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Rianne E. van Outersterp, Jonathan Martens, Giel Berden, Valerie Koppen, Filip Cuyckens, Jos Oomens

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Distinguishing positional isomers, such as compounds having different substitution patterns on an aromatic ring, presents a significant challenge for mass spectrometric analyses and is a frequently encountered difficulty in, for example, drug metabolism research. Here, we demonstrate infrared ion spectroscopy (IRIS) as a promising new mass spectrometry-based technique that easily differentiates between positional isomers of disubstituted phenyl-containing compounds. By analyzing different substitution patterns over several sets of isomeric compounds, we show that IRIS produces a highly consistent and distinct pattern of IR bands, especially in the range between 650 and 900 cm\(^{-1}\), that are mostly independent of the specific chemical functionality contained in the substituent group. These patterns are accurately predicted by quantum-chemically computed IR spectra and correspond well with tabulated IR group-frequencies known from conventional absorption spectroscopy. Therefore, we foresee that this method will be generally applicable to disubstituted phenyl-containing compounds and that direct interpretation of experimental IRIS spectra in terms of ortho-, meta- or para-substitution is possible, even without comparison to experimental or computationally predicted reference spectra. Strategies for the analysis of larger compounds having more congested IR spectra as well as of compounds having low (electrospray) ionization efficiencies are presented in order to demonstrate the broad applicability of this methodology.

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Mass spectrometry-based identification of ortho-, meta- and para-isomers using infrared ion spectroscopy

Rianne E. van Outersterp¹, Jonathan Martens¹, Giel Berden¹, Valerie Koppen², Filip Cuyckens²* and Jos Oomens¹,³*

¹Radboud University, Institute for Molecules and Materials, FELIX Laboratory, Toernooiveld 7, 6525ED Nijmegen, The Netherlands
²Drug Metabolism & Pharmacokinetics, Janssen R&D, B-2340, Beerse, Belgium
³van’t Hoff Institute for Molecular Sciences, University of Amsterdam, 1098XH Amsterdam, Science Park 908, The Netherlands

ABSTRACT: Distinguishing positional isomers, such as compounds having different substitution patterns on an aromatic ring, presents a significant challenge for mass spectrometrical analyses and is a frequently encountered difficulty in, for example, drug metabolism research. Here, we demonstrate infrared ion spectroscopy (IRIS) as a promising new mass spectrometry-based technique that easily differentiates between positional isomers of disubstituted phenyl-containing compounds. By analyzing different substitution patterns over several sets of isomeric compounds, we show that IRIS produces a highly consistent and distinct pattern of IR bands, especially in the range between 650 and 900 cm⁻¹, that are mostly independent of the specific chemical functionality contained in the substituent group. These patterns are accurately predicted by quantum-chemically computed IR spectra and correspond well with tabulated IR group-frequencies known from conventional absorption spectroscopy. Therefore, we foresee that this method will be generally applicable to disubstituted phenyl-containing compounds and that direct interpretation of experimental IRIS spectra in terms of ortho-, meta- or para-substitution is possible, even without comparison to experimental or computationally predicted reference spectra. Strategies for the analysis of larger compounds having more congested IR spectra as well as of compounds having low (electrospray) ionization efficiencies are presented in order to demonstrate the broad applicability of this methodology.

Full molecular structure identification of detected compounds is a common bottleneck in the application of mass spectrometry (MS)-based analytical workflows.¹⁻³ Often, many isobaric candidate structures may correspond to a detected m/z and the structural information yielded from MS-based experiments (such as retention times and MS/MS fragmentation patterns) is insufficient to distinguish between them and challenging to predict in silico.³⁻⁴ Synthesis of multiple reference standards is therefore often required to achieve full identification, which is usually the most time-consuming step in the analysis. Compounds containing a substituted aromatic ring form a particular challenge in their identification, as the positional isomers (ortho, meta, para) that result from a different substitution pattern are by definition isobaric and often have both very similar retention times and MS/MS fragmentation patterns. As a result, analysis of such compounds by MS often gives only partially resolved structures (i.e. Markush structures)⁵ and complete molecular identification must fall back on alternative techniques, such as nuclear magnetic resonance (NMR) spectroscopy that lack the sensitivity of MS and are thus challenging to apply to low-abundance components.

In the field of drug discovery and development, studies on metabolism focusing on degradation and detoxification of drug compounds in the body are crucial and rely strongly on MS for the detection and identification of drug metabolites in in vitro or in vivo matrices.⁶⁻⁷ While the type of metabolic reaction that occurs can most often be derived from the mass differences between the drug and its metabolites as detected by LC-MS, the exact site of the metabolic transformation is often difficult to determine. In particular, determining the site of oxidation on aromatic moieties in the molecule, the most common phase I metabolic transformation, is a commonly encountered challenge.

In contrast to mass spectrometry, infrared (IR) spectroscopy is well known as a sensitive structural diagnostic, able to differentiate between the positional isomers of substituted phenyl groups.⁸⁻¹⁰ The 2- (ortho), 3- (meta) and 4- (para)substituted isomers each possess a characteristic pattern of IR absorption bands, particularly in the spectral range between 650 and 900 cm⁻¹. Vibrational bands in this range are attributed to the out-of-plane CH bending modes of the substituted phenyl ring. However, IR spectroscopy is generally not applicable in the identification of low-abundance species in complex mixtures, such as in incubates or body fluids in drug metabolism studies, due to the lack of (mass-) selectivity: spectra are obtained for the complex mixture as a whole rather than for a single molecular component of the mixture. Circumventing this limitation, infrared ion spectroscopy (IRIS)¹¹⁻¹⁶ records IR spectra of mass-isolated ions trapped in an ion storage mass spectrometer. This yields IR information of individual MS-features while maintaining the full sensitivity and selectivity of analytical LC-MS workflows¹⁷⁻²⁰. Previous studies have shown that IR spectra of ions commonly detected in typical electrospray ionization MS experiments (i.e. protonated, deprotonated, metal adducts etc.) can differentiate between closely related isomeric structures.¹⁷⁻²⁰ This enables the identification of small molecules in complex mixtures, such as urine, plasma, cerebral spinal fluid and liver microsomes.¹⁷⁻²¹

Even in the absence of reference standards, IR spectra can relate to molecular structure by comparison with quantum-chemically computed spectra for candidate structures. In silico prediction of IR spectra using density functional theory (DFT) is generally reliable for most small molecules and in this sense gives IRIS a significant advantage over identification based on LC-MS/MS.¹⁹

In this study, we describe the differentiation of sets of positional isomers of several molecules using IRIS in order to
demonstrate that this method can be generally applied for the identification of substituted phenyl compounds. We demonstrate strategies for the analysis of relatively large (>300 Da) or non-ionizable compounds and observe (completely analogous to classical IR absorption spectroscopy) that ortho-, meta- and para-substituted positional isomers present a highly predictable pattern of vibrational bands in the 650-900 cm⁻¹ region. Calculated IR spectra are used to attribute these absorption bands to specific out-of-plane CH-bending vibrations. While very sensitive to the positional isomerism, the pattern of bands is rather insensitive to the nature of the phenyl-substituent, the full molecular structure of the compound and the nature of the ion ( protonated, deprotonated or metal adduct). Therefore, we suggest this method is generally applicable in the differentiation of non-ionizable compounds and observe (completely analogous to compounds selected previously in an investigation by ion mobility). The compounds differ in the nature of the phenyl ring can be analyzed independently. Five compounds were 2-hydroxylated (ortho) (with groups R₁ and R₂), two were 3-hydroxylated (meta) (with groups R₃ and R₄) and three were 4-hydroxylated (para) (with groups R₅ and R₆). Distinguishing the positional isomers with the same R-group on the basis of MS has been shown previously to be very challenging, even when multiple stages of fragmentation were applied.

**Fourier transform infrared spectroscopy.** Gas-phase FTIR spectra were obtained using a Bruker VERTEX 80v FTIR spectrometer and a home-built 15-cm long gas cell with ZnSe windows. The cell was evacuated using a vacuum pump and liquid samples were evaporated into the cell by heating the cell to 70°C. A background subtraction and baseline correction was applied to all spectra.

**Computational procedure.** In order to obtain theoretical IR spectra we performed quantum-chemical calculations using the Gaussian 16 software package. For the m/z 202 phenylethylpiperidine fragment ions, geometry optimizations and harmonic IR frequency calculations were performed using density functional theory (DFT) at the B3LYP/6-31++g(d,p) level. Input structures for these calculations were generated with a molecular mechanics/molecular dynamics (MM/MD) computational approach using AMBER 12. Geometries of initial guess structures were optimized at the B3LYP/6-31++g(d,p) level and, after an initial minimization within AMBER, a simulated annealing procedure up to 500 K was used to explore the potential energy landscape of the ions. Throughout the procedure, molecular structures were saved periodically as snapshots yielding 500 structures. These structures were clustered based on similarity (yielding ~10 structures) and used as input structures for the DFT calculations. The results were sorted on the basis of their free energies relative to the ground state conformer and among the lowest-energy structures (relative free energy <2 kJ/mol) the conformer with the theoretical IR spectrum that showed the best match with the experimental IR spectrum was selected. The selected conformers of the ortho-, meta- and para-isomers have a free energy of 1.5 kJ/mol, 0.5 kJ/mol and 0.1 kJ/mol relative to the ground-state conformer, respectively. This is well within the error range expected for energy calculations at this level of theory. Input structures for the caesium-adducts of the fluorobenzyl alcohols and neutral fluorobenzyl alcohols were generated using chemical intuition. DFT calculations were performed at the B3LYP/def2-TZVPP and B3LYP/6-31+g(d,p) level of theory for the caesium adducts and neutrals, respectively. All vibrational spectra were scaled using a linear scaling factor of 0.975 and the vibrational stick spectra were convoluted with a Gaussian function of 20 cm⁻¹ full width at half maximum to facilitate comparison with the experimental spectra.

**RESULTS AND DISCUSSION**

Our study focuses on a group of 10 compounds sharing a common phenylethylpiperidine substructure (see Figure 1a), which is analogous to compounds selected previously in an investigation by ion mobility. The compounds differ in the nature of the group R and the site of hydroxylation of the phenyl ring. The different R-groups are not further specified here as they are not relevant and only present to show that the dissubstituted phenyl ring can be analyzed independently. Five compounds were 2-hydroxylated (ortho) (with groups R₁, R₂, R₃, R₄ and R₅), two were 3-hydroxylated (meta) (with groups R₁ and R₂), and three were 4-hydroxylated (para) (with groups R₁, R₂ and R₃). Distinguishing the positional isomers with the same R-group on the basis of MS has been shown previously to be very challenging, even when multiple stages of fragmentation were applied.
IR spectra were recorded for the [M+H]+-ions of all compounds. Figure 1b compares the IR spectra of the three compounds (ortho, meta and para-hydroxylated) with the same substituent group, R. The three spectra are indeed distinct, especially in the low-frequency range, but the differences are small. Furthermore, the spectra are crowded and partially unresolved due to the relatively large size of the ions (m/z 460), possessing many partially overlapping IR bands (1000-1500 cm⁻¹). This makes isomer assignment difficult especially on the basis of computed reference spectra.

The extensive MS² capabilities of ion trap mass spectrometers can be used to simplify IR spectra by recording IRIS spectra not for the precursor analyte, but instead for one of its MS/MS fragment ions produced by collision-induced dissociation (CID). We expect that their IR spectra not only are less crowded, but may also contain sharper IR peaks due to reduced conformational flexibility. A similar approach to reduce conformational flexibility and thereby improve differentiation of drug metabolites was used with ion mobility spectrometry. Phase I drug metabolites were shown to be particularly well suited for this strategy as most of the molecular structure is known (i.e. the precursor drug) and only a small part of the structure requires elucidation: the site of the biotransformation. IR spectra of small fragment ions that contain the biotransformation site but not unmetabolized parts of the molecule should therefore allow us to target the structural area of interest without interference from uninteresting parts of the molecule.

Figure 2a shows the CID MS/MS spectrum of the ortho-hydroxylated compound, which is very similar to those of the corresponding meta- and para-hydroxylated compounds (see Figure S1). For all three analytes a fragment ion at m/z 202 is observed, corresponding to the phenylethylpiperidine ion resulting from loss of the R₁-group (see Figure 2b). This fragment retains the hydroxyl-group but lacks the relatively bulky R₁-group that is identical for each of the references. IR spectra for the three m/z 202 fragment ions are shown in Figure 2c. Figure 2d compares the IR spectrum of the m/z 202 fragment of the ortho-hydroxylated metabolite with the IR spectrum of its precursor. Although the two spectra have similar overall IR features, the spectrum of the MS/MS fragment indeed shows sharper and better resolved peaks.

Spectra for the three m/z 202 fragment ions are clearly distinct and allow one to distinguish the three compounds, particularly in the 600-900 cm⁻¹ range of the spectrum. This spectral region is well-known to be diagnostic for the distinction between ortho-, meta- and para-isomers in conventional FTIR spectroscopy. The isolated nature of the IR bands in the spectra also suggests that it is possible to decompose their fractional abundances in a mixture of isomers, for instance in cases where the drug metabolism yields more than one of the positional isomers. Moreover, it suggests that the three ions may be distinguished from the IR profile over only this approximately 100 cm⁻¹ range, which paves the way towards the development of rapid screening methodologies for pharmaceutical laboratories.

In order to explore the general applicability of our method, the compounds that contain different R-groups were investigated as well. Four compounds, R₁ with m/z 460 (ortho, meta, para) and R₂ with m/z 347 (ortho), were found to yield the m/z 202 fragment and their hydroxylation-site could be correctly determined based on their fragment ion IR spectra, which were identical to the ones shown in Figure 2c (see Figure S2). Due to its smaller size, the IR spectrum of the R₂-precursor ion is much better resolved than the IR spectra of the other precursor ions, probably allowing one to distinguish the positional isomers without CID fragmentation. CID of the remaining three compounds (ortho R₃ with m/z 315 and ortho/para R₅ at m/z 303) did not yield the m/z 202 fragment ion. However, as a result of the low mass of these ions, the precursor IR spectra show well-resolved diagnostic features and the two R₅-isomers could be clearly distinguished (see Figure S3).

An additional advantage of investigating the smaller fragment ions instead of their precursors involves the prediction of their IR spectra, which are both less demanding to compute and easier to assign. The IR spectra of the ortho-, meta- and para
hydroxylated m/z 202 fragment ions were computed using DFT at the B3LYP/6-31++G(d,p) level. Figure 3a shows a comparison between the experimental spectra of the m/z 202 fragments of the R₁-compounds and the theoretical IR spectra of the three ions. Although computed band intensities are not entirely in agreement with experimental data, the main spectral features differentiating the three experimental spectra are well reproduced. This suggests that in the absence of reference standards, this method may be used to identify ortho-, meta- and para-isomers with the R₁-group. As an example, Figure S4 compares the experimental IR spectrum of the m/z 202 fragment ion of the ortho-isomer to calculated spectra for each of the three isomers. Clearly, the best match is obtained for the ortho-substituted system.

The quantum-chemical calculations also allow us to assign molecular normal mode vibrations to the experimentally observed IR bands. We focus on the diagnostic CH out-of-plane modes in the 600-900 cm⁻¹ region. The most prominent of these bands are labelled in Figure 3a and their normal modes are visualized in Figure 4. Their frequencies are listed in Table 1 and compared to tabulated IR group frequencies of out-of-plane CH vibrations of aromatic groups. It is seen that a single vibrational mode involving all CH-oscillators of the phenyl-group is predicted and observed for the ortho- and para-isomers, whereas three bands (involving one or three CH-oscillators) are predicted for the meta-isomer. Two bands labeled as mₐ and mₚ are clearly observed. The mₗ-band is not observed, suggesting that its intensity is insufficient to induce dissociation of this (relatively stable) fragment ion. Spectra for the ortho- and para-isomers show some intensity at the position of the low-frequency band of the meta-isomer (labeled mₘ) as well, but this is due to an overlapping out-of-plane CH bending vibration of the other ring in the molecule. Both the number of these IR bands as well as their frequency positions correspond well to the known group-frequencies of out-of-plane CH vibrations. Note that such an interpretation of the CH vibrations is not possible for the precursor ions themselves due to overlapping bands from the R-group in the 800 cm⁻¹ region.
Figure 3. Comparison of the experimental and predicted IR spectra of (a) the m/z 202 fragments of the ortho- (left), meta- (middle) and para-metabolites (right), (b) the caesium-adducts of 2-fluorobenzyl alcohol (left), 3-fluorobenzyl alcohol (middle) and 4-fluorobenzyl alcohol (right) obtained using IRIS and (c) of the three neutral gaseous fluorobenzyl alcohols obtained using FTIR spectroscopy. The most prominent CH-out-of-plane vibrations in the predicted spectra are indicated with green, red and blue for the ortho-, meta-, and para-isomers, respectively. In (b) and (c) the C-OH stretching vibrations are also indicated (yellow).
To further explore the generality of structural diagnostics based on the out-of-plane CH-bending vibrations, we selected a set of simple and readily available positional isomers: 2-, 3- and 4-fluorobenzyl alcohol. The relatively high vapor pressure of these compounds allowed gas-phase FTIR spectra to be recorded of the neutral species for comparison. The methoxy- and fluoro-substituents were chosen to assess the effect of the functional groups on the vibrational signatures. For the IRIS measurements in the mass spectrometer, these analytes are not easily protonated or deprotonated by ESI, but their alkali metal-ion adducts can easily be produced. IRIS spectra were therefore recorded for the [M+Cs]+ ions of the ortho-, meta- and para-isomers. Caesium has a relatively low binding-energy as compared to smaller alkali metal ions and [M+Cs]+ adducts should therefore easily undergo IR induced dissociation. Moreover, the mass of Cs+ (m/z 133) is well above the low-mass cut-off of the ion trap mass spectrometer, which allows for its detection as a fragmentation product upon IR induced dissociation.

Figure 3b shows the experimental spectra of the three ions and compares them with calculated spectra obtained using DFT. Again, the three ions can be spectroscopically differentiated, especially based on their distinctive pattern of vibrational bands in the 600-900 cm⁻¹ region, attributed to the out-of-plane CH bending normal modes. These vibrations are indicated with colors in Figure 3b. Their normal mode displacements are visualized in Figure 4, showing that they are identical to the modes of the phenylethylpiperidine fragment ions. Comparing Figure 3a and 3b, it is clear that although two rather different classes of molecules are being investigated, the pattern of out-of-plane CH bending modes is very similar for corresponding positional isomers (see also Table 1). This suggests that the band patterns do not strongly depend on the functionality of the substituents.
Moreover, these out-of-plane CH-vibrations are especially well modelled by the calculations. These results suggest that it is possible to identify the substitution pattern without computational reference spectra, simply based on group frequency arguments for this spectral region. We note however that other (non CH-bending) vibrations can also appear in the 600-900 cm\(^{-1}\) region and therefore possibly interfere with a direct interpretation of the CH-bending vibrations (for example, see the comparison above of precursor/fragment IR spectra in Figure 2d) highlighting the benefit of working with fragment IR spectra.

The results shown in Figure 3b suggest that the interaction of the analyte with the Cs\(^{+}\)-ion does not have a large effect on the frequency of the out-of-plane CH-bending vibrations. In order to directly probe the influence of Cs\(^{+}\)-coordination, we compare these spectra with the gas-phase FTIR spectra of the three neutral fluorobenzyl alcohol isomers (Figure 3c) and their DFT calculated spectra. Figure S5 provides a direct comparison of the FTIR and IRIS spectra of each of the isomers. The IRIS and FTIR spectra of the fluorobenzyl alcohols are highly similar and mainly differ in the relative intensities of several bands. The most significant effect of the interaction with the Cs\(^{+}\)-ion is a red-shift of the strong band around 1000 cm\(^{-1}\), yellow shaded in Figure 3b and 3c. From the calculations, we attribute this band to the C-OH stretching vibration, which shifts to lower frequencies due to the interaction of the O-atom with the Cs\(^{+}\) ion. Experimental and theoretical frequencies of the out-of-plane CH bending normal modes of the neutral fluorobenzyl alcohols are also listed in Table 1 and the normal modes are visualized in Figure 4. Generally, these modes appear to be shifted by 10 – 15 cm\(^{-1}\) to higher frequencies due to the interaction with the Cs\(^{+}\) ion. Moreover, in neutral 3-fluorobenzyl alcohol the \(m_1\)-normal mode (which was observed for the ionic meta-isomers) is coupled to another out-of-plane CH bending mode, leading to two, partially overlapping bands (see Figure 3 and 4). However, the location of this doublet is comparable to the other \(m_r\)-bands. This confirms that the patterns of out-of-plane CH bending modes observed for substituted phenyl-isomers are very similar for IRIS and FTIR spectroscopy.

CONCLUSIONS

IRIS is a valuable method for the differentiation and identification of positional isomers of substituted phenyl-containing compounds. Ortho-, meta- and para-substitutions of the phenyl-ring leave distinct signatures on the vibrational bands in the 650-900 cm\(^{-1}\) region, which remain fairly unaffected by the type of substituents and the nature of ionization (neutral, protonated, deprotonated, metal adducts etc.). This pattern corresponds well with tabulated IR group frequencies for conventional (FT)IR spectra of neutral molecules with positional isomerism and is well predicted by quantum-chemical calculations. This consistency suggests that IRIS-based identification is generally applicable in the MS-analysis of ortho, meta and para-isomers, e.g. in the analysis of phase I drug metabolites, but also in wider analytical MS identification approaches. The predictability of the patterns of CH out-of-plane bending modes across molecular systems makes it possible to directly assign substitution patterns, regardless of the remaining part of the molecular system and without the need for reference IR spectra. This highlights a clear advantage of IR spectroscopy over other MS-hypenated methods such as ion mobility spectrometry or chromatography: retention times and collisional cross sections naturally contain information of a full molecular system, whereas regions of the IR spectrum contain information on relatively isolated functional groups contained in a molecule.

To resolve issues of spectral congestion for larger molecular systems, we show that IRIS can also be performed on CID fragments of the precursor ion containing the substituted phenyl ring. Moreover, for species with low ionization efficiency that are hard to detect as protonated/deprotonated ions in ESI-MS, IRIS spectra of Cs\(^{+}\)-adducts can be recorded. Their CH out-of-plane vibrational frequencies are similar to those of their gas-phase neutral counterparts. In addition, the relatively low threshold to dissociation of Cs\(^{+}\)-adducts may be exploited in the development of IRIS-based identification strategies using IR laser sources with lower output power, which may eventually extend the applicability of this method to laboratories beyond FEL facilities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figures S1-S5 (PDF)

AUTHOR INFORMATION

Corresponding Author

*Correspondence to: fcuycken@its.jnj.com and jos.oomens@ru.nl

Notes

The authors declare no competing financial interest.

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Supporting information:

Mass spectrometry-based identification of ortho-, meta- and para-isomers using infrared ion spectroscopy

Rianne E. van Outersterp¹, Jonathan Martens¹, Giel Berden¹, Valerie Koppen², Filip Cuyckens²* and Jos Oomens¹,³*

¹Radboud University, Institute for Molecules and Materials, FELIX Laboratory, Toernooiveld 7, 6525ED Nijmegen, The Netherlands
²Drug Metabolism & Pharmacokinetics, Janssen R&D, B-2340, Beerse, Belgium
³van’t Hoff Institute for Molecular Sciences, University of Amsterdam, 1098XH Amsterdam, Science Park 908, The Netherlands

Figure S1. CID MS/MS spectra showing the fragmentation of the (a) \( R_1 \)-meta-isomer \( m/z \) 460 and (b) \( R_1 \)-para-isomer \( m/z \) 460.

![Figure S1](image-url)
Figure S2. (a) Comparison of the IR spectra of the ortho-, meta- and para-isomers with the \( R_2 \)-group (m/z 460) (b) IR spectrum of the ortho-isomer of the \( R_3 \)-compound (m/z 347) (c) Comparison of the IR spectra of the m/z 202 fragments of the ortho-, meta- and para-isomers from the \( R_2 \)-precursor ions. (d) IR spectrum of the m/z 202 fragment of the ortho-isomer of the \( R_3 \)-precursor ion.
Figure S3. (a) IR spectrum of the ortho-isomer with the $R_4$-group ($m/z$ 315) (b) Comparison of the IR spectrum of the ortho- and para-isomers with the $R_5$-group ($m/z$ 303)
Figure S4. Comparison of the IR spectrum of the m/z 202 fragment of the R_1-ortho-isomer to calculated IR spectra for the (a) ortho-, (b) meta- and (c) para-phenylethylpiperidine fragment ions. Qualitatively, the best match is indeed found in panel a.
Figure S5. Comparison of the IRIS spectra (of caesium-adducts) and FTIR spectra (of neutrals) of (a) 2-fluorobenzyl alcohol, (b) 3-fluorobenzyl alcohol and (c) 4-fluorobenzyl alcohol.
