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Infrared ion spectroscopy: New opportunities for small-molecule identification in mass spectrometry - A tutorial perspective

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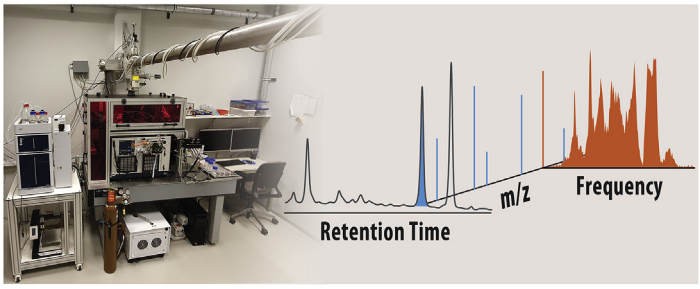
HIGHLIGHTS

- IR ion spectroscopy (IRIS) provides a fingerprint for peaks in a mass spectrum.
- The IR fingerprint is a sensitive diagnostic for the molecular structure.
- Identification through reference spectra from standards or from computation.
- IRIS can be integrated in analytical MS workflows including MS^n and LC-MS.
- A tutorial on IRIS and selected applications in untargeted metabolomics is presented.

GRAPHICAL ABSTRACT



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ABSTRACT

Combining the individual analytical strengths of mass spectrometry and infrared spectroscopy, infrared ion spectroscopy is increasingly recognized as a powerful tool for small-molecule identification in a wide range of analytical applications. Mass spectrometry is itself a leading analytical technique for small-molecule identification on the merit of its outstanding sensitivity, selectivity and versatility. The foremost shortcoming of the technique, however, is its limited ability to directly probe molecular structure, especially when contrasted against spectroscopic techniques. In infrared ion spectroscopy, infrared vibrational spectra are recorded for mass-isolated ions and provide a signature that can be matched to reference spectra, either measured from standards or predicted using quantum-chemical calculations. Here we present an overview of the potential for this technique to develop into a versatile analytical method for identifying molecular structures in mass spectrometry-based analytical workflows. In this tutorial perspective, we introduce the reader to the technique of infrared ion spectroscopy and highlight a selection of recent experimental advances and applications in current analytical challenges, in particular in the field of untargeted metabolomics. We report on the coupling of infrared ion

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spectroscopy with liquid chromatography and present experiments that serve as proof-of-principle examples of strategies to address outstanding challenges.

1. Introduction

Mass spectrometry has developed into one of the key analytical technologies, indispensable in detailed analyses of complex mixtures in fields ranging from (bio-)chemistry and medical diagnostics to environmental and forensic sciences. Developments in mass spectrometry (MS) are one of the major drivers of the current revolution in omics fields [1]. The selectivity as well as the sensitivity of modern MS instrumentation is virtually unrivalled by any other analytical method. A wide range of MS instrumentation in terms of ion sources, ion manipulation, mass analyzers, etc. provides flexible platforms to study analytes of nearly any chemical class with sensitivities down to low nano-molar levels. Accurate mass data is available from several types of high-end instruments and unambiguously defines the elemental composition of a compound. However, the primary weakness of MS relates to the limited structural information that is contained in its data, *i.e.* in a molecular formula, in sharp contrast with spectroscopic analyses. The challenge to distinguish between structural and stereo isomers found at the same mass-to-charge ratio (*m/z*) has spurred the development of orthogonal separation techniques hyphenated to MS. Chromatography as well as collision-induced dissociation (CID) MS/MS are historically among the most prominent sources of orthogonal information used in MS-based structural elucidation. Chromatography coupled-mass spectrometry (LC-MS, GC-MS) is currently one of the primary analytical methods used for small molecule analysis. Closely related isomers are often not separable by chromatography and can give the same tandem MS (and MS^n in general) fragments. As well, retention times and MS fragmentation patterns are primarily used in an empirical fashion in database comparisons, but provide relatively little help towards elucidating the structure of a completely unknown *m/z* feature, so that major challenges remain in untargeted analyses and identification of unknowns. Ion mobility spectrometry (IMS), where the drift velocity of a gaseous molecular ion through a buffer gas is measured, is a more recent technique that – in different implementations – is now available on various commercial MS platforms; the drift velocity can be related to the collisional cross section (CCS) of the ion in an empirical fashion (especially for differential mobility IMS implementations) or also based on a more fundamental molecular approach (especially for drift-tube, travelling-wave and trapped IMS implementations) [2]. However, identification of unknowns on the basis of comparison versus a computed CCS value relies on a single number that is susceptible to small variations in temperature or pressure.

In the field of ion chemistry, focusing on the fundamentals of ion structure and kinetics in mass spectrometry, the relatively novel technique of infrared ion spectroscopy has rapidly become recognized as a valuable method in structural elucidation. Although the density of ions in any type of mass spectrometer is far below what is necessary for a conventional transmission spectrum to be recorded, the myriad of tandem mass spectrometers with wavelength-tunable infrared (IR) laser sources enables photodissociation spectroscopy as an alternative route towards an IR fingerprint of the ions. Proof-of-concept studies showing the viability of this technique date back to the 1980's [3–5], but it was not until the turn of the millennium that widely tunable IR laser sources with sufficient pulse energies became available, which truly kick-started the new field of infrared ion spectroscopy (IRIS).

Our group was the first to demonstrate the potential of a widely tunable infrared free-electron laser (FEL) in the study of the IR spectroscopy of mass-selected ions in an ion trap mass spectrometer [6]. Ion spectroscopy evolved quickly with the introduction of more sophisticated MS platforms [7–11] and other tunable IR laser sources, including especially the FELs in Orsay [12], Tokyo [9] and Berlin [13] as well as sources based on optical parametric oscillation (OPO) [7,8,14]. However, for many years, the applications of IRIS remained limited to structural questions in fundamental ion chemistry, involving for instance the determination of metal-ion coordination geometries [12,15], cluster structures [16], effects of ion hydration [14,17], protonation [13] and deprotonation [18] sites, reaction intermediates [19–21], and product ion structures in MS/MS methods [22], especially applied to peptide dissociation [23–26].

The integration of selectivity and sensitivity as provided by MS with the structural diagnostics of IR spectroscopy obviously has great potential in analytical settings as well. We as well as others have recently begun to explore analytical applications of IRIS [27–35], particularly in small-molecule identification. Our focus has mainly been on (bio)medical applications, most notably in the field of metabolomics. Our groups were the first to obtain IRIS spectra for individual metabolites in patient samples, including urine, plasma and cerebrospinal fluid (CSF) [28]. This article presents an overview of recent IRIS-based studies that aim to advance the technique as an analytical method, and presents several perspectives of analytical IRIS. The combination of IRIS with orthogonal separation methods commonly hyphenated to MS, most notably high-pressure liquid chromatography (HPLC) and ion mobility [29,36–38], further pushes towards realizing the full analytical potential of IRIS. We present here an overview of several approaches currently being pursued to combine IRIS and HPLC for the identification of small molecules directly from complex samples, such as body fluids or plant extracts.

Identification of structural and stereo-chemical properties of unknown compounds by MS is a commonly encountered bottleneck in the (bio)analytical sciences [39]. This challenge has several levels of uncertainty related to the degree of known versus unknown character of a given analyte. Targeted analytical approaches focus on known compounds that are expected (or not expected) in a sample based on, for example, (bio)chemical or metabolic knowledge, and have structures that are well established. The goal of a targeted analysis is to confirm the presence/absence of a particular compound(s) and often to quantify the actual amount present in the sample. On the other hand, untargeted analyses aim at a more generalized, holistic screening. The goal is to detect everything and pick out the knowns by database matching, which is fairly routine. The biggest challenge is then to identify the remaining unknown but detected compounds without *a priori* knowledge of their chemical structures.

Infrared (vibrational) spectroscopy is an analytical technique that probes the frequencies of vibrational resonances of molecules, which are determined by the nature of chemical bonding in the molecular system. These frequency values are characteristic for the molecular structure and the technique is therefore frequently

applied, for example, in organic chemistry laboratories to determine the presence (or absence) of specific functional groups in a molecule in order to characterize the products of a chemical reaction. Functional groups carry unique and predictable vibrational resonances that are normally limited to a narrow range of the overall IR spectrum. Also, for nearly any molecular structure, vibrational frequencies can be reliably and efficiently predicted on the basis of modern quantum-chemical calculations, particularly when molecules are isolated in the gas phase.

For samples in solid (*e.g.* KBr pellet), liquid (*e.g.* solution) or gas phases, Fourier Transform infrared spectrometers (FTIR) are convenient instruments. Sample density and optical pathlength can usually be adjusted such as to bring the attenuation of the IR beam into an observable range. However, this is not the case for the gaseous ions inside an ion trap: due to Coulombic repulsion and the absence of a dielectric solvent and counterions, densities are limited to values that are about 10 orders of magnitude below what is used for typical FTIR experiments. Therefore, IRIS makes use of tunable IR lasers and is not based on monitoring light attenuation. Instead, IRIS is a tandem MS technique based on wavelength-dependent IR photo-fragmentation of mass-selected ions inside an (ion trap) MS. The detection of MS/MS fragment ions correlates with the absorption of IR photons occurring when the frequency of the laser is resonant with one of the allowed vibrational transitions of the precursor ion population.

It is interesting to note that the sensitivity of IRIS experiments is thus exactly the same as for any other tandem MS/MS experiment, *i.e.* an IR spectrum can be generated for any *m/z* peak having sufficient intensity for an MS/MS measurement. IRIS can be regarded as an additional MS^n stage that can be tagged onto the end of any MS-based protocol. The general workflow is illustrated in Fig. 1 .

Fig. 1 illustrates the analytical gain in coupling an IRIS workflow to an MS-based method for small molecule identification (see Fig. 2). Panel **a** shows an *m/z* feature of possible interest in a sample of a patient having N-Acetylneuraminic Acid Synthase (NANS) deficiency [28,40], which is absent in a control set (not shown), as may be encountered for instance in an untargeted small molecule screening experiment. The *m/z* of interest is mass-isolated in the MS (**b**) and its IR spectrum can be measured *in situ* using IRIS (**c, d**). This IRIS spectrum can subsequently be matched to IRIS spectra of reference compounds obtained by direct infusion of the standards (**e**). This allows the unambiguous identification of, in this case, one of three N-acetylhexosamines. Clearly, IRIS identifies N-acetylmannosamine as the metabolite, which is indeed the substrate of the defective NANS-enzyme and which confirms identification obtained recently by NMR [40]. Note that these molecules are not distinguishable based on common LC-MS/MS methods in research-level analytical labs – the molecules are not retained, and thus co-elute, using reversed-phase liquid chromatography and have nearly identical MS/MS fragmentation spectra. An interesting opportunity of IRIS based identification, is that the reference spectra may also be generated by quantum-chemical predictions of the IR fingerprints, which would mitigate the need for reference compounds that – unlike the situation illustrated in Fig. 1 – may not always be available.

Here, we present an overview of recent progress made in extending the use of IRIS as a generalized analytical workflow for small-molecule identification. Focusing on the recent application of IRIS in the field of metabolomics, we will offer a perspective on future directions and applications of IRIS as an analytical technique, especially in combination with routine separation methods such as liquid chromatography. Furthermore, the combination with computational chemistry is elaborated upon and we show an example of reference-free identification using predicted IR spectra *in lieu* of spectra of physical reference materials.

2. Methods

2.1. A brief tutorial on infrared ion spectroscopy

Classical infrared absorption spectroscopy is based on detecting the extent of attenuation of an IR beam after it is passed through a sample. For molecular ions in a (storage) mass spectrometer, this attenuation is not measurable as the maximum attainable number density of ions is many orders of magnitude too low. In place of directly measuring absorption, the mass spectrometer is used to detect the effect – or "action" – of IR absorption: dissociation of the molecular ion.

The energy of an infrared photon is small compared to typical bond dissociation energies; with typical covalent bond energies on the order of several hundreds of kJ mol^{-1} and IR photon energies in the range of ~10–40 kJ mol^{-1}, the absorption of tens or even hundreds of IR photons is required to induce the action. Fundamentals of the process of IR multiple-photon excitation in polyatomic molecules have been extensively studied experimentally and theoretically [41–44]. Vibrational potentials are inherently anharmonic and absorption of tens-to-hundreds of photons occurs in a non-coherent manner; ladder-climbing in a single vibrational well is excluded as resonance with the laser frequency would rapidly be lost as the system is excited. Instead, intramolecular vibrational redistribution (IVR) quickly diffuses the energy deposited in the excited vibrational coordinate over all other vibrational degrees of freedom before the next photon is absorbed. This results in a slow heating of the molecule, typically leading to dissociation along the lowest-energy fragmentation pathway(s). Importantly, there is no relation between the excited normal mode and the dissociation channel observed; memory effects are completely washed out by the IVR process.

IR multiple-photon excitation therefore operates on the premises that the IVR rate ($\gtrsim 10^{12}$ s^{-1}) is much faster than the photon absorption rate. With moderately intense laser sources and molecules with a reasonable density of states, this is usually the case. In fact, IR multiple-photon dissociation (IRMPD) using fixed-frequency laser sources, commonly a CO_2 laser, is a well-known alternative to CID in MS/MS experiments. With the parameters of the FELIX laser [45], we estimate the absorption rate to be on the order of 10^9 - 10^{10} s^{-1} during a micropulse. Another important condition for efficient excitation is a photon absorption rate that is higher than the deactivation, or quenching, rate. Hence,

$$k_{IVR} \gg k_{abs} \gg k_{quench}$$

Although the first condition is nearly always met because of the extremely fast inherent IVR rates, the second condition is dependent on the design of the experiment. Radiative cooling is typically very low in the IR range (<100 s^{-1}), so that it is usually not a show stopper. However, competition from collisional decay becomes relevant at pressures above ~10^{-5} mbar, such as in quadrupole ion traps [46]. This can be offset by using laser sources with high (instantaneous) power levels and tight focusing of the laser beam. Depending on the size of the ion cloud, a tight laser focus may reduce the residence time of the ions in the focus; the use of pulsed laser sources can be advantageous as the instantaneous power during the residence time of an ion in the focus is many orders of magnitude higher than from a continuous-wave laser source. Also, the use of ion storage mass spectrometers has the advantage of allowing for longer, and adjustable irradiation times.

IR multiple-photon excitation thus results in statistical heating analogous to the mechanism involved in low-energy CID, so that CID and IRMPD MS/MS fragment spectra are usually similar. However, it is also noted that differences may be observed due to

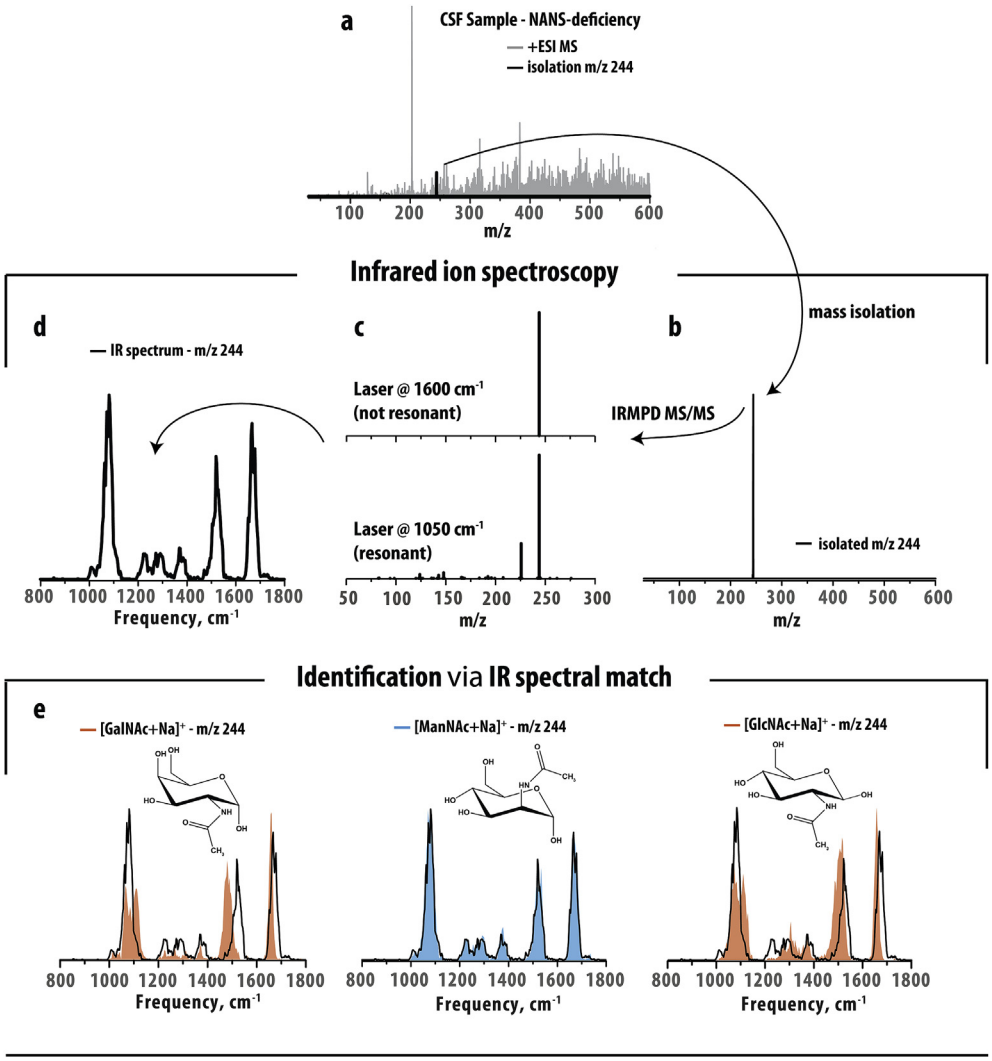

Fig. 1. Illustration of a typical experiment using IRIS to distinguish isomers. Panel **a)** presents a sample MS spectrum of the CSF of a NANS-deficiency patient. A potential biomarker is identified based on the absence of this mass in control samples (not shown). Panel **b)** illustrates mass isolation, which is followed by irradiation by the IR laser in panel **c)**. Upon resonance, photo-fragmentation is observed. Plotting the extent of fragmentation in the mass spectrum at each frequency of the IR laser produces the IR spectrum in panel **d)**. An analogous workflow can be used to generate reference IR spectra from directly-infused standards, allowing structural assignment based on comparison with the IR spectrum of the unknown (panel **e**) [28].

secondary dissociations in IRMPD, as a result of fragment ions having vibrational resonances at frequencies identical to that of the precursor ion. This is uncommon in resonant, *i.e.* mass-selective CID in quadrupole ion traps or in FTICR instruments (sustained off-resonance irradiation, SORI-CID). Taking the ratio of the sum of all fragment ion intensities to the total ion intensity (precursor + fragments) gives a normalized fragmentation signal referred to as the yield (Y)

$$Y(\lambda) = \frac{\sum I_{frg}}{I_{pre} + \sum I_{frg}}$$

Recording the yield for a series of mass spectra taken with the laser stepping through the wavelength range of interest then allows one to plot an IRMPD spectrum. One may realize that the fragment yield can saturate ($Y \approx 1$) because of depletion of the precursor ion population on strong transitions, so that it is advised to take the natural logarithm of the yield as the signal [47–49].

$$S(\lambda) = -\ln[1 - Y(\lambda)]$$

This signal, which is directly proportional to the fragmentation rate of the ions, can be interpreted as the vibrational spectrum of the precursor ion population. Many IRMPD spectroscopy experiments and comparisons with computed IR spectra (which are linear absorption spectra) have shown that an IRMPD spectrum is usually a good surrogate for a true absorption spectrum (see e.g. Refs. [8,13,15,19,21,23,50–55]).

Nonetheless, an IRMPD spectrum inherently relies on the absorption of many photons by a system whose internal energy is not constant. This induces line shifts and broadenings that depend on the topology of the underlying potential energy hypersurface, which cannot be known exactly. Empirically, many studies have shown that IR bands in an IRMPD spectrum typically undergo a small red-shift, due to anharmonicity, of a few percent at most and that bandwidths are in the range of 25 cm^{-1} [43,44], with larger bandwidths observed especially for systems with large-amplitude motions [56,57]. In systems with high barriers to dissociation, weak IR bands may become unobservable resulting in an apparent threshold in their IRMPD spectra [58–61].

Spurious effects on the spectra due to the multiple-photon

nature of the IRMPD process can be mitigated by reducing the energy required to induce dissociation of the precursor ion, ultimately down to the energy of a single IR photon. Weakly bound complexes of the ion of interest, typically formed by the non-covalent attachment of a rare gas atom or an H_2 or N_2-molecule, are investigated *in lieu* of the ion itself, and the detachment of this "messenger" [62] is used as a probe of photon absorption. The small ion/induced-dipole binding energies allow for one-photon IR dissociation and moreover perturb the system of interest only marginally. However, to stabilize these weakly bound systems, low temperatures are required such as in supersonic expansions or cryogenic ion trap mass spectrometers, which are not available commercially. These methods date back to the pioneering experiments by Okumura and Y.T. Lee [62,63], which constitute some of the earliest comprehensive IR spectroscopic investigations of mass-selected ions, but a detailed description of these methods is beyond the scope of this perspective and the reader is referred to reviews on the topic [20,64–68].

2.2. Some practical aspects of infrared ion spectroscopy

Infrared ion spectroscopy experiments have been implemented in several types of ion trap-based MS platforms, including both FTICR and linear as well as 3D multipole ion traps [6,10,12,43,46,69–72]. Originally, home-built experiments were developed offering flexibility in hardware design and synchronization between MS and laser systems. However, over the past decade the coupling of commercial MS instruments with tunable IR lasers has been implemented in several laboratories, where the efficiency and sensitivity of state-of-the-art MS platforms is a crucial aspect of the analytical application of IRIS.

In order to conduct laser spectroscopy inside the ion trap, several modifications to the MS are required. Optical access to the trapped ion population necessitates the installation of IR transparent windows on the MS vacuum housing (common materials include diamond, BaF_2, MgF_2, and ZnSe and KRS-5 among others). Depending on the type of ion trap instrument used, additional modifications are often required.

Our implementation is based on a Bruker Amazon quadrupole ion trap mass spectrometer [46,73] and the free-electron laser FELIX [45], see Fig. 2. FELIX delivers tunable IR radiation as macropulses of energies up to 150 mJ and neutral-density filters can be inserted into the beam to reduce this energy to the level required by the experiment. Each ~5 μs-long macropulse consists of a train of ~5 ps-long micropulses spaced by 1 ns. The bandwidth of the radiation is transform-limited to about 0.5% of the central frequency, which amounts 5 cm^{-1} at $\lambda = 10$ μm. Reflective optics are used to focus the beam in the center of the Paul-type ion trap via two 3-mm diameter holes in the ring electrode [6]. In addition, two gold-coated mirrors are mounted below the ion trap to guide the laser beam back out of the instrument and onto an external pulse energy detector. The mirror mount is attached to a newly introduced flange on one of the sides of the vacuum housing so that the mirrors can easily be accessed.

In addition to hardware modifications, synchronization of the MS sequence (accumulation, mass isolation, trapping delay, detection, etc.) with the (typically pulsed) laser irradiation is required [46]. This can be accomplished using a combination of hardware and software triggering. Commonly, an optical shutter is used, allowing irradiation only after the desired ion population is prepared, mass-selected and stored. Finally, data analysis often requires additional software customization, as the time dependent MS data (chromatograms) must be correlated with the IR frequency as the laser is scanned.

While the development of IRIS was most definitely driven by the development of tunable mid-IR FELs, there is significant interest in conducting these experiments using more widely available mid-IR lasers. Until now, this has mainly been accomplished using table-top optical parametric oscillators/amplifiers (OPO/OPA), producing sufficient pulse energies only in the 3 μm range and thus accessing only the hydrogen stretching vibrations. The versatility of OPO/OPA lasers for IRIS is further limited by the maximum pulse energies that are typically on the order of 20 mJ. However, the trade off between limitations of accessibility (IR-FEL) and versatility (OPO/OPA) is likely to be overcome in the upcoming years with the rapidly and continually advancing mid-IR laser technology.

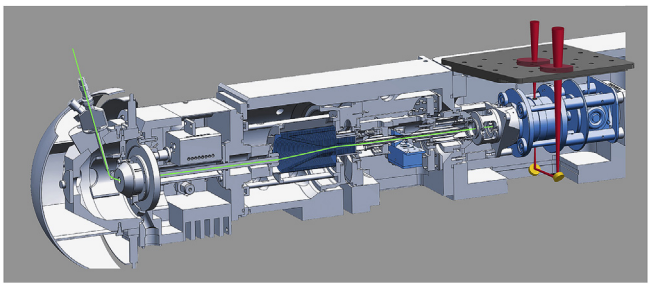

Fig. 2. Schematic drawing of a modified 3D quadrupole ion trap (Bruker AmaZon) allowing for the irradiation of the trapped ion cloud with an IR laser. Ions are formed in an ESI source and follow the path of the green trace to the trapping region. The only significant modifications to the instrument from its factory state are the installation of IR transparent windows in the vacuum housing above the ion trap and two 3 mm holes in the ring electrode of the ion trap creating an optical path for the laser to pass through the ion cloud and finally be out-coupled from the instrument. Despite a larger outflow of helium buffer gas from the ion trap itself, recalibration of the helium flow controller allows the instrument to meet all factory specifications related to sensitivity and mass resolution after these modifications for laser access [28,46]. Figure reproduced from Ref. [28] under a Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. LC-IRIS

Currently, direct infusion electrospray ionization is the most widely used ionization method for IRIS experiments. However, any ion source that can be attached to the MS can be used and other ionization sources such as electron ionization, chemical ionization and laser desorption ionization have been demonstrated. Typical LC-MS workflows use an ESI source to interface the two systems (in rare cases, apolar compounds can be ionized using an atmospheric pressure chemical ionization source, APCI). The main difference between direct infusion ESI and LC-ESI is typically related to the flow rate. Direct infusion ESI typically runs at 1–2 μL min^{-1}, while LC-ESI typically requires flow rates of 100–500 μL min^{-1}. For a typical LC method, such as discussed in the results below, 1–5 μL of sample is injected onto a column, while for direct infusion ESI experiments sample volumes of more than 20 μL are typically used.

LC-IRIS is presented here in both offline and online fashions (discussed below). In the simplest approach, automated fractionation of an LC run can be conducted over fixed time intervals (~5–10 s) into, for example, a well-plate, giving 15–85 μL fractions (when using flowrates of 200–500 μL min^{-1}). Direct infusion ESI of one of the collected fractions can then provide up to 40 min of MS signal for an "offline" IRIS experiment (40 μL at 1 μL min^{-1} flow rate) [29,55]. More sophisticated approaches using a split-flow valve, sending a small percentage of the flow to the MS and the majority to the fraction collector, enables feature detection by the MS and more accurate fraction collection (less dilution) over a desired peak. An alternative coupling of LC-IRIS using a several

minute long stop-flow method has also been shown, however the drawbacks to such an approach are significant reductions in sensitivity and separability, limiting its applicability for complex samples and overall analytical utility [33]. In all results presented here, separations were performed using a Bruker Elute HPLC system and a Waters Acquity HSS T3 C18 column (100 × 2.1 mm i.d., 1.8 μm particle size and 100 Å pore size) held at 40 °C. To fractionate more polar metabolites, hydrophilic interaction LC (HILIC) is employed using an amide column (Waters Acquity amide column, 100 × 2.1 mm i.d., 1.7 μm particles, 130 Å pore size).

2.4. Quantum-chemical calculations

In contrast to tandem mass spectrometry fragmentation spectra, IR spectra can be quickly and reliably predicted for nearly all classes of chemical compounds – and particularly well for gaseous, organic molecules – on the basis of quantum-chemical calculations. Various commercial as well as open-source software packages are now available that run density functional theory (DFT) calculations, which are particularly efficient in predicting high-fidelity IR spectra for small to medium-sized molecular structures. On a modern multiple-core computer node, a DFT geometry optimization plus harmonic frequency calculation for a medium-sized molecule (say ~200 u) takes on the order of 10 min. To address the challenge of finding the lowest-energy conformers on the hyperdimensional potential energy surface especially for flexible molecules, molecular mechanics/molecular dynamics (MM/MD) based sampling approaches have been developed to generate a comprehensive set of input structures for optimization at the DFT level. Structures within approximately 10 kJ mol^{-1} from the global minimum structure can be expected to be populated to some extent at room temperature and their predicted IR spectra have been found to match experimental spectra in the large majority of cases. Kinetic trapping in higher-lying local minima is exceptional, but occurs for instance in cases where the solution-phase structure, presumably present prior to evaporation of the ESI droplets, is very different from the gas-phase minimum [13,18,50]. Also, MS/MS fragments may adopt non-equilibrium structures when the dissociation reaction is under kinetic rather than thermodynamic control, as is for instance well known in the dissociation of protonated peptides [22,51,53].

Vibrational frequency calculations are mostly based on the harmonic approximation, corrected with an empirical scaling factor of a few percent for anharmonicity. Although implicit anharmonic frequency calculation is available in most software packages, its computational cost is significantly higher and scaled harmonic frequencies are in nearly all cases about as reliable, especially given the typical spectral resolution of IRIS of about 20 cm^{-1}. In exceptional cases severe deviations are encountered, for instance where the potential is intrinsically non-harmonic, such as for double-well potentials due to proton sharing structural motifs [14,56,57,74,75].

In the studies described here, molecular structures have been manually defined based on chemical intuition and known gas-phase ion chemistry and optimized using DFT. Specifically, we use the B3LYP functional with the 6–31++G(d,p) basis set as implemented in the Gaussian09 software package [76]. This method, and especially the B3LYP functional, has been shown to outperform most other functionals in the accurate prediction of IR spectra [77]. For each optimized molecular geometry, harmonic vibrational frequencies were computed and scaled using a factor of 0.975. Vibrational line spectra were broadened using a Gaussian line shape with a full-width-at-half-maximum (FWHM) of 25 cm^{-1} to facilitate comparison with our experimental spectra.

As we will illustrate below, the ability to identify compounds on the basis of matching spectra of the unknown metabolite with computed reference spectra, instead of experimental spectra, is one of the most interesting opportunities of IRIS analyses. It may identify unknowns without having a physical reference compound available; even if exact identification is ambiguous, matching IRIS spectra with predicted IR spectra can significantly narrow down the list of potential candidates for an unknown.

3. Results

3.1. Distinguishing closely related isomers by IRIS

Prostaglandins, as part of the larger group of eicosanoids, are well-known lipid inflammatory signal markers, both pro-inflammatory and anti-inflammatory depending on the specific compound. They are also associated with a horde of other effects, such as renal diseases, vasodilation, pain sensation, bronchoconstriction, tumor initiation as well as the recruitment of dendritic cells and neutrophils during inflammation resolution. They act as sensitive markers of pharmacodynamics and have therefore been widely investigated. Prostaglandins have a high efflux from cells to plasma and are highly reactive, so that they probe quickly and are also rapidly broken down again. Furthermore, their biological activity is often related to subtle changes in molecular structure, such as the α- or β-orientation of hydroxyl groups. In conjunction with their low concentrations, this forms an analytical challenge that often results in lengthy analytical run times.

Prostaglandin lipid compounds contain 20 carbon atoms, including a five-membered carbon ring. While playing diverse roles physiologically, in terms of chemical structures there are several highly similar isomers that are challenging to separate. Prostaglandins E2 (PGE2) and D2 (PGD2) are positional isomers (Fig. 3a) that are nearly indistinguishable by their MS/MS fragmentation spectra (Fig. 3b). Moreover, the separation of these prostaglandins using reversed-phase LC (RPLC) is a major challenge, and complicated two dimensional-LC protocols are required to isolate them from a complex matrix such as a body fluid.

On the other hand, as illustrated in panels **c** and **d** of Fig. 3, we find PGE2 and PGD2 to be readily distinguishable on the basis of their IR spectra. In Fig. 3d, an overlay of their IR spectra in the vibrational fingerprint region is presented.

PGE2 and PGD2 show differences in the intensities as well as in the frequencies of their IR bands, presenting several positions where IR features are well resolved from each other. Two wavelength positions, at ~1650 and just above 1700 cm^{-1}, are diagnostic of PGD2, and a band at ~1750 cm^{-1} is diagnostic of PGE2, which enables one to distinguish these isomers from one another by using a fixed-frequency IR laser at one of these diagnostic features. Additionally, the black trace in panel **d** of Fig. 3 represents the spectrum of a 50:50 mixture of PGE2 and PGD2, which suggests that the relative abundances of the two isomers in a mixture can be established by recording this two-peak IR feature, resulting from the two slightly different C=O stretch bands. This example suggests that the spectrum of a mixture of isobars can be chemometrically deconvoluted into its individual contributions if spectra of the isolated reference compounds are available. The presence of resonant frequencies diagnostic for one of the two isomers but not for the other also allows for a two-laser isomer-selective IR-IR scheme that offers the possibility to measure the IR spectrum of a single component in a mixture of isobaric ions; in this case, a high-power IR laser fixed at 710 cm^{-1} would photo-dissociate, and thus remove, all ions absorbing at that frequency (PGD2), so that a second, tuneable, IR laser can selectively record the IR spectrum of the remaining ions (PGE2). This method has been demonstrated for an enol/aldehyde isomer pair in Ref. [54]. In cases where two isomers show different MS/MS fragments, individual IR spectra may be extracted from the IR-induced dissociation into each of the

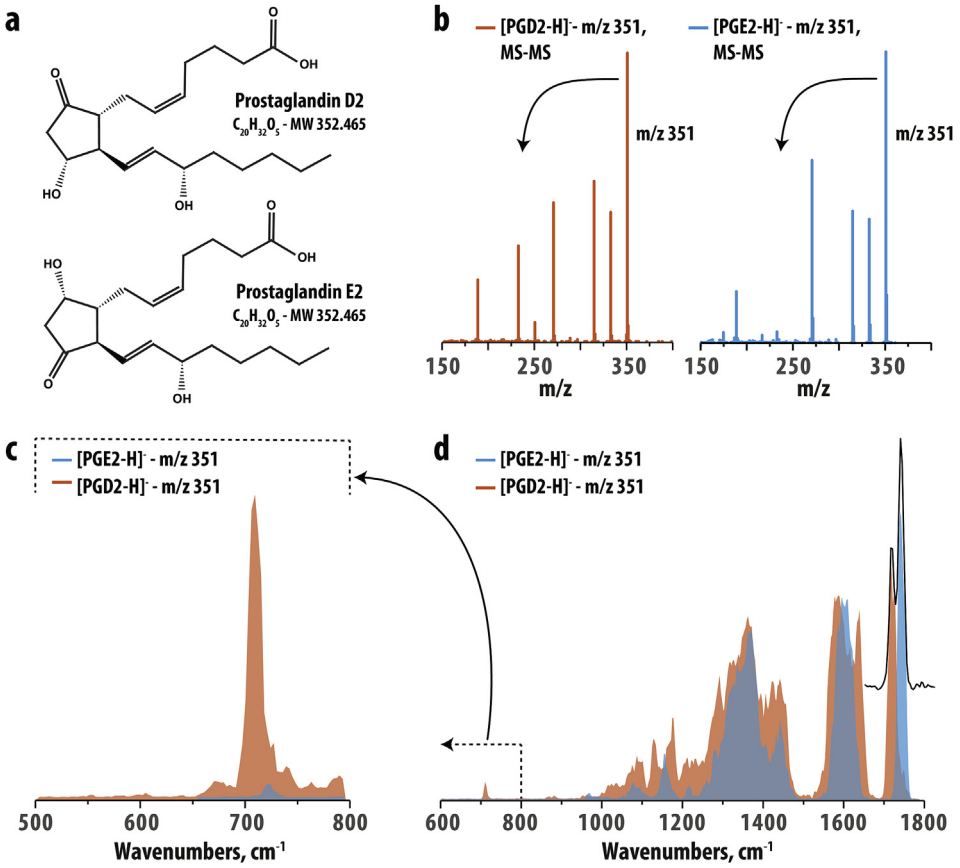

Fig. 3. Panel **a)** contains the structures of prostaglandins D2 and E2. Their nearly identical tandem MS/MS fragmentation spectra are present in panel **b)**. Panels **c)** and **d)** show the infrared spectra of deprotonated PGE2 and PGD2 in the spectral range of 500–1800 cm^{-1}. The black trace in panel **d)** is measured for a 50/50 mixture of the two prostaglandins.

different fragment channels; see Ref. [78] for an example.

3.2. Online combination of LC and IRIS illustrated using 2,5-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid

Even in cases where two isobaric compounds can be separated using LC, their identification is often not straightforward. Many analytical workflows attempt to identify molecular structures via retention time matching using reference compounds. A major limitation of this approach is that retention times are poorly reproducible between instruments and labs, thus making the use of retention time databases challenging. Additionally, retention times are challenging to predict *in silico*, making identification in absence of physical standards nearly impossible.

In order to demonstrate different approaches for the online coupling of (U)HPLC and IRIS we selected two structurally similar isobaric phenolic acids involved in human metabolism, 2,5-dihydroxyphenylacetic acid (homogentisic acid, biomarker for Alkaptonuria) and a positional isomer 3,4-dihydroxyphenylacetic acid (DOPAC, a metabolite of dopamine), as shown in Fig. 4a. Alkaptonuria is an inborn error of human metabolism caused by the deficiency of the enzyme homogentisic acid 1,2-dioxygenase that plays a role in tyrosine metabolism. Patients accumulate large amounts of homogentisic acid, the substrate of the enzyme, which is excreted in their urine. While these compounds can be separated using a standard RPLC method, they cannot be identified by their MS/MS fragmentation spectra. Here we illustrate the potential of LC-IRIS in such cases.

The (resonant) CID MS/MS spectra presented in Fig. 4b show that the deprotonated ions of homogentisic acid and DOPAC (*m/z* 167) give identical fragmentation patterns, both undergoing decarboxylation yielding a fragment ion at *m/z* 123. Panel **c** shows, in sharp contrast, the unique IR spectral features of the two ions. The most prominent difference in the two IR spectra is the presence of an intense and well-resolved carbonyl stretching feature just below 1800 cm^{-1} in the spectrum of DOPAC, which is absent for homogentisic acid. This indicates that the carboxylic acid group is not deprotonated in DOPAC to give a carboxylate anion, as is apparently the case for homogentisic acid. Deprotonation of DOPAC likely occurs on one of the adjacent hydroxyl moieties, leaving the remaining proton shared between the two O-atoms. In homogentisic acid, the negatively charged carboxylate possesses a red-shifted carbonyl stretch at approximately 1650 cm^{-1}.

In contrast to LC retention times, vibrational frequencies are intrinsic to a molecular structure and independent of experimental conditions or platforms; IR spectra are therefore suitable for reference databases. Often, a database search (such as from the Human Metabolome Database, HMDB [79]) of a feature detected in an LC-MS experiment returns multiple isobaric compounds. IR spectra reveal the presence (or absence) of specific functional groups that can aid in the identification of an unknown from a list of candidate structures. As well, comparison of the experimental IR spectrum of an unknown versus quantum-chemically predicted spectra for arbitrary candidate structures can be used to quickly screen the candidates and narrow down the list of potential structures. For instance, a search of the chemical formula of homogentisic acid ($C_8H_8O_4$) in the HMDB, METLIN and ChemSpider results in 18, 127 and 494 distinct candidate structures, respectively. If this were an unknown compound, the functional group information contained in its IR spectrum would offer the possibility

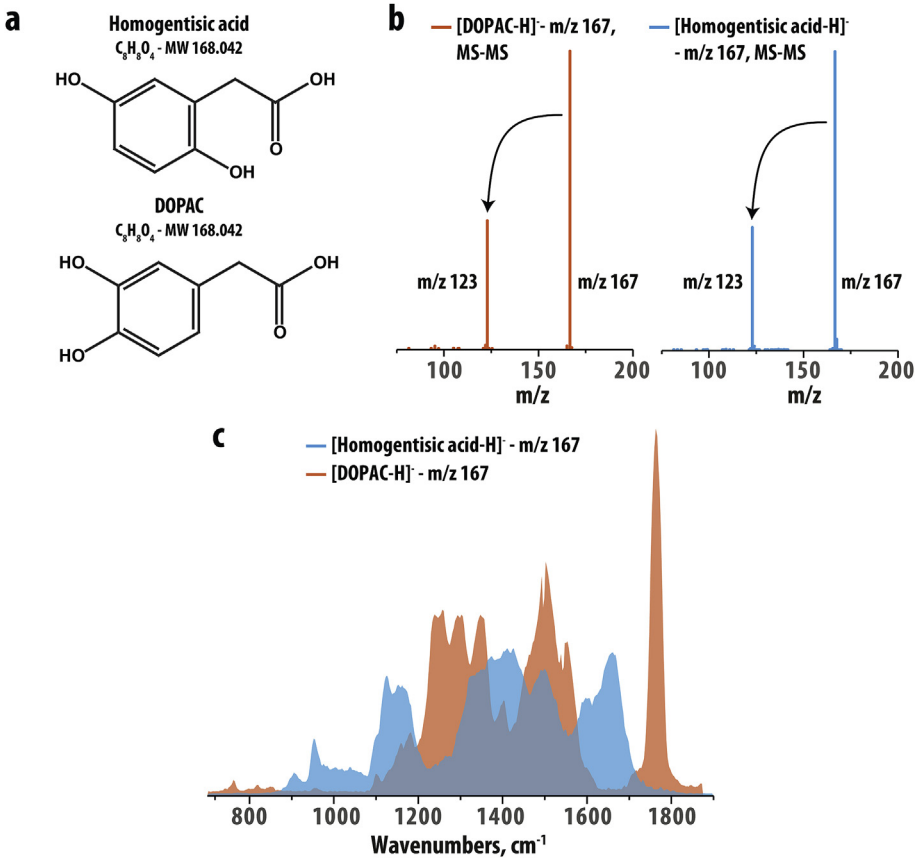

Fig. 4. Structures of 2,5-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid are shown in panel **a)** and their indistinguishable tandem MS/MS fragmentation spectra in panel **b)**. Panel **c)** contains the infrared spectra of deprotonated 2,5-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid in the spectral range of 750–1850 cm^{-1}.

to quickly and significantly narrow down this list.

The main challenge combining IRIS and analytical (U)HPLC in an online fashion is to find a compromise between the time-scales of the two experimental techniques. A full IR spectral scan on our analytical IRIS set-up, typically from 1000 to 1800 cm^{-1}, requires approximately 10 min, which is much longer than a typical LC transient peak (typically 10–30 s on our system). Different approaches to mitigate this mismatch have been investigated in proof-of-principle experiments [29,33]. For the targeted identification of metabolites (*i.e.* to distinguish between two or more known metabolites), one or a few diagnostic wavelengths may be used instead of scanning across a broad IR spectral range as already mentioned for the examples of PGD2 and PGE2. Fig. 4 suggests that homogentisic acid can be distinguished from DOPAC by IR-induced photo-fragmentation at both 1650 or 1760 cm^{-1}, corresponding to selective absorption frequencies of either one of the isomers. Recording a single-wavelength IR photodissociation MS/MS spectrum is readily compatible with the transient ion signals in an HPLC-MS run. A significant level of IR-induced dissociation is typically already obtained from irradiation with a single laser pulse, enabling the integration of HPLC-MS with IRIS in an online fashion. Fig. 5 demonstrates the distinction of homogentisic acid and DOPAC using resonant IR photo-fragmentation at frequencies selective for either one or the other isomer. Panel **a** shows an extracted ion chromatogram (EIC) of *m/z* 167 giving two features resulting from the separation of a mixture of homogentisic acid and DOPAC. The identity of each of the peaks cannot be determined using CID MS/MS of *m/z* 167 since both isomers give the same fragment (*m/z* 123). However, IR induced photo-fragmentation at either 1650 cm^{-1} or 1760 cm^{-1} leads to the selective fragmentation of only one of the two species at each frequency. Fig. 5b shows the EIC of the *m/z* 123 IR induced photo-fragment ion during the chromatographic run with the IR laser at either one of the diagnostic wavelengths. Fig. 5c and d shows the underlying IR photo-fragmentation MS spectra at the center of each of the LC peaks. Single-wavelength IRIS thus offers particularly interesting possibilities for the development of table-top platforms employing commercially available low-cost laser sources with a limited wavelength tuning range for the identification of targeted metabolites.

When metabolites are not distinguishable based on one or a few single wavelengths, but rather based on subtle spectral differences, a scan over a broader wavelength range is typically required. As well, full-range spectral scans over the entire vibrational fingerprint region are necessary in order to identify previously unknown metabolites. Therefore, in order to integrate such experiments with LC timescales, the speed of a spectral scan needs to be increased in order to obtain a full spectrum during the elution of an LC-peak. Standard IR experiments on our IR-FEL [45] are performed using a step-scan protocol, in which the FEL wavelength is stepped to the next value after recording each photodissociation MS/MS spectrum. The time-limiting step is the accurate positioning of the laser frequency (undulator magnet arrays) at each step. Alternatively, one can let the FELIX wavelength scan continuously over the fingerprint region in less than 1 min. An IRIS spectrum can then be obtained by continually acquiring IR photo-fragmentation MS/MS spectra over an HPLC peak while freely tuning the FELIX frequency. In our demonstrative experiment presented here, no MS averaging was used and ions were irradiated with two pulses from FELIX per MS/

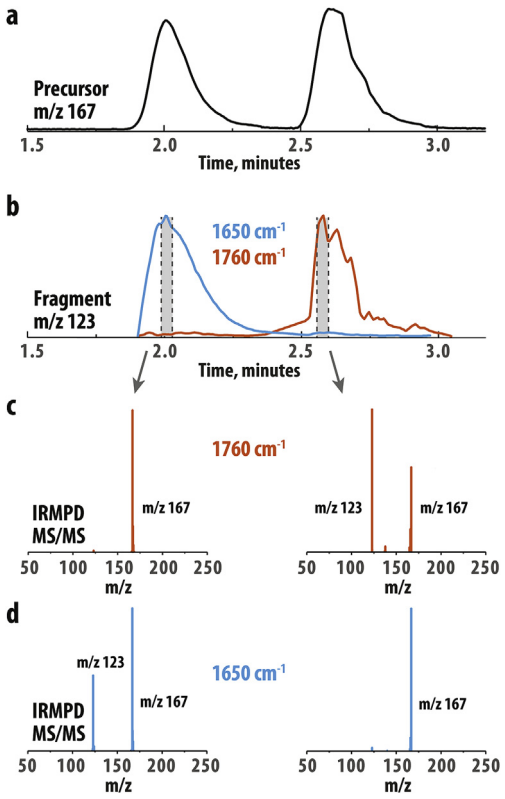

Fig. 5. LCMS trace of the *m/z* 167 EIC of a mixture of homogentisic acid and DOPAC (panel **a**). Although the MS/MS fragmentation patterns are identical (neutral loss of 44 mass units), panels **b-d** demonstrate that the identity of the two features can be established using their photo-dissociation response at two single IR wavelengths, which are selected to be absorbed by either one of the two isomers only, as derived from their full-range IRIS spectra displayed in Fig. 4.

MS spectrum. This results in three IR photo-fragmentation mass spectra per second.

Fig. 6a contains the LC-MS data for a separation of an alkaptonuria patient urine sample where homogentisic acid (*m/z* 167) elutes from 6.8 to 7.2 min as the base *m/z* peak. An IRIS spectrum was obtained by freely tuning the laser frequency from 5.74 to 9.48 μm in the ~40 s elution time window. An overlay between the resulting IRIS spectrum and a reference IRIS spectrum obtained using the regular step-scan experimental protocol is shown in Fig. 6b. Despite the fact that averaging of MS spectra is no longer possible in this quick-scan protocol, the two spectra are reasonably identical, which holds promise for the further development of online LC-IRIS using the IR-FEL or alternative laser sources.

3.3. Reference-free small molecule identification using in-silico predicted IR spectra

Untargeted analyses using (tandem) MS rely heavily on database matching and hence on the availability of reference compounds. This presents a challenge when considering the identification of an unknown, as reference compounds may not be available and their MS/MS spectra are by definition not known. While efforts have gone into predicting MS/MS fragmentation spectra for arbitrary chemical structures, both from scoring algorithms based on known fragmentation patterns for various functional groups and chemical classes as well as from methods based on quantum-chemical simulations, until now the inaccuracy of these methods prevents their widespread use for accurate molecular structure identification. Additionally, the synthesis of potential reference compounds is often very time consuming and expensive. In comparison, IRIS offers a convenient alternative, since IR spectra can be reliably and routinely predicted for nearly all relevant classes of chemical compounds with the use of widely available computational protocols. Quantum-chemically predicted spectra can then be compared to the IR spectrum measured for an unknown detected in an (LC) MS experiment.

Here we present an example from an untargeted LC-MS experiment to highlight how this approach can be used for reference-free molecular identification within an IRIS based workflow.

LC-MS analysis on plasma samples of two siblings with a neurological disease of unknown etiology repeatedly showed a feature of unknown molecular identity with *m/z* 100.0757 ($[C_5H_9NO + H]^+$) and retention time of 3.9–4.1 min on RPLC in both children. The plasma samples were obtained from a hospital abroad. MS/MS experiments, database searches and whole-exome sequencing were not helpful in determining the identity of the compound giving rise to this feature. Entering the chemical formula into the METLIN database returned 92 potential matches.

IRIS-spectra were recorded using our LC-MS/MS platforms and FELIX to find the identity of the unknown compound in the samples from the two siblings. IR spectra were calculated for the protonated forms of the compounds indicated by the METLIN search, starting

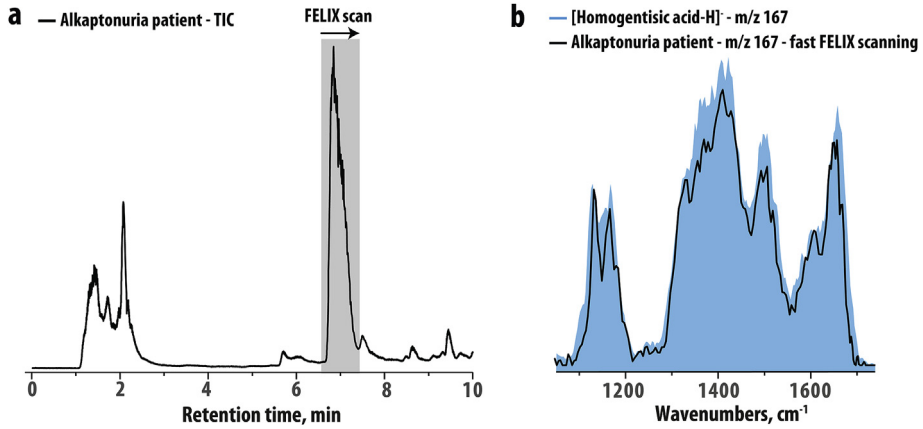

Fig. 6. The TIC of a standard analytical LC-MS experiments of a urine sample from an alkaptonuria patient (panel **a**). Panel **b** shows the IR spectrum measured online in 40 s for the isolated *m/z* 167 feature with RT 7.1 min. This spectrum is compared to an IR spectrum measured for the homogentisic acid (*m/z* 167) reference compound using the standard step scanning protocol of the IR laser.

with the most likely structures according to the database ranking. Fig. 7 displays comparisons of the experimental IR spectrum of the unknown feature versus the calculated IR spectra of four of the candidate molecular structures. The calculated spectrum of N-methyl-2-pyrrolidinone in panel **d** is seen to give the best match, especially in terms of the band frequencies, which suggests that this is the mostly likely candidate for the unknown feature. With this information in hand, a reference sample of N-methyl-2-pyrrolidinone, which is commercially available, was purchased and its IRIS spectrum was recorded using direct infusion of a 100 nM solution of the compound. Comparison with the IRIS spectrum of the unknown in the patient sample in panel **e** shows a definitive match.

Clearly, narrowing down the list of potential candidates based on the comparison of the IRIS spectrum of the unknown with the *in silico* predicted spectra for candidate structures suggested by the database before comparison with a physical standard greatly reduced the time, effort and cost required to identify this feature. N-methyl-2-pyrrolidinone has thus far not been identified as a human metabolite and moreover, further analyses on plasma of a healthy brother and from an unrelated person from abroad revealed the presence of the same compound in similar concentration. The identification of this feature as N-methyl-2-pyrrolidinone thus allowed us to determine that its presence is in all likelihood an artifact of the blood plasma sampling procedure. N-methyl-2-pyrrolidinone is used as a solvent in the industrial production of polymers.

3.4. IRIS combined with MS^n

IRIS is typically implemented on storage mass spectrometers, for instance on Paul-type ion traps such as in this work, or on linear ion traps or FTICR-MS platforms. These instruments typically have extensive possibilities for MS/MS and MS^n, using collision-induced dissociation (CID) or other fragmentation methods, such as electron-induced dissociation (ETD, ECD) and photodissociation (UVPD, IRMPD). Interestingly, IRIS can also be applied to these MS/MS fragments, and even to MS^n fragments. From a fundamental ion chemistry perspective, identification of product ion structures may provide insight into the dissociation mechanisms and guide computational investigations of the transition states involved in the reaction. As an example, many IRIS investigations have addressed the fragmentation products of protonated peptides to elucidate the dissociation mechanisms underlying MS-based peptide sequencing methods [22–25,51,52,80,81]. From an analytical viewpoint, resolving these fragment molecular structures can aid in a bottom-up approach to identify the full molecular structure. Moreover, it can be used to correctly annotate fragment mass spectra.

As proof-of-principle of such a bottom-up structure elucidation, a recent study [55] reported identification of metabolites in the plasma sample of a patient having hyperlysinemia, an inborn error of metabolism characterized by high plasma L-lysine levels. Using HILIC chromatographic separation, we indeed observed elevated levels of an ion at the mass of lysine ($C_6H_{14}N_2O_2$ at m/z 147) and collected fractions of eluent containing this ion. Dominant CID

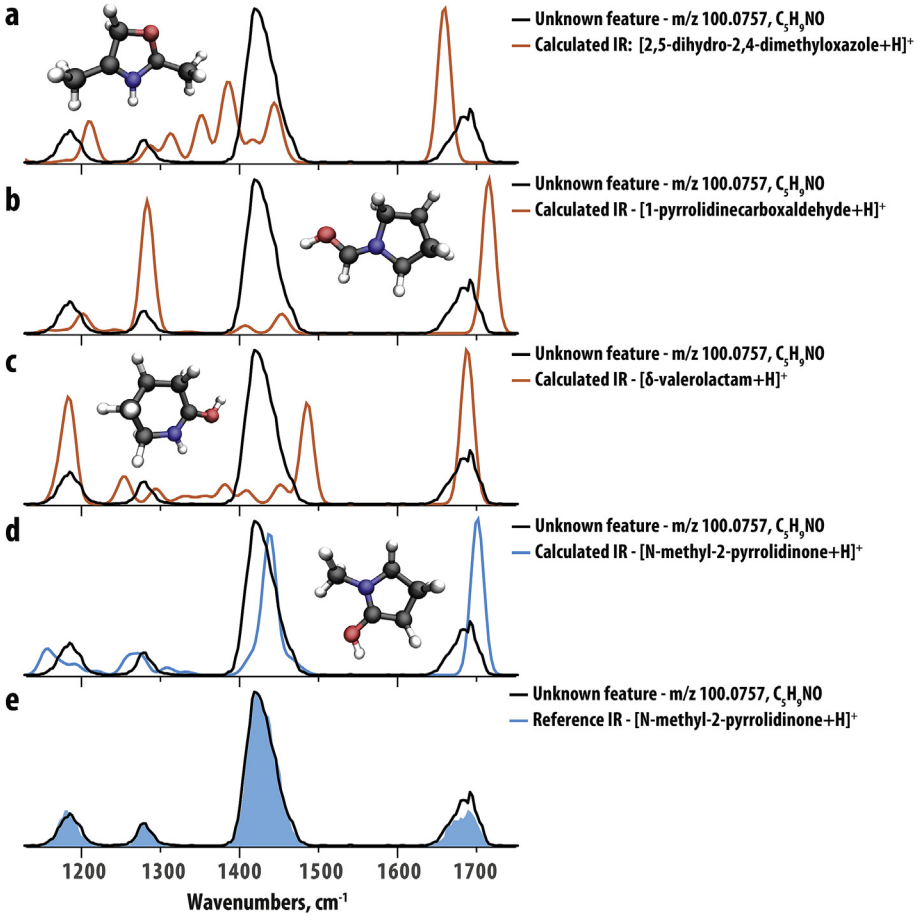

Fig. 7. Computed IR spectra (coloured traces, panels **a-d**) of potential candidate structures resulting from a database search for an unknown feature at m/z 100.0757 compared to the IRIS spectrum of the unknown feature (black trace) from the patient sample. Panel **e** compares the IR spectrum of the reference compound N-methyl-2-pyrrolidinone identified by the match found for the predicted spectrum.

fragmentation by neutral loss of ammonia (−17) forming *m/z* 130 is observed (Fig. 8a), which may suggest L-lysine, but does not exclude various isobaric alternatives. Fig. 8b compares the IRIS spectrum of the *m/z* 147 ion to the quantum-chemically predicted spectrum of protonated L-lysine. The experimental spectrum shows relatively broad and poorly defined features, which likely finds its origin in the strong hydrogen bonds involving the added proton and the anharmonic nature of the potential energy surface for such moieties in combination with the IRMPD method [56,57]. Identification based on this spectral comparison is therefore less confident. An IR spectrum was also recorded for the *m/z* 130 fragment of the precursor isolated from the patient sample. This spectrum was compared to quantum−chemically computed IR spectra for several candidate structures for the fragment ion, where a reasonably good match was found with protonated pipecolic acid (Fig. 8c). Fig. 8d rationalizes the formation of this ion from protonated L-lysine. Of particular interest here is the observation that the fragment ion spectrum shows sharper and better defined features, likely due to the higher rigidity of the cyclic structure and the absence of proton sharing. Taken all together, the MS and MS/MS data, the IRIS spectra of precursor and fragment ions, their resemblance to the computed IR spectra, and a plausible precursor-to-fragment reaction pathway present strong evidence in favor of identification of the metabolite as lysine.

4. Conclusions and outlook

We have presented several perspectives of the application of IRIS in MS-based metabolomics, and in particular in the identification of low-abundance unknowns in body fluids. Several proof-of-principle experiments show the diagnostic power of this combination of mass spectrometry and IR laser spectroscopy, which can be incorporated in existing MS-based workflows. Combination with HPLC-MS methods enables individual IR spectra to be recorded for isomeric species coexisting in a sample. Applications combining IRIS with ion mobility mass spectrometry (including also field-asymmetric IMS, FAIMS) have also been demonstrated recently, further increasing the separation capabilities, enabling for instance IR spectra to be recorded for individual tautomers, diastereomers or conformers [13,82]. Also, the predictive power of quantum chemistry is currently far greater for IR spectra than for MS/MS spectra, emphasizing the perspective of reference-free identification, as explicitly exemplified in this contribution. This is also one of the main advantages of IRIS over its UV/vis analogue, UV photodissociation ion spectroscopy (UVPD, see e.g. Ref. [83]): an IR spectrum generally contains more diagnostic features that are also more reliably predictable as compared to UV spectra. Moreover, UV/vis spectroscopy is limited to species with UV chromophores, and spectral features are generally significantly broadened at room temperature (comparable to solution-phase UV spectra). Cryogenic UV ion spectra can reach good spectral resolution [83], which has also been exploited in analytical settings [84], but the liquid He-cooled MS instrumentation required for such experiments is (commercially) not widely available. An advantage is the good availability of tunable UV/vis laser sources suitable for UVPD applications.

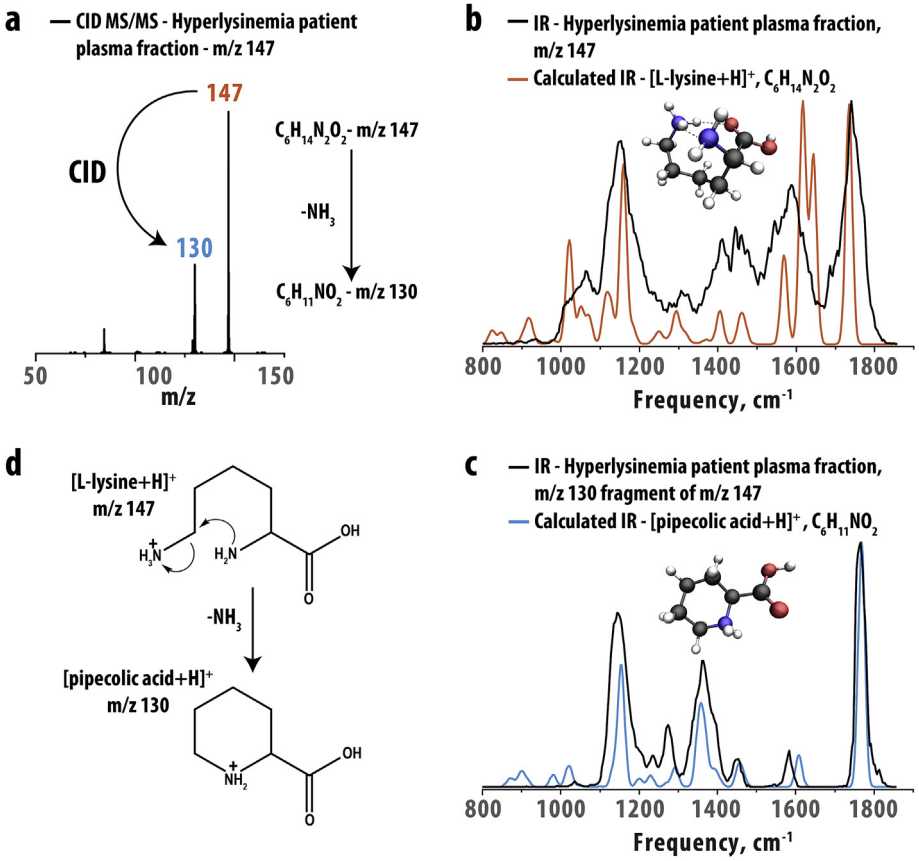

Fig. 8. Proof-of-principle IRIS analysis of a metabolite at *m/z* 147 ($C_6H_{14}N_2O_2$) in the plasma of a hyperlysinemia patient. The accurate mass information and the MS/MS fragmentation (**a**) suggest lysine as a possible candidate structure, but this cannot be securely established on this basis alone. The IRIS spectrum of *m/z* 147 suffers from a relatively poor spectral resolution and cannot be unambiguously matched to the computed spectrum of protonated lysine (**b**). The IRIS spectrum of its *m/z* 130 CID fragment matches well with the DFT computed spectrum for protonated pipecolic acid (**c**), which is a plausible NH_3-loss fragment from protonated lysine (**d**).

Until recently, a large majority of IRIS-based studies have utilized one of the IR-FEL facilities with MS platforms coupled to the IR beamline (Nijmegen, Orsay, Berlin, Tokyo). Other facilities operate IR FELs that are likely suitable for IRIS if appropriate MS instrumentation is installed (Dresden, Osaka, Kyoto, Hefei). Alternatively, table-top tuneable mid-IR laser sources, such as optical parametric oscillators (OPO), can be utilized, although most systems currently available have relatively low output power and limited tuning ranges. This together with the requirements for a relatively high level of technical expertise for operation have limited the extent of application. However, with the quickly advancing development of new mid-IR laser sources (such as several already commercially available quantum cascade laser systems [85], robust OPO systems [86], and hybrid fiber-bulk chalcogenides based laser systems [87]), and the progress to make these systems "turn-key", the outlook for placement of IRIS-enabled MS-systems in state-of-the-art analytical laboratories requiring the most advanced techniques for small molecule identification looks rather positive. This is especially true for labs that do not require large wavelength tuning ranges, such as in targeted screening experiments as outlined above. In order to facilitate such developments, our group is working towards the inclusion of both IRIS generated IR spectra as well as *in silico* predicted IR spectra for sets of isobaric metabolites in the human metabolome database (HMDB [79]). The inclusion of full-range reference IR spectra (using the IR-FEL) will enable the advance of IRIS experiments using table-top infrared sources with more limited tuning ranges through the identification of spectral regions of interest that are selective to one (or a set of) target compound(s). Additionally, the demonstration of examples of isobaric compounds that are challenging to distinguish by standard LC-MS approaches, but distinguishable by IRIS, will enable other researchers to evaluate if their questions of small-molecule identification can be resolved on the basis of IRIS.

Summary

A perspective on the analytical potential for small-molecule identification by infrared ion spectroscopy.

Ethical approval

The work described in this study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All patients (or their guardians) approved of the possible use of their left-over samples for method validation purposes, in agreement with institutional and national legislation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Jonathan Martens, Rianne E. van Outersterp, Rob J. Vreeken, Filip Cuyckens, Karlien L. M. Coene, Udo F. Engelke, Leo A. J. Kluijtmans, Ron A. Wevers, Lutgarde M. C. Buydens, Britta Redlich, Giel Berden, and Jos Oomens declare no conflicts of interest.

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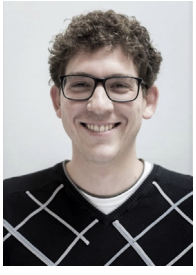

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