- and mass-selected m/z 104 ions separated by modifier-assisted DMS (methanol at a $1.5 \mu\text{L}\cdot\text{min}^{-1}$ flow rate). IRMPD spectra corresponding to $\text{CV} = -24.6$, -20.0 , and -16.9 V are shown in panels a), b), and c), respectively. All the extracted ion chromatograms are normalized to the maximum. The theoretical spectra are given in black as stick bars. Optimized structures of protonated AABA, BABA, and GABA. Selected bond lengths are given in Angstrom unit.

For this purpose, quantum chemical calculations were performed. The lowest energy optimized structure of the three isomers are given in Figure 2. For the three isomers, the lowest energy structure has a hydrogen bond (HB) between the ammonium and the carboxylic C=O group. This type of intramolecular HB allows for charge solvation and is observed for protonated aminoacids,³⁴ for example. In the present case, the HB gives rise to the formation of a five-, six-, and seven-membered ring for protonated AABA, BABA, and GABA, respectively. As expected, the (ammonium)H \cdots (carbonyl)O distance varies from one isomer to another: 1.95, 1.77, and 1.71 Å for AABA, BABA, and GABA, respectively. The carbonyl C=O bond distance is also sensitive to the HB distance. One can thus expect the vibrational modes associated with the carbonyl C=O and the ammonium to be different from one isomer to the other.

IRMPD of the DMS- and mass-selected ions are shown in Figure 2. The C=O stretching region is the most structurally diagnostic region, and only this region is shown in this Figure. The experimental IRMPD spectra of the individual standards were also recorded for comparison and presented in Figure S1, along with the calculated IR absorption spectra. Identification of the three DMS peaks and structural identification of the isomeric forms can be done based on the relative position of the C=O stretching band. The corresponding IRMPD band observed when the CV value is fixed at -24.6, -20 and -16.9 V are centered at 1775, 1730, and 1710 cm⁻¹, respectively. This trend nicely reflects the one predicted for the protonated AABA, BABA, and GABA for which the C=O stretching band predicted at 1769, 1722, and 1703 cm⁻¹.

Identification of the DMS peaks using a methanol flow rate (Figure 1b) can be made based on the MS/MS(CID) spectra of the DMS-selected ions, *i.e.* using an approach similar to the so-called Single Reaction Monitoring (SRM) employed in LC-MS/MS protocols. Indeed, each ion has a specific fragment at *m/z* 44.05, 58.07, and 87.05 for protonated AABA, BABA, and GABA respectively. In addition, peak identification can also be made based on the DMS spectra of the individual standards (Figure S2). These results support the assignments made based on the IRMPD spectra of the DMS- and mass-selected *m/z* 104 isomers (Figure 2).

The lower energy part of the IR spectra seems to be less diagnostic (Figure S1), although some subtle differences can be observed. In the 1350-1550 cm⁻¹ range, a broadband is observed for the three protonated isomers. These bands correspond to C-H and N-H bending modes, and in particular the ammonium umbrella mode. As can be seen in Table 1, the latter mode is sensitive to the nature of the isomer. Finally, the third cluster band is observed between 1050 and 1250 cm⁻¹. As can be seen in Table 1, this band can be assigned to the C-O stretching and C-O-H bending modes.

Table 1: Experimental and calculated vibrational frequencies for protonated AABA, BABA, and GABA. Vibrational frequencies are given in units of cm^{-1} . Calculated intensities (in units of km.mol^{-1}) are given in parentheses.

description	AABA		BABA		GABA	
	exp	theory	exp	theory	exp	Theory
C-O st.	1130-	1166 (124)		1099 (97)	1177	1176 (213)
C-O-H bend	1195	1174 (87)	1177	1182 (155)		
CH bend	1385-	1393 (109)	1380-	1412 (186)	1400-	1419 (99)
-NH ₃ ⁺ umb	1480	1447 (169)	1495	1462 (181)	1545	1464 (118)
C=O st.	1780	1769 (279)	1740	1722 (273)	1725	1703 (353)

It can thus be concluded that the C=O stretching range is the most structurally diagnostic region. In particular, the protonated AABA and GABA isomers are likely to be selectively fragmented if the IR laser is tuned to 1780 and 1690 cm^{-1} , respectively (vide infra).

In the following, we will turn our attention to the assignment of the DMS peaks obtained in the absence of modifier gas (Figure 1a) using the DMS in scanning mode by recording DMS-MS²(IRMPD) at a selected wavelength. DMS-MS²(IRMPD) spectra recorded at four selected IR FEL wavenumbers are given in Figure 3 along with the DMS-MS spectrum, *i.e.* without IR activation. In the left part of the figure, the intensity of the m/z 104 parent is given as a function of the CV. In the right-hand part of the figure, the abundances of the fragments ions, *i.e.* m/z 44, 58, 86, and 87 are given.

Based on the IR spectra of the protonated isomers previously shown, the C=O stretching region appears to be highly diagnostic. The corresponding IRMPD band is found to be the least red-shifted in the case of protonated AABA, with a maximum near 1780 cm^{-1} . The DMS-MS²(IRMPD@1780 cm^{-1}) spectra are given in Fig. 3e. As can be seen in the right-hand part, only the m/z 58 fragment is observed, and the Gaussian fit is centered at -7.0 V, which is a clear signature of the specific fragmentation of the protonated AABA isomer. A depletion of the m/z 104 parent ion is also observed at -7.0 V (Fig. 3e, left).

Specific depletion of the protonated GABA isomer can be observed when the laser wavenumber is fixed at 1690 cm^{-1} . The corresponding DMS-MS²(IRMPD@1690 cm^{-1}) spectra are given in Figure 3c. Both the m/z 86 and 87 fragment ions were observed, and the two

Gaussian fits are centred at -5.6 V (Figure 3c, right). Inspection of the DMS spectrum in the m/z 104 parent channel reveals that the Gaussian fit of the broad band reveals a depletion of the component centered at -5.5 V. By tuning the laser wavenumber in the C=O stretching region, selective fragmentation of the BABA isomer could not be observed. As illustrated in Figure 3d with the DMS-MS²(IRMPD@1730 cm⁻¹) spectra, simultaneous fragmentation of both protonated BABA and GABA isomers was observed at wavenumber values between 1690 and 1780 cm⁻¹.

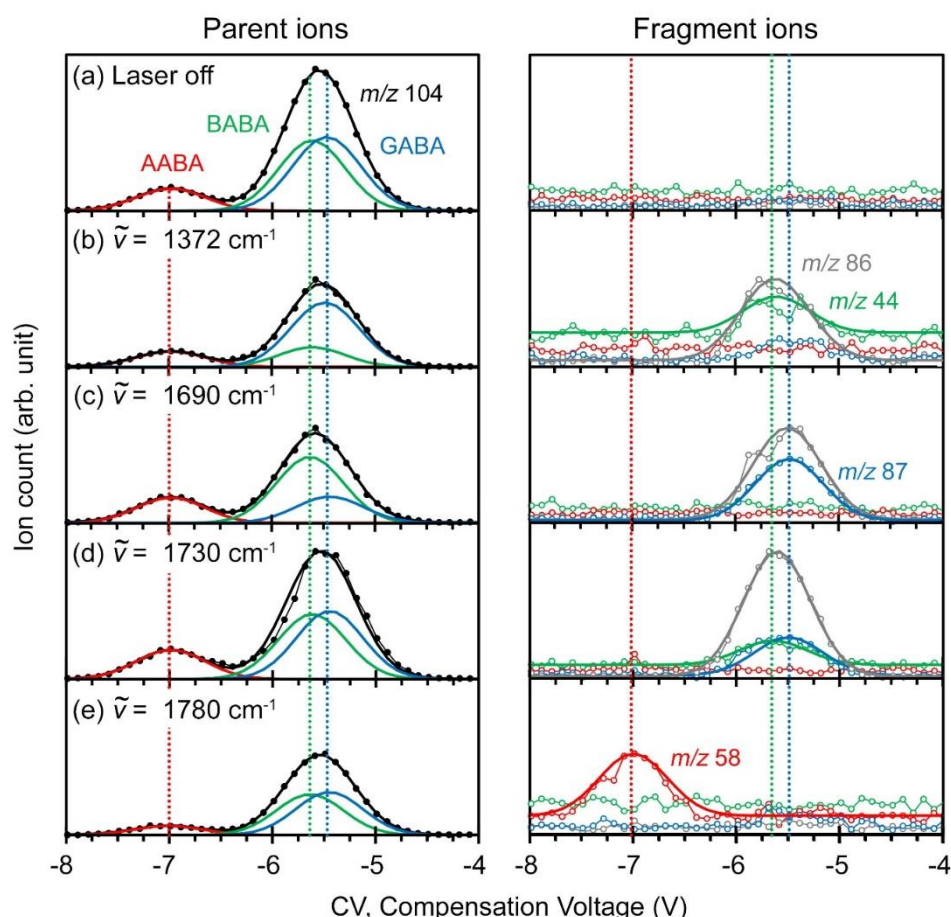


Figure 3: DMS-MS²(IRMPD) spectra of an equimolecular mixture of AABA, BABA, and GABA. In each panel (a-e), in addition to the extracted ion chromatograms (EIC) of the parent m/z 104 ion (left), the EICs of the four fragments are shown on the right. Specific fragments of protonated AABA, BABA, and GABA are shown in red (m/z 58.07), green (m/z 44.05), and blue (m/z 87.05). All data were fitted with a single Gaussian, except for the EIC corresponding to the m/z 86.06 for which a two Gaussian fit was used.

Specific depletion of the protonated BABA isomer could however be obtained at 1372 cm⁻¹ (Figure 3b). At this laser wavenumber, the fit of the broadband centered near -5.5 V reveals a

depletion of the BABA contribution, which is consistent with the Gaussian fit of the m/z 44 and 86 fragments (Figure 3b, right).

Overall, DMS-MS²(IRMPD) at three selected laser wavenumbers allows for the identification of the three isomers, and in particular for the resolution of two overlapping components. These results are consistent with those which can be drawn from the comparison of the DMS spectrum of the equimolecular mixture with those of the individual standard DMS spectra given in Figure S3. The broad peak in Figure 1a results from the overlap of two contributions centered at -5.6 and -5.5 V associated with protonated BABA and GABA isomer, respectively. The -7.0 V peak can be assigned to the protonated AABA isomer. The width of the DMS peaks is ~0.80 V for the individual standards.

Conclusion

Wavelength-selective IR activation is integrated in two workflows for the separation and identification of isomers of small molecules such as metabolites using tandem mass spectrometry. Separation is performed using a homebuilt DMS device which has been specifically designed for the coupling with our high resolution FT-ICR MS/MS. Performance of the two workflows is illustrated on the separation of aminobutyric acid isomers.

Our objectives here range from fundamental understanding of the DMS separation process to developing complementary methodology for improving the identification of isomeric species in the context of targeted metabolomics analyses. From a fundamental point of view, IRMPD spectroscopy is a particularly interesting tool since it does not only offer identification but also structural information on DMS-selected ions. This is particularly useful since the ordering of DMS peaks is not easily a priori predictable, especially when modifier gases are seeded in the carrier gas. The first IRMPD spectra of modifier-assisted DMS separated isomers have been recorded. They match well with the IR absorption spectra predicted by DFT. In particular, a clear trend is observed in the C=O stretching region, which is consistent with isomer-specific hydrogen bonding motifs predicted for the lowest energy isomers.

A second operation mode of the DMS-MS/MS(IRMPD) instrument consists selective or even specific depletion of DMS peaks. At each step of the DMS scan, DMS- and mass-selected ions are irradiated by the IR beam at a selected wavelength. This second workflow would be particularly well suited for a rapid screening of target compounds within complex mixture, as in targeted metabolomics. Indeed, with the CV parameter set for the optimal transmission of a targeted ion to the ion trap, high ion count can be obtained allowing for subsequent infrared interrogation.

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