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Document Classification

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Document History

Name	Date	Comment
P4 UHRO – Christopher P. Rüger	2021-09-15	First final version of the deliverable, iteration #2 for the standardized protocols with focus towards new examples covering other sample types and methodologies not in focus in iteration #1
P4 UHRO – Christopher P. Rüger, Anika Neumann	2021-11-05	After the 2nd periodic report meeting, the reviewer suggested to revise this deliverable. Hence, the first version was rejected and a new version accordingly to the reviewer comments and suggestions herein created, significantly increasing the level of detail per standard protocol and focusing towards common samples investigated within TNAs.
P1 CNRS – Christian Rolando	2023-01-31	Added 3 new protocols from P1 CNRS and P2 Uliege.

Document Validation

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P1 CNRS – Christian Rolando	2023-10-09	christian.rolando@univ-lille.fr

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Document Abstract

The following deliverable provided in the form of a public report document is the second iteration (#3) of the deliverable “Standardized protocols for the more common samples”. The motivation of this deliverable is to expand and increase the level of detail of the set of protocols reported within the first iteration (updated protocols). Consequently, this report has to be used together with the deliverable “Standardized protocols for the more common samples -Iteration#1” 4.2.1, and 4.2.2 which gives a brief introduction to the consortium expertise and fundamentals as well as on the general protocol structure. Particularly, also data analysis and visualization aspects are reported in more detail therein.

The topics and sample types were related to common sample materials investigated within trans-national access proposals, covering ion molecule reactions, metabolomics, and various fossil petroleum sample materials, such as vacuum gas oils, bitumen and asphaltenes. Hence, this iteration encompasses 3 additional protocols, leading to an experimental library with 10 standardized protocols scheme in expanded level of detail.

- ion molecular reactions by FT-ICR MS
- direct infusion ESI FT-ICR MS for the characterization of food matrices (metabolomics)
- direct infusion (+)- and (-)-ESI-FT-ICR MS for vacuum gas oils (VGOs)
- direct infusion APPI- and APCI-FT-ICR MS for crude oil-derived samples
- direct infusion ESI FT-ICR MS for trace polar species in diesel samples
- GC-APCI-FT-ICR MS for polar and semi-polar species in diesel samples and similar distillation cuts
- TG-APCI-FT-ICR MS for solid or highly viscous petroleum fractions, such as asphaltenes and bitumen
- Taxonomy and classification of Upper Pleistocene bones by ultrahigh resolution MALDI-FT ICR
- Direct infusion (+)-nano-ESI-FT-ICR MS for analysis of insoluble polydienes
- comprehensive analysis and workflow for mass spectrometry imaging for brain tissue

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1. Ion-Molecule Reactions by FT-ICR MS

Introduction

Application field

Solvation energies of ionic species are very large and can mask differences in intrinsic reactivity. Within the vacuum system of a FT-ICR cell, a wide array of reactions can be performed in a solvent free environment with significant impact in the fields of environment, health, catalysis, and fundamentals. In this highly dilute medium charged species may be isolated and their increased lifetime allows sampling and characterization of elusive species and otherwise fleeting intermediates. The ions and charged complexes are sampled as naked species which allows a direct relationship with computational data, disregarding solvation effects. In turn, a thorough assay in a bare state represents a reference for estimating local effects and perturbations when the ionic species of interest is examined in solution or in solid matrices. In short the study of ion-molecule reactions by FT-ICR MS is expected (as proven in a large variety of instances) to have an impact in all areas where (i) structural characterization of analytes, (ii) mechanisms of ionic reactions, and (iii) identification of elusive intermediates are significant goals.[1,2] The long lived condition of ions to be assayed is also a very suitable situation for ion-molecule reactions to attain thermodynamic parameters, e.g. gas phase basicities, ligand binding energetics, data regarding non-covalent interactions.[3]

Selected applications

An example is provided in the following sequence showing the formation of an elusive complex by an ion-molecule reaction (IMR). It is noteworthy that while the precursor ion, the iron(III) protoporphyrin IX complex $[(PP\text{-}IX)Fe^{III}]^+$, is present in solution and released in the gas phase by ESI, the oxo complex $[(PP\text{-}IX)^{+\cdot}Fe^{IV}=O]^+$ can be formed as long lived ion only in the gas phase.[4] Only in this medium a thorough assay of its reactivity features has become feasible. In solution its reactivity is too high, even towards its same precursor, to allow its detection.

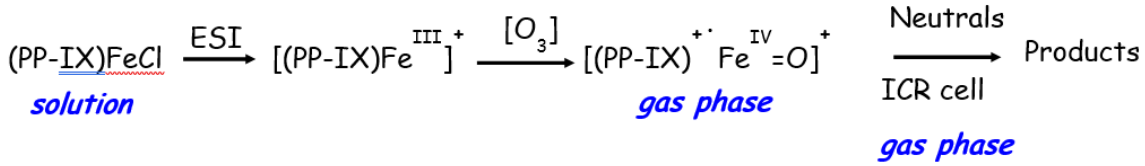

Figure 1. 1) Positive mode ESI of a solution of Iron Protoporphyrin IX Chloride. 2) Formation of an oxo iron(IV) protoporphyrin IX radical cation (m/z 616), the naked core of Compound I, by reaction with O_3 diluted in O_2. 3) Mass-selection of m/z 616 and O-atom transfer (OAT) reactivity with selected neutrals leaked at a constant pressure in the range of 10^{-8}-10^{-7} mbar in the FT-ICR cell.

As a second example the reaction between neutral and protonated nitric oxide is illustrated in Figure 2.[5] Nitroxyl (HNO) and nitric oxide (NO) play relevant roles in various contexts, e.g. behaving as signalling molecules.

$$[HNO]^+ + NO \rightarrow NO + HNO^+ \quad \text{PT} \quad (1)$$
$$[HNO]^+ + NO \rightarrow HNO + NO^+ \quad \text{ET} \quad (2)$$
$$[HNO]^+ + NO \rightarrow NO^+ + HNO \quad \text{HAT} \quad (3)$$

Figure 2. Proton, electron, and hydrogen atom transfer processes (PT/ET/HAT) are elementary paths available to the HNO+/NO couple that can be discriminated only by resorting to isotopic labelling. Usage of D- and 15N-labelling in the reagent species allows to unequivocally observe an exclusive ET reactivity.

Materials

Reagents

Chemicals

Each experiment requires specific gases, solvents and reagents. Listed herein are the ones most generally used and the ones used in the above reported exemplary experiments

- Ammonia solution 25% (Sigma Aldrich, for HPLC-MS, > 99.9%) used in dilute solution (10^{-3}M) to achieve basic conditions for optimal electrospray ionization Caution! Ammonia solution 25% is harmful if inhaled and causes severe skin burns and eye damage
- Formic acid 98-100 % (Sigma Aldrich, for HPLC-MS, > 99.9%) used in dilute solution (10^{-3}M) to achieve acidic conditions for optimal electrospray ionization Caution! Formic acid is corrosive and toxic if inhaled
- Hemin chloride (Sigma Aldrich)
- Hydroxylamine phosphate salt ($3NH_2OH \bullet H_3PO_4$ Sigma-Aldrich)
- High purity (>98%) volatile (b.p.< 250°C) neutrals (olefins, aromatics, ketones, amines, sulphides, phosphites).

Solvents

- Water (LC/MS grade)
- Methanol (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Methanol is highly flammable and toxic
- Acetonitrile (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Acetonitrile is highly flammable and toxic

- Dichloromethane (Merck ≥ 99.8%) Caution! Dichloromethane causes irritation and is suspected of causing cancer

Gases

- High purity gases (O_2, Ar, N_2)
- Nitric oxide, NO (Sigma Aldrich ≥98.5%) Caution! Fatal if inhaled

Equipment

Instruments

- FT-ICR MS and software (4.7 T Bruker BioApex equipped with an Apollo I ESI source, a cylindrical infinity cell, two needle and two pulsed valves, and XMass Data Analysis software package, Bruker Daltonics GmbH)
- cold-cathode sensor (IKR Pfeiffer Balzers S.p.A., Milan, Italy)
- syringe pump for direct infusion of sample solutions into the ESI source

Consumables

- Glass flasks with sealed tap (500 ml, handmade by the glassmaker)
- Glass vials, with screw caps (1.5 ml, Supelco Analytical Products)
- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)
- Glass syringe for ESI (250 µl, blunt needle, Hamilton)

Reagent Setup

Preparation of sample solution for electrospray ionization typically involve careful weighing of mg amounts of liquid/solid compounds to obtain ca. 10^{-3} M stock solutions. Mixing in proper ratios (if required) and dilution yields ca. 10^{-5} M solutions of the selected analyte(s) to be directly infused in the ESI source by means of a syringe pump at a flow rate of 120 µL h^{-1}.
Peculiar sample ions may need individual procedures to be planned in each case. For example in order to generate $[HNO]^+$ ions, a sample of hydroxylamine phosphate salt ($3NH_2OH \bullet H_3PO_4$) is introduced in the electron ionization/chemical ionization (EI/CI) external ion source using a direct insertion probe heated at 80-100°C.[6] Gaseous hydroxylamine is generated by decomposition of the salt in the inlet system. Ions were

produced either by EI or by CI using N_2 as charge transfer reagent gas. Alternatively, $[HNO]^+$ is formed by CI(CH_4) of gaseous NO, exploiting a mildly endothermic proton transfer reaction from CH_5^+.[7] The labeled ion $[DNO]^+$ is obtained by treating hydroxylamine phosphate salt several times with D_2O. Labeled ^{15}NO is prepared allowing to react $Na^{15}NO_2$ and $FeSO_4$ dissolved in water with a solution of H_2SO_4, using vacuum line procedures.[8] Passage through two traps at differential low temperatures yields ^{15}NO, found to be 99.9% pure by mass spectrometric analysis.

Equipment Setup

To perform IMRs, a commercial ESI ion source (Bruker Daltonics GmbH) is used for delivering the ionic reactant to the gas-phase. A counter current flow of heated dry gas (nitrogen typically at 130°C) is used to desolvate the ions. After an accumulation interval of 0.5 s in a rf-only hexapole, the ions of interest are pulsed into the ICR cell at room temperature (300 K). Here, ions are mass-selected using broad band excitation or single radiofrequency pulses to eject few ions of minor intensity and quenched of any excess kinetic energy

by an argon pressure pulse using a magnetic valve. Then ions are allowed to react with the neutral leaked by the needle valves at a stationary pressure (Figure 3).

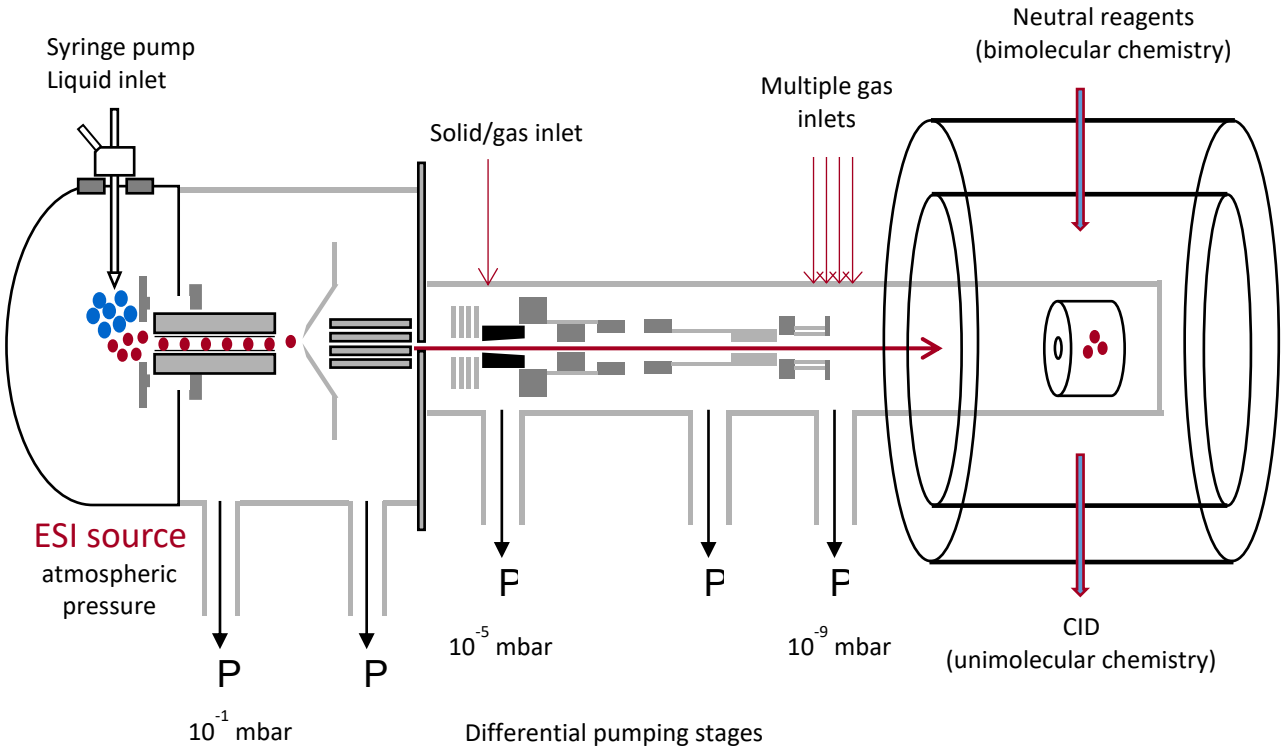

Figure 3. Schematics of 4.7 T Bruker BioApex set up suitably modified to perform gas phase IMR.

Procedure

Sample preparation:

1-10 µM solutions are prepared as described above in an appropriate solvent (methanol, acetonitrile, water and their mixtures) by stepwise dilution.

Introduction of neutrals:

The selected neutrals are admitted into the ICR cell by :

- a needle valve, operating in a pressure range of 10^{-8}-10^{-7} mbar. If the neutral is in a solid or liquid, freeze-thaw cycles in liquid nitrogen are necessary to degas the sample; if the sample is a gas, a vacuum line is used to fill a glass flask with a sealed tap.
- a pulsed valve, reaching a peak pressure of ca. 10^{-6} mbar for 1s. This procedure is usually applied to admit an inert gas (N_2, Ar) and perform collision induced dissociation (CID) assay of the sampled ions. If ions are not kinetically activated, thermalizing collisions occur between ion and bath gas molecules whose end effect is to bring the ion to thermal equilibrium with the cell walls.

Pressure calibration:

The partial pressures of the neutrals are measured by a cold-cathode sensor calibrated before and after each set of IMR experiments by using the rate constant k= 1.1 x 10^{-9} cm^3s^{-1} for the reference reaction: $CH_4^{+\cdot}$ + CH_4 → CH_5^+ + $CH_3^{\cdot}$, and then corrected using individual response factors.[9,10] The estimated error that is typically associated to the bimolecular rate constants (± 30%) is largely caused by the uncertainty of neutral pressure.[11]

Data Handling

Measurements of thermal bimolecular rate constant:

The kinetic progress of the IMRs is monitored by recording ca. 5-20 averaged scans for each mass spectrum in series of runs corresponding to increasing reaction time. Pseudo first-order rate constants are achieved from the slope of the semilogarithmic decrease with time of the parent ion abundance. The remarkable ion storage capability of FT-ICR allows to follow the reaction kinetics till several hundred seconds. At least three

replicates are collected at each neutral pressure. Bimolecular rate constant (k_{exp}) are obtained by the ratio between pseudo first-order rate constants and the substrate concentration expressed in cm^3 $molecole^{-1}$.

Kinetics of IMRs are recorded normally at three different values of neutral pressure. In those few cases where the IMR does not proceed to the total disappearance of the reagent ion but the system comes to a constant ratio of the abundances of reagent and product ion, the kinetics are analyzed according to the pattern for a reversible reaction.

Calculation of collision rate constant and reaction efficiency:

The collision rate constant (k_c)are calculated according to the parametrized trajectory theory.[12] The reaction efficiencies (Φ) are calculated as % ratio of k_{exp} relative to the collision rate constant (k_c). Dipole moments were obtained from current literature or obtained by quantum chemical calculations using the Spartan program suite (Wavefunction, Inc.). $\Phi = k_{exp} / k_c$

Data visualization:

An example of kinetic plot is illustrated in Figure 4 where the selected ion $[(PP\text{-}IX)^{+\cdot}Fe^{IV}=O]^+$ reacts with NO.[4] The reaction follows pseudo first-order kinetics. $[(PP\text{-}IX)^{+\cdot}Fe^{IV}=O]^+$ promotes an oxygen atom transfer to NO, forming the reduced species $[(PP\text{-}IX)Fe^{III}]^+$ which subsequently undergoes ligand addition with NO yielding the adduct ion $[(PP\text{-}IX)Fe(NO)]^+$.

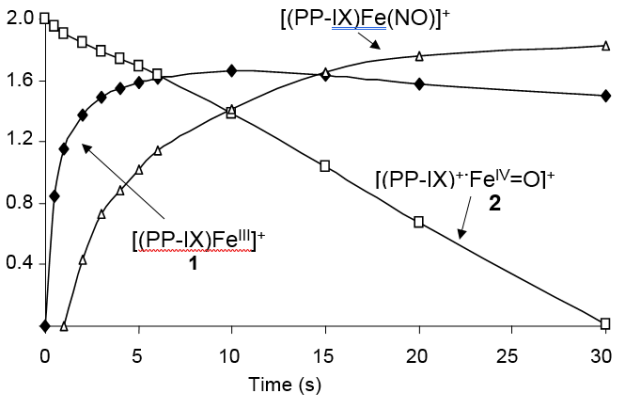

Figure 4. Kinetic (semilog) plot of relative ion abundances versus time for the reaction of $[(PP\text{-}IX)^{+\cdot}Fe^{IV}=O]^+$ ions with NO at the stationary pressure of 3.0 x 10^{-8} mbar.

An additional example regards the HNO^+/NO system.[5] Based on the reaction of unlabeled species, ambiguities remain between the three possible elementary processes of equations (1-3) shown in Figure 4. Thus, labeled species, namely $[DNO]^+$ and neutral (^{15}NO), have been sampled. The reaction of either $[HNO]^+$ or $[DNO]^+$ with ^{15}NO does not reveal any $[H^{15}NO]^+$ or $[D^{15}NO]^+$ being formed. The occurrence of a proton transfer event (equation (1)) is therefore excluded. The very efficient formation of NO^+ from the $[HNO]^+$ reaction with NO may be traced to either a charge transfer process (equation (2)) or a hydrogen atom transfer reaction (equation (3)). However, allowing both $[HNO]^+$ and $[DNO]^+$ to react with labeled ^{15}NO leads to the predominant formation of $^{15}NO^+$ supporting the occurrence of electron transfer between the reacting partners. The minor formation of NO^+, which could suggest the contribution of an overall hydrogen atom transfer process, is not deemed reliable in consideration of a minor background process forming NO^+ in inert argon. Figure 5 shows an example of the kinetic progress of the reaction.

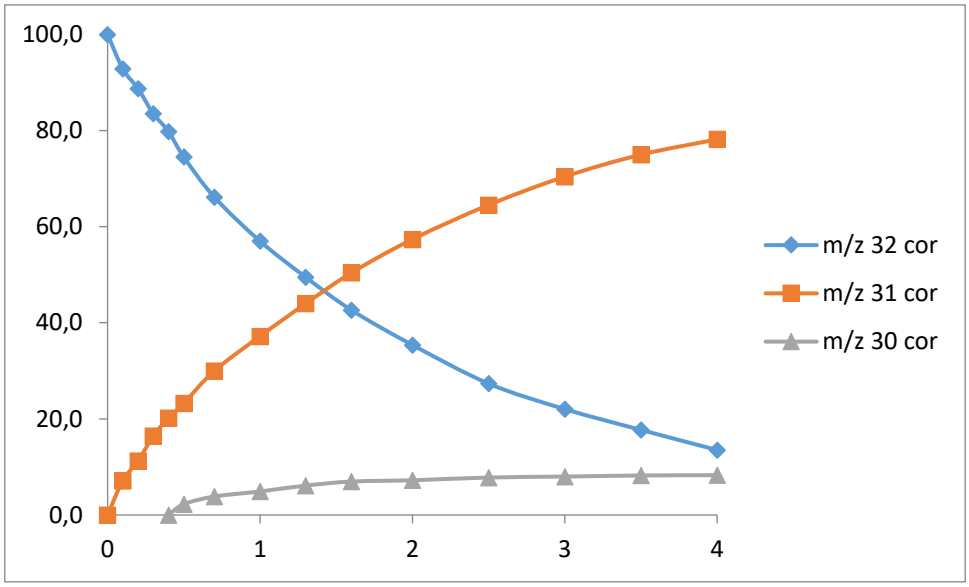

Figure 5. Time dependence of ion abundances after selection of $[DNO]^+$ ions at *m/z* 32 in the presence of ^{15}NO at 3.8×10^{-9} mbar in the FT-ICR cell at room temperature.

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2. Direct infusion ESI FT-ICR MS for the characterization of food matrices

Introduction

Application field

A non-targeted fingerprinting approach is required for a full metabolic characterization of any food sample. In order the ensure a comprehensive profiling, a high-resolution FT-ICR MS method provides ultimate accuracy of mass determination, limit of detection and wide dynamic range and has been recognized to allow a reliable authentication strategy, circumventing the need for chromatographic separations prior to MS analysis even for highly complex samples. The low ppm mass accuracy achieved with FT-ICR MS implies that metabolites can often be identified based on their accurate mass alone.
As an exemplary application we now refer to the characterization of Torpedino di Fondi (TF) tomatoes at both pink and red ripening stages and comparison with traditional San Marzano (SM) tomatoes.

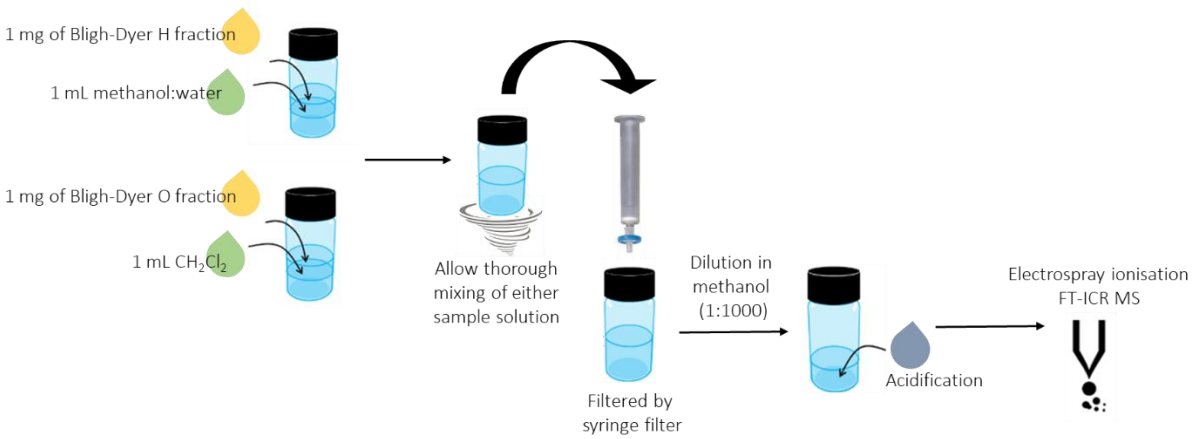

Figure 6; Graphical illustration of the preparation procedure. 1) Dissolution of 1 mg of each dried Bligh–Dyer hydroalcoholic (H) and organic (O) fraction of TF and SM cultivars in 1 mL (1:1) methanol/water and CH_2Cl_2, respectively. 2) Mixing of either one by vortex for 3 min and filtering. 3) Dilution in methanol (1:1000) to obtain a final dilute concentration suitable for MS analysis. 4) Addition of formic acid solution to allow analyses in positive mode. 5) ESI-MS measurements.

Materials

Reagents

Chemicals

- Formic acid 98-100 % (Sigma Aldrich, for HPLC-MS, > 99.9%) used in dilute solution (10^{-3}M) to achieve acidic conditions for optimal electrospray ionization Caution! Formic acid is corrosive and toxic if inhaled

Solvents

- Water (LC/MS grade)
- Methanol (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Methanol is highly flammable and toxic
- Acetonitrile (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Acetonitrile is highly flammable and toxic
- Dichloromethane (Merck ≥ 99.8%) Caution! Dichloromethane causes irritation and is suspected of causing cancer

Gases

- Nitrogen 5.0 (for FT ICR MS)

Equipment

Instruments

- Shaker & mixer (Heidolph Instruments Gmbh&Co.)
- Apollo II electrospray ionization (ESI) coupled with Bruker SolariX XR (7T with infinity cell, Bruker Daltonics GmbH; Bruker Compass Data Analysis 5.0, Bruker Daltonics GmbH). Lisbon
- FT-ICR MS (4.7 T Bruker BioApex equipped with an Apollo I ESI source, a cylindrical infinity cell. Roma

Consumables

- Glass vials, with screw caps (4 ml, Sigma-Aldrich)
- Glass syringe for ESI (10 µl, 250 µl, 1 mL, blunt needle, Hamilton)
- Syringe filter 0.45 µm hydrophobic polypropylene Acrodisc (VWR)

Reagent Setup

Dilute the arginine solution (2 mg/ml) 1:10 with methanol to obtain an arginine solution of 0.2 mg/ml for quality check and pre-calibration of the instrument. This procedure is common to other described protocols.

Equipment Setup

For the ESI measurements, a commercial ESI ion source (Bruker Daltonics GmbH) is used. The general set-up can be abstracted from **Erreur ! Source du renvoi introuvable.**.

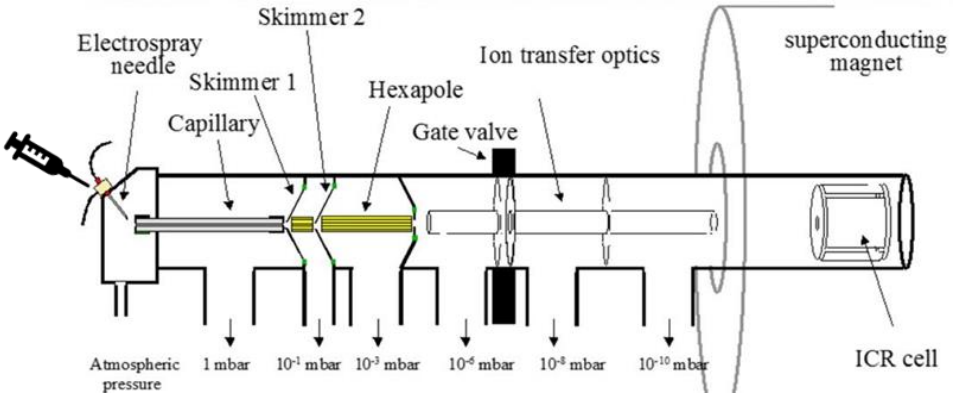

Figure 7. Schematics ESI FT-ICR MS setup for direct infusion measurements.

For pre-calibration of the instrument, take 200 µl of the diluted arginine mixture with the glass syringe. The parameters for pre-calibration should be the similar to the parameters used for the measurements and can be therefore taken from the section "procedure". The pre-calibration should be repeated every day before the measurements. This procedure is common to other described protocols.

Procedure

In order to avoid degradation, stock solutions were kept at -20°C and analysed within 24 h after final dilution.

Sampling

Fresh fruits of *Solanum lycopersicum* L. TF variety were grown and collected both in Fondi (Latina, Italy). Samples were harvested at two different ripening stages according to market demand, namely pink (P) stage (from 30% to 60% of not green tomato skin) and red stage (R) (about 90% of not green tomato skin) showing a red colour.

Extraction

Fifteen fresh whole fruits from pink TF (TFP), red TF (TFR), pink SM (SMP), and red SM (SMR) were frozen and ground in liquid nitrogen to obtain a homogeneous pool and subjected to the Bligh–Dyer extraction method, which allows to extract both water-soluble and liposoluble metabolites in a semi-quantitative manner.

Dilution

1) Dissolve 1 mg of Bligh-Dyer hydroalcoholic (H) fraction of TF and SM cultivars in 1 mL (1:1) methanol/water and 1 mg of Bligh-Dyer organic (O) fraction of both cultivars in 1 mL CH2Cl2.
2) Mix by vortex for 3 min and filter through a 0.45 µm polypropylene Acrodisc (VWR) syringe filter to remove debris.
3) Subsequently dilute in methanol to obtain a final concentration of 100 µg L^{-1}.

4) For each extract, three distinct solutions are prepared according to the above procedure and submitted to analysis.
5) Add 1 volume-% of formic acid for acidification to the extract for positive ESI measurements.
6) Add an internal reference to all samples at a final concentration of 0.5 µg L^{-1} to calibrate the spectra by means of the on-line calibration tool (Data Analysis 5.0, Bruker Daltonics); e.g. leucine enkephalin (YGGFL, $C_{28}H_{37}N_5O_7$) is revealed as $[M+H]^+$ at *m/z* 556.27657 in positive mode and as $[M-H]^-$ at *m/z* 554.26202 in negative mode.

Measurement

For direct infusion ESI, fill approximately 200 µl of the diluted extract into the syringe. Be careful, that there are no air bubbles left in the syringe. The measurement parameters are given below. The total analysis time amounts to 2.5 min. This procedure is common to other described protocols.

Cleaning

After each measurement, it is necessary to clean the ESI syringe and capillary. Therefore, flush the syringe with methanol. Subsequent, flush the capillary several times with methanol with the help of the cleaned syringe. If there are sticky contaminants, use different solvents in the order of polarity to prevent a clocking of the ESI needle. This procedure is common to other described protocols.

ESI Injection Parameters

capillary exit voltage	200 V
drying gas flow	4.0 L min^{-1}
flow rate (syringe)	120 µl h^{-1}
nebulizer gas pressure	1.0 bar
temperature	200°C

FT-ICR Mass Spectrometry Parameters

mass-to-charge ratio (m/z) range	100-3000 Da
resolution	650 000 @*m/z* 400
spectra number	200
free induction decay (FID)	1,973 s
run time	10 min

Data Handling and Anticipated Results

Calibration

The mass spectra can be calibrated by adding leucine enkephalin (YGGFL, $C_{28}H_{37}N_5O_7$) to all samples at a final concentration of 0.5 µg L^{-1} as an internal reference (revealed as $[M+H]^+$ at m/z 556.27657 in positive mode and as $[M-H]^-$ at m/z 554.26202 in negative mode) so as to calibrate the spectra by means of the on-line calibration tool (Data Analysis 5.0, Bruker Daltonics). For further internal calibration one may refer to a list of ubiquitous metabolites, including hexose/monosaccharides, citric and palmitic acids. Routine mass accuracy should be lower than 0.2 ppm.

Peak assignment

The list of *m/z* values is exported with a cut-off signal-to-noise ratio (S/N) of 4 and submitted to the free tool MassTRIX, taking into account protonated, sodiated, and potassiated (ESI(+)), and deprotonated and chlorinated (ESI(-)) ions, with a maximum deviation range set to ±1ppm. An accurate check of the isotopic pattern based on the natural abundances of ^{13}C, ^{15}N, ^{18}O, ^{34}S and ^{37}Cl isotopes, is also performed to minimize false positive results. Only singly charged species are revealed, in both polarity modes. A large number of unambiguous molecular formulas, for which several isomers are possible, admitting the presence of the elements C, H, O, N, P, and S, could be assigned by both ESI(+) and ESI(-) analyses and are further filtered by application of several chemical constraints. Additional information is obtained by acquisition of collision

induced dissociation (CID) spectra, though limited to components of adequate abundance, further verified against fragmentation patterns of reference compounds or data inserted into a specialized database.

Relative frequency distribution

Specific data analysis allows to organize the vast amount and complexity of detected formulas to uncover interesting information. Among the detected molecular formulas, the relative frequency distribution was investigated showing that all tomato extracts contain a majority of CHO species followed by CHON, CHOP and, in smaller amount, CHNOP and CHNOS. In particular, CHO components correspond mainly to polyphenols, steroids, and fatty acids, followed by di- and tri-glycerides, terpenoids, organic acids, and arachidonic derivatives. When considering CHON components, they can be ascribed mainly to amino fatty acids, amino-sugars, amines, N-acylamines, followed by amino acids, solanidines, nucleosides, and vitamins.

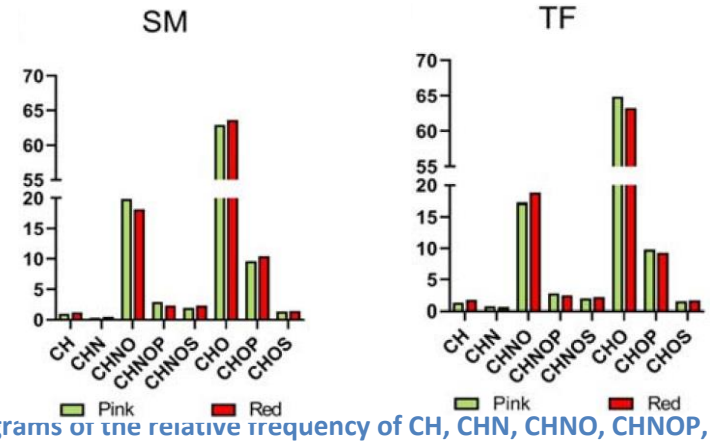

Figure 8. Histograms of the relative frequency of CH, CHN, CHNO, CHNOP, CHNOS, CHO, CHOP, CHOS compounds

H/C ratio and van Krevelen plots

The formulas generated from each sample are transposed to two-dimensional van Krevelen diagrams, known as elemental ratio analysis, constructed by plotting the molar hydrogen to carbon ratio (H/C) vs. the molar ratio of oxygen to carbon (O/C) for each data point. According to their own characteristic H/C and O/C ratios, main classes of compounds are specifically localized as areas in the plot, thus allowing a depiction of a sample's composition. So detected molecular formulas have been classified in different groups of natural compounds such as lipids, terpenoids, carbohydrates, amino acids, aminosugars, nucleic acids, polyphenols, polyketides, unsaturated hydrocarbons and condensed hydrocarbons as show in figure 3.

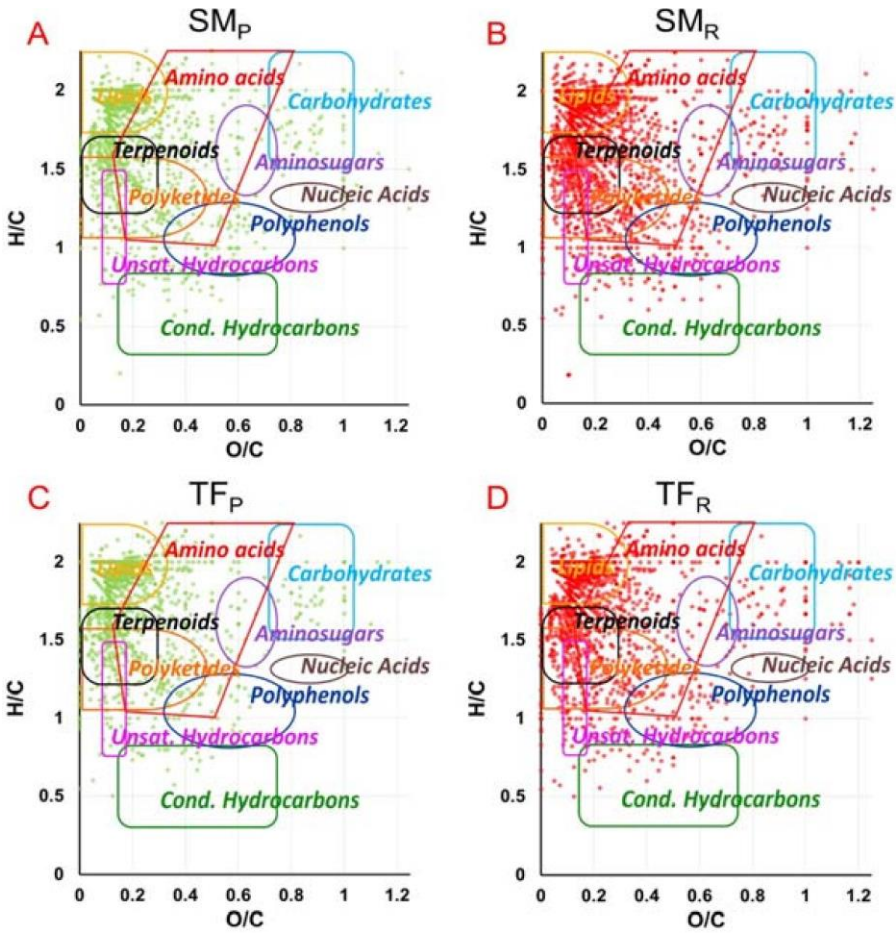

Figure 9. Van Krevelen plot of the molecular formulas obtained by ESI FT-ICR MS analysis of total hydroalcoholic and organic *fractions.*

Statistical analysis

The principal component analysis (PCA) is an essential tool for the exploratory data analysis and to summarize their main characteristics. It is commonly used for dimensionality reduction by projecting each data point onto only the first few principal components, wherein principal component can be described as a direction that maximizes the variance of the projected data. This point allows to obtain a lower-dimensional data while preserving as much of the data's variation as possible. An additional advantage is the obtaining of a graphic visualization of the data.

3. Direct infusion (+)- and (-)-ESI-FT-ICR MS for vacuum gas oils (VGOs)

Introduction

Application field

Vacuum gas oils are high complex mixtures containing species with high molecular weight and a high content of heteroatoms, such as oxygen, sulphur and nitrogen or organometallic compounds. During catalytic hydrocracking, the activity of the conversion catalyst is decreased in the presence of heteroatoms and therefore, their removal is essential.

Materials

Reagents

Chemicals

- Formic acid 98-100 % (Merck LiChropur for HPLC)
- Ammonia solution 25% (Merck LiChropur for HPLC)

Solvents

- Methanol (Romil-UpS Ultra Gradient for HPLC-MS,>99.9%)
- Toluene (Sigma–Aldrich Chromasolv Plus for HPLC,≥99.9%)

Gases

- Nitrogen 5.0 (for FT ICR MS)

Equipment

Instruments

- FT-ICR MS (12T Solarix XR with para cell, Bruker Daltonics GmbH)

Consumables

- Glass vials, with screw caps (1.5 ml, BGB analytics)
- Single-use glass pipettes

Further equipment

- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)
- Glass syringe for ESI (250 µl, blunt needle, Hamilton)
- spatula

Equipment Setup

For the ESI measurements, a commercial ESI ion source (Bruker Daltonics GmbH) is used. The general set-up can be abstracted from **Erreur ! Source du renvoi introuvable.**.

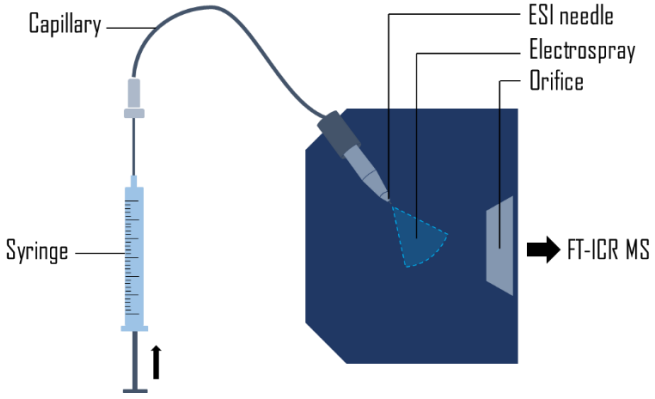

Figure 10: Schematic ESI setup for direct infusion measurements.

Procedure

Besides the sample preparation, it is recommended to perform the same preparation on a blank sample for blank value correction or for removing contaminants. Record a blank spectrum prior each sample introduction.

Dilution of the sample

1) Wear gloves to avoid contamination of the samples and the equipment.
2) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.
3) Take a new, clean 1.5 ml brown glass vial with cap and sealing. Weigh the vial and write down the exact value of the empty vial.
4) Take a clean spatula or a single-use glass pipette to fill 10 mg of the vacuum gas oil into the before weighed vial.
5) Weigh vial+sample and write down the exact value.
6) Add now toluene to obtain a dilution of 1:100. In case of 10 mg sample, add 990 µl toluene to the sample with an adjustable Eppendorf pipette.
7) Close the vial with sealing and cap.
8) Gently shake the mixture by hand for approximately 1 min.

Prepare a mixture of methanol/toluene (50:50 v/v).

9) Take a clean 250 ml conical flask with plug.
10) Add 100 ml of toluene and 100 ml of methanol to the flask and close the flask with the plug.
11) Gently shake the mixture by hand to mix both solvents.

Now, the sample can be further diluted to a final concentration of 0.5 mg/ml.

12) Take two new, clean 1.5 ml brown glass vials with caps and sealings.

13) Use an adjustable Eppendorf pipette to add 995 µl of the toluene/methanol mixture, prepared in step 9-11), to each vial.
14) Add 5 µl of the diluted sample obtained in step 8) to each vial.
15) Close the vials with sealings and caps.
16) Gently shake the mixtures by hand for approximately 1 min.

For better ionisation of the components, an acid has to be added for positive ESI measurements, whereas negative ESI measurements require the addition of a base.

17) For positive ESI measurements, add 1 volume-% of formic acid for acidification to the sample. Therefore, add 10 µl formic acid with a pipette to 1 ml of the diluted sample.
18) For negative ESI measurements, add 3 volume-% of ammonia to the sample. Therefore, add 30 µl ammonia with a pipette to 1 ml of the diluted sample.

Preparation of blank sample

19) Wear gloves to avoid contamination of the samples and the equipment.

The blank is prepared with the same procedure as used for the samples. The blank measurement can be also later used for background correction in sample spectra and should be measured between each sample.

20) Take a new, clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 1000 µl of the toluene/methanol (50:50 v/v) mixture prepared in steps 9) to 11) to the vial.
21) Close the vial with a sealing and a cap.
22) Gently shake the mixture by hand for approximately 1 min.
23) Take two other clean 1.5 ml glass vials with caps and sealings. Use an Eppendorf pipette to add 995 µl of the toluene/methanol (50:50 v/v) mixture to each vial.
24) Add 5 µl of toluene prepared in steps 20-22) to each vial.
25) Close the vials with sealings and caps.
26) Gently shake the mixtures by hand for approximately 1 min.
27) For positive ESI measurements, add 1 volume-% of formic acid for acidification to the blank sample. Therefore, add 10 µl formic acid with a pipette to 1 ml of the blank mixture.
28) For negative ESI measurements, add 3 volume-% of ammonia to the blank sample. Therefore, add 30 µl ammonia with a pipette to 1 ml of the blank mixture.

Measurement

Before each sample, a solvent blank has to be acquired.

29) Wear gloves to avoid contamination of the samples and the equipment.
30) Clean the syringe by flushing the whole volume with toluene for 10 times.
31) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
32) Fill the syringe with approximately 200 µl of the blank sample prepared in step 27) or step 28). Make sure that no air bubbles are left inside the syringe.
33) Set the parameters for the FT-ICR MS as given below.
34) Place the syringe into the syringe pump and connect the capillary.
35) Start tune modus at the FT-ICR MS.
36) Press “fast forward” until a stable signal is obtained.

37) Start the measurement.

Measurement of the sample.

38) Wear gloves to avoid contamination of the samples and the equipment.
39) Clean the syringe by flushing the whole volume with toluene for 10 times.
40) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
41) Fill the syringe with approximately 200 µl of the sample prepared in step 17) or step 18). Make sure that no air bubbles are left inside the syringe.
42) Set the parameters for the FT-ICR MS as given below.
43) Place the syringe into the syringe pump and connect the capillary.
44) Start tune modus at the FT-ICR MS.
45) Press “fast forward” until a stable signal is obtained.
46) Start the measurement.

Cleaning

After each sample measurement, it is necessary to clean the syringe and capillary.

47) Wear gloves to avoid contamination of the equipment.
48) Clean the syringe by flushing the whole volume with toluene for 10 times.
49) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
50) Switch on “tune” modus at the FT-ICR MS.
51) Flush the capillary using the syringe several times with toluene and toluene/methanol mixture. If there are sticky contaminants, use different solvents in the order of polarity to prevent a clocking of the ESI needle.
52) Flush the capillary until the capillary is clean, which can be checked online in the tune mode.
53) The instrument is now ready for the next measurement.

ESI Injection Parameters

Positive mode

capillary voltage	-4500 V
dry gas flow	4 l/min
flow rate (syringe)	400 µl/h
nebulizer gas flow	0.5 l/min
source temperature	146 °C

Negative mode

capillary voltage	+4500 V
dry gas flow	4 l/min
flow rate (syringe)	400 µl/h
nebulizer gas flow	0.5 l/min
source temperature	150 °C

FT-ICR Mass Spectrometry Parameters

Positive mode

accumulation time	0.025 s
mass-to-charge ratio (m/z) range	147-1300 Da
mode	broadband
octopole energy	350 V_{pp}
quadrupole collision energy	1200 V_{pp}
quadrupole lower cut-off	200
resolution	500 000-900 000 @m/z400
spectra number	400
transient length	3.4 s *
Time-of-flight	0.8 ms

*For a 12 T FT-ICR MS with para cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Negative mode

accumulation time	0.035 s
mass-to-charge ratio (m/z) range	147-1300 Da
mode	broadband
octopole energy	350 V_{pp}
quadrupole collision energy	1200 V_{pp}
quadrupole lower cut-off	200
resolution	500 000-900 000 @m/z400
spectra number	400
transient length	3.4 s *
Time-of-flight	0.8 ms

*For a 12 T FT-ICR MS with para cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Data Handling and Anticipated Results

Calibration

The mass spectra can be externally m/z calibrated by standards from quality measurements. For higher mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.). For calculation, we recommend to limit the assignment boundaries as following: Signal-to-noise ratio above 6, even electron configuration, a double bond equivalent of -1.5 to 30, a mass accuracy of 0.2-1 ppm and sum formula parameters of $C_{6\text{-}50}H_{4\text{-}100}N_{0\text{-}2}O_{0\text{-}2}S_{0\text{-}2}$ (positive mode), and of $C_{6\text{-}50}H_{4\text{-}100}N_{0\text{-}2}O_{0\text{-}4}S_{0\text{-}1}$. For positive ion mode, 0-1 Na atom can be considered due to possible adduct formation.

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion ESI measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

4. Direct infusion APPI- and APCI-FT-ICR MS for crude oil-derived samples

Introduction

Application field

Atmospheric pressure chemical ionization (APCI) is a gas phase ionization technique, which is able to ionize polar to semi-polar species. In APCI, especially oxygen-containing molecules are pronounced. Atmospheric pressure photo ionisation (APPI) is a gas phase ionisation technique which is able to ionize semi-polar species and pronounces preferentially sulphur-containing and aromatic species.

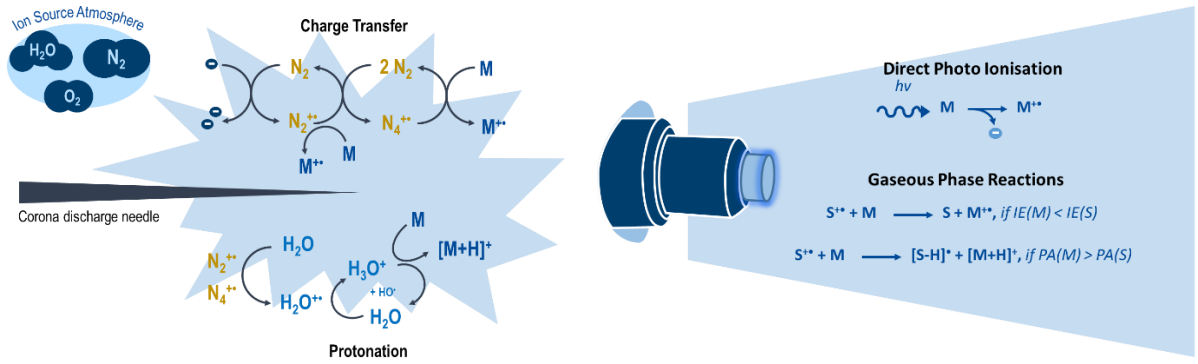

Figure 11. Schematic operating principle of the APCI and APPI ionisation mechanisms

Materials

Reagents

Solvents

- Methanol (Romil-UpS Ultra Gradient for HPLC-MS,>99.9%)
- Toluene (Sigma–Aldrich Chromasolv Plus for HPLC,≥99.9%)

Gases

- Nitrogen 5.0 (for FT ICR MS)

Equipment

Instruments

- FT-ICR MS (12T Solarix XR with para cell, Bruker Daltonics GmbH, Bremen, Germany)
- Sprayer for direct infusion APCI/APPI (Bruker Daltonics GmbH, Bremen, Germany)
- APCI needle (Bruker Daltonics GmbH)
- APPI lamp (Syagen Technology Inc. California, USA)

Consumables

- Glass vials, with screw caps (1.5 ml, BGB analytics)
- Glass syringe for sample introduction (250 µl, blunt needle, Hamilton)

Further equipment

- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)

Equipment Setup

For the APCI measurements, a commercial APCI ion source (Bruker Daltonics GmbH) with vaporizer is used. The general set-up can be abstracted from **Erreur ! Source du renvoi introuvable.** a). For APPI measurements, an additional Krypton discharge lamp with 10.0 and 10.6 eV is needed. The general set-up is shown in **Figure 11** b).

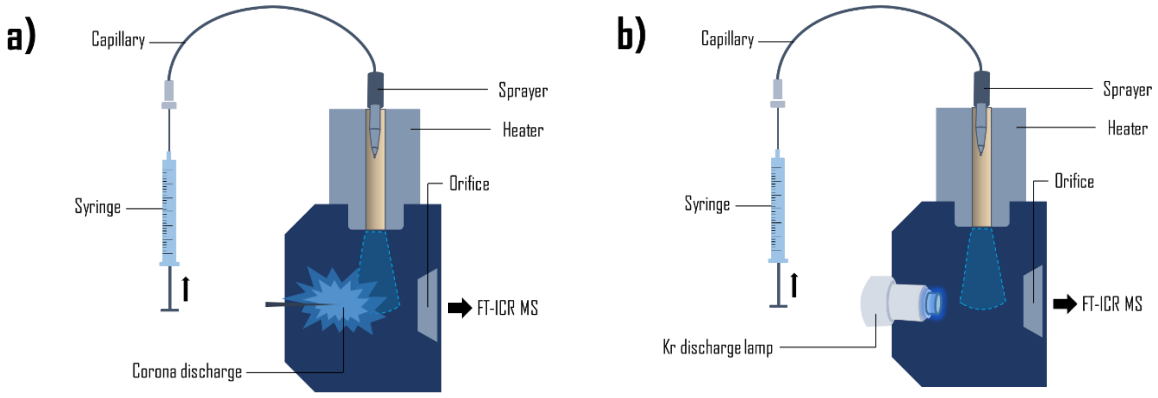

Figure 12. a) Schematic APCI-source setup for direct infusion measurements. b) schematic APPI-source setup for direct infusion measurements.

Procedure

Besides the sample preparation, it is recommended to perform the same preparation on a blank sample for blank value correction or for removing contaminants. Record a Blank spectrum prior each sample introduction.

Dilution of the sample

1) Wear gloves to avoid contamination of the samples and the equipment.

For solid to highly viscous samples:

2) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.

3) Take a new, clean 1.5 ml brown glass vial with cap and sealing. Weigh the vial and write down the exact value of the empty vial.
4) Take a clean spatula or a single-use glass pipette to fill 10 mg of the solid or highly viscous sample into the before weighed vial.
5) Weigh vial+sample and write down the exact value.
6) Add now toluene to obtain a dilution of 1:100. In case of 10 mg sample, add 990 µl toluene to the sample with an adjustable Eppendorf pipette.
7) Close the vial with sealing and cap.
8) Gently shake the mixture by hand for approximately 1 min.

For liquid samples:

9) Take a new, clean 1.5 ml brown glass vial with cap and sealing.
10) Add 990 µl of toluene with an Eppendorf pipette to the vial.
11) Add 10 µl of the liquid fuel sample with an Eppendorf pipette into the vial.
12) Close the vial with sealing and cap.
13) Gently shake the mixture by hand for approximately 1 min.

Prepare a mixture of methanol/toluene (50:50 v/v).

14) Take a clean 250 ml conical flask with plug.
15) Add 100 ml of toluene and 100 ml of methanol to the flask and close the flask with the plug.
16) Gently shake the mixture by hand to mix both solvents.

Now, the sample can be further diluted to a final concentration of 0.5 mg/ml.

17) Take a new, clean 1.5 ml brown glass vials with cap and sealing.
18) Use an adjustable Eppendorf pipette to add 995 µl of the toluene/methanol mixture, prepared in step 14-16), to the vial.
19) Add 5 µl of the diluted sample obtained in step 8) or 13) to the vial.
20) Close the vial with a sealing and a cap.
21) Gently shake the mixture by hand for approximately 1 min.

Preparation of blank sample

The blank is prepared with the same procedure as used for the samples. The blank measurement can be also later used for background correction in sample spectra and should be measured between each sample.

22) Wear gloves to avoid contamination of the samples and the equipment.
23) Take a new, clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 1000 µl of the toluene/methanol (50:50 v/v) mixture prepared in steps 14-16) to the vial.
24) Close the vial with a sealing and a cap.
25) Gently shake the mixture by hand for approximately 1 min.
26) Take another clean 1.5 ml glass vials with cap and sealing. Use an Eppendorf pipette to add 995 µl of the toluene/methanol (50:50 v/v) mixture to the vial.
27) Add 5 µl of toluene prepared in steps 23-25) to each vial.
28) Close the vials with sealings and caps.
29) Gently shake the mixtures by hand for approximately 1 min.

Measurement

Before each sample, a solvent blank has to be acquired.

30) Wear gloves to avoid contamination of the samples and the equipment.
31) Clean the syringe by flushing the whole volume with toluene for 10 times.
32) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
33) Fill the syringe with approximately 200 µl of the blank sample prepared in step 29). Make sure that no air bubbles are left inside the syringe.
34) Set the parameters for the FT-ICR MS as given below.
35) Check that the vaporizer has reached the desired temperature.
36) Place the syringe into the syringe pump and connect the capillary.
37) Start tune modus at the FT-ICR MS.
38) Press “fast forward” until a stable signal is obtained.
39) Start the measurement.

Measurement of the sample.

40) Wear gloves to avoid contamination of the samples and the equipment.
41) Clean the syringe by flushing the whole volume with toluene for 10 times.
42) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
43) Fill the syringe with approximately 200 µl of the sample prepared in step 21). Make sure that no air bubbles are left inside the syringe.
44) Set the parameters for the FT-ICR MS as given below.
45) Check that the vaporizer has reached the desired temperature.
46) Place the syringe into the syringe pump and connect the capillary.
47) Start tune modus at the FT-ICR MS.
48) Press “fast forward” until a stable signal is obtained.
49) Start the measurement.

Cleaning

After each sample measurement, it is necessary to clean the syringe and capillary.

50) Wear gloves to avoid contamination the equipment.
51) Clean the syringe by flushing the whole volume with toluene for 10 times.
52) Clean the syringe by flushing the whole volume with toluene/methanol mixture for 10 times.
53) Switch on “tune” modus at the FT-ICR MS.
54) Flush the capillary using the syringe several times with toluene and toluene/methanol mixture. If there are sticky contaminants, use different solvents in the order of polarity to prevent a clocking of the sprayer needle.
55) Flush the capillary until the capillary is clean, which can be checked online in the tune mode.
56) The instrument is now ready for the next measurement.

APCI Injection Parameters

capillary voltage	-4000 V
corona needle	9000 nA
dry gas flow	3 l/min
flow rate (syringe)	600 µl/h
nebulizer gas flow	2.5 l/min
source temperature	220 °C
vaporizer temperature	300

APPI Injection Parameters

capillary voltage	-900 V
dry gas flow	3 l/min
flow rate (syringe)	600 µl/h
nebulizer gas flow	2.5 l/min
source temperature	220 °C
vaporizer temperature	300 °C

FT-ICR Mass Spectrometry Parameters

Positive mode

accumulation time	0.025 s
mass-to-charge ratio (m/z) range	147-1300 Da
mode	broadband
octopole energy	350 V_{pp}
quadrupole collision energy	1200 V_{pp}
quadrupole lower cut-off	200
resolution	300 000 – 900 000 @ m/z400 (depending on sample)
spectra number	150
transient length	3.4 s * (depending on sample)
Time-of-flight	0.8 ms

*For a 12 T FT-ICR MS with para cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Data Handling and Anticipated Results

Calibration

The mass spectra can be externally m/z calibrated by standards from quality measurements. For higher mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.).

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

5. Direct infusion ESI FT-ICR MS for trace polar species in diesel samples

Introduction

Application field

Polar species in diesel contribute significantly to the fuels' physical properties, such as lubricity and stability and are therefore important to analyse.

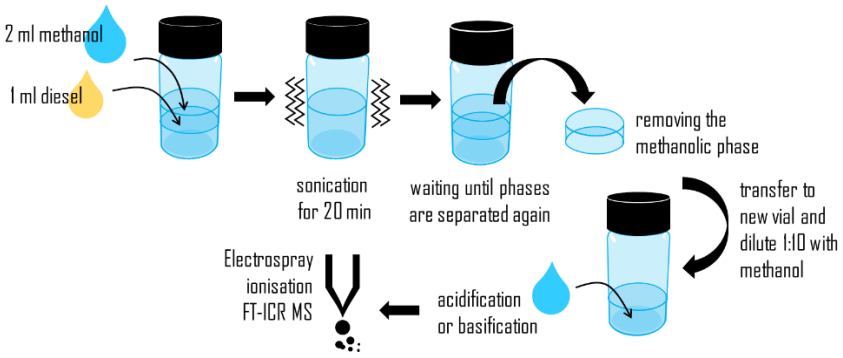

Figure 13. Graphical illustration of the preparation procedure. 1) Methanolic extraction of the fuel. 2) Sonication of the extraction mixture. 3) Remove of the methanolic phase. 4) Dilution of the extract 1:10 with methanol. 5) Adding of 1 volume-% formic acid/ammonia solution. 6) ESI positive/negative measurement.

Materials

Reagents

Chemicals

- Arginine in methanolic solution (2 mg/ml)
- Ammonia solution 25% (Merck LiChropur for HPLC)
- Formic acid 98-100 % (Merck LiChropur for HPLC)

Solvents

- Methanol (Romil-UpS Ultra Gradient for HPLC-MS,>99.9%)

Gases

- Nitrogen 5.0 (for FT ICR MS)

Equipment

Instruments

- FT-ICR MS and software (7T Solarix with infinity cell, Bruker Daltonics GmbH and Bruker Compass Data Analysis 4.0 SP 5 software package, Bruker Daltonics GmbH)
- Sonication bath

Consumables

- Glass vials, with screw caps (8 ml, BGB analytics)
- Glass vials, with screw caps (1.5 ml, BGB analytics)
- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)
- Glass syringe for ESI (250 µl, blunt needle, Hamilton)

Equipment Setup

For the ESI measurements, a commercial ESI ion source (Bruker Daltonics GmbH) is used. The general set-up can be abstracted from **Erreur ! Source du renvoi introuvable.**.

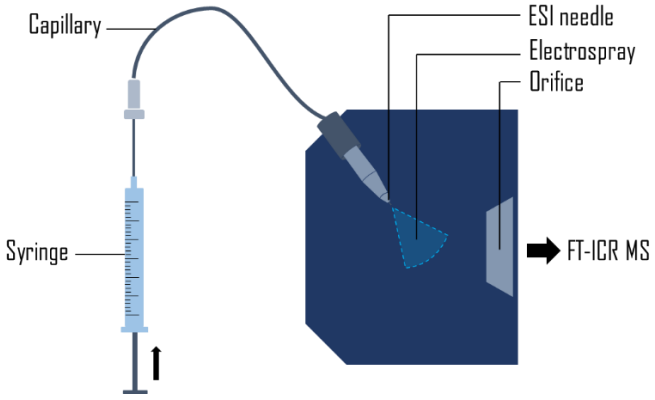

Figure 14. Schematic ESI setup for direct infusion measurements.

Procedure

Dilution of the arginine standard

1) Wear gloves to avoid contamination of the samples and the equipment.
2) Take a new, clean 1.5 ml brown glass vial with cap and sealing. Fill 990 µl of methanol into the vial using an adjustable Eppendorf pipette (1000 µl).
3) Add 10 µl of methanolic arginine solution (2 mg/ml) to the vial using an adjustable Eppendorf pipette (10 µl).
4) Close the vial with a sealing and a cap.
5) Gently shake the mixture by hand for approximately 1 min.

6) Take another new, clean 1.5 ml brown glass vial with cap and sealing. Fill 900 µl of methanol into the vial using an adjustable Eppendorf pipette (1000 µl).
7) Add 100 µl of the diluted arginine solution from step 2-5) to the vial using an adjustable Eppendorf pipette (100 µl).
8) Close the vial with a sealing and a cap.
9) Gently shake the mixture by hand for approximately 1 min.

Extraction of the fuel

See also section “preparation of blank sample”.

10) Wear gloves to avoid contamination of the samples and the equipment.
11) For the extraction of polar components from light, petroleum-derived fuels, fill 1 ml of the neat fuel with a pipette into an 8 ml glass vial.
12) Add with a pipette 2 ml of methanol to the vial.
13) Close the vial with a sealing and a cap.
14) Choose a small beaker, in which the sample vial cannot fall down.
15) Add water to the beaker until it has approximately 1/3 of the height of the sample vial.
16) Place the beaker with the sample vial in a sonication bath.
17) Sonicate the mixture for 20 min.
18) Take the vial out of the sonication bath and place the vial on a flat surface in the dark.
19) Wait for 2 hours until both phases are separated.
20) Transfer the top layer (methanol phase) into a new, clean 1.5 ml brown glass vial with cap and sealing.

Dilution of the sample

21) Wear gloves to avoid contamination of the samples and the equipment.
22) Take two new, clean 1.5 ml brown glass vials with caps and sealings. Fill 900 µl of methanol into each vial using an adjustable Eppendorf pipette (1000 µl).
23) Using an adjustable Eppendorf pipette (100 µl), add 100 µl of the extract obtained in step 10-20) to each vial.
24) Close the vial with a sealing and a cap.
25) Gently shake the mixture by hand for approximately 1 min.

For better ionisation of the components, an acid has to be added for positive ESI measurements, whereas negative ESI measurements require the addition of a base.

26) For positive ESI measurements, add 1 volume-% of formic acid for acidification to the sample. Therefore, add 10 µl formic acid with a pipette to 1 ml of the diluted sample.
27) For negative ESI measurements, add 1 volume-% of ammonia to the sample. Therefore, add 10 µl ammonia with a pipette to 1 ml of the diluted sample.

Preparation of blank sample

Besides the sample preparation, it is recommended to make the same preparation with a blank sample for blank value correction or for removing contaminants.

28) Wear gloves to avoid contamination of the samples and the equipment.
29) Take a new, clean 8 ml vial and add 2 ml of methanol with a pipette.
30) Close the vial with a sealing and a cap.
31) Choose a small beaker, in which the sample vial cannot fall down.
32) Add water to the beaker until it has approximately 1/3 of the height of the sample vial.
33) Place the beaker with the sample vial in a sonication bath.
34) Sonicate the mixture for 20 min.
35) Take the vial out of the sonication bath and place the vial on a flat surface in the dark.
36) Wait for 2 hours until.
37) Transfer the methanol to a new, clean 1.5 ml glass vial with cap and sealing.
38) Take two new, clean 1.5 ml brown glass vials with caps and sealings. Fill 900 µl of methanol into each vial using an adjustable Eppendorf pipette (1000 µl).
39) Using an adjustable Eppendorf pipette (100 µl), add 100 µl of the blank sample finally obtained in step 35) to each vial.
40) Close the vial with a sealing and a cap.

41) Gently shake the mixture by hand for approximately 1 min.
42) For positive ESI measurements, add 1 volume-% of formic acid for acidification to the blank. Therefore, add 10 µl formic acid with a pipette to 1 ml of the blank sample.
43) For negative ESI measurements, add 1 volume-% of ammonia to the blank. Therefore, add 10 µl ammonia with a pipette to 1 ml of the blank sample.

Calibration of the Instrument

For calibration of the instrument, the 0.002 mg arginine solution is used. Due to the relatively high concentration of the arginine, it forms clusters allowing for a calibration from m/z 174 to approximately m/z 700.

44) Wear gloves to avoid contamination of the samples and the equipment.
45) Clean the syringe 15 times by flushing the whole volume with methanol.
46) Fill the syringe with approximately 100 µl of the arginine standard prepared in steps 1-9). Make sure that no air bubbles are left inside the syringe.
47) Set the parameters for the FT-ICR MS as given below. Average 50 spectra for the calibration.
48) Place the syringe into the syringe pump and connect the capillary.
49) Start tune modus at the FT-ICR MS.
50) Press “fast forward” until a stable signal is obtained.
51) Start the measurement.
52) Calibrate the m/z values at the instrument using the acquisition software.

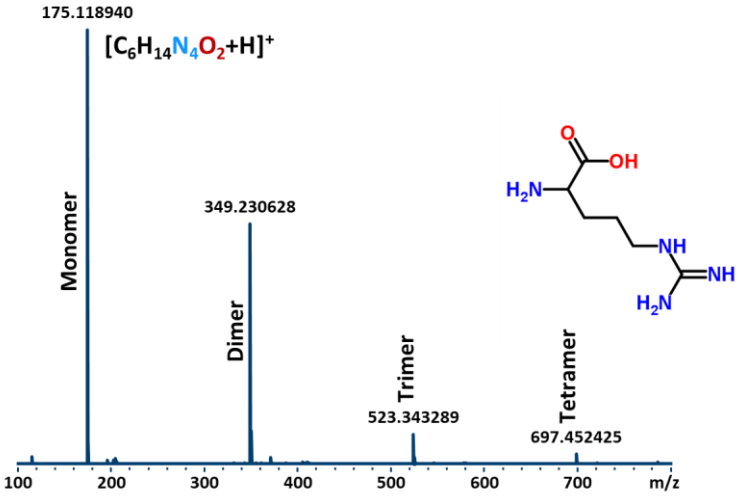

Figure 15. Typical arginine spectrum used for mass calibration of the instrument.

Measurement

Before each sample, a solvent blank has to be acquired.

53) Wear gloves to avoid contamination of the samples and the equipment.
54) Clean the syringe 15 times by flushing the whole volume with methanol.
55) Fill the syringe with approximately 200 µl of the blank sample prepared in step 42) or step 43). Make sure that no air bubbles are left inside the syringe.
56) Set the parameters for the FT-ICR MS as given below.
57) Place the syringe into the syringe pump and connect the capillary.
58) Start tune modus at the FT-ICR MS.
59) Press “fast forward” until a stable signal is obtained.
60) Start the measurement.

Measurement of the sample.

61) Wear gloves to avoid contamination of the samples and the equipment.
62) Clean the syringe 15 times by flushing the whole volume with methanol.
63) Fill the syringe with approximately 200 µl of the sample prepared in step 26) or step 27). Make sure that no air bubbles are left inside the syringe.
64) Set the parameters for the FT-ICR MS as given below.
65) Place the syringe into the syringe pump and connect the capillary.
66) Start tune modus at the FT-ICR MS.
67) Press “fast forward” until a stable signal is obtained.
68) Start the measurement.

Cleaning

After each sample measurement, it is necessary to clean the ESI syringe and capillary.

69) Wear gloves to avoid contamination of the equipment.
70) Clean the syringe 15 times by flushing the whole volume with methanol.
71) Switch on “tune” modus at the FT-ICR MS.

72) Flush the capillary using the syringe several times with methanol. If there are sticky contaminants, use different solvents in the order of polarity to prevent a clocking of the ESI needle.
73) Flush the capillary until the capillary is clean, which can be checked online in the tune mode.
74) The instrument is now ready for the next measurement.

ESI Injection Parameters

Positive mode

collision induced dissociation	45 V
dry gas flow	adjust for stable electrospray
flow rate (syringe)	2.5 µl/min
nebulizer gas flow	adjust for stable electrospray
spray voltage	+4 kV

Negative mode

collision induced dissociation	45 V
dry gas flow	adjust for stable electrospray
flow rate (syringe)	5 µl/min
nebulizer gas flow	adjust for stable electrospray
spray voltage	-3 kV

FT-ICR Mass Spectrometry Parameters

mass-to-charge ratio (m/z) range	100-1000 Da
resolution	150 000 @m/z 200 *
spectra number	200
transient length	0.490 s *
Time-of-flight	0.6-0.7 ms

*For a 7 T FT-ICR MS with infinity cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Data Handling and Anticipated Results

Calibration

The mass spectra can be externally m/z calibrated by arginine and its oligomeric clusters from quality measurements. For higher mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.). For calculation, we recommend to limit the assignment boundaries as following:

Signal-to-noise ratio above 6, even electron configuration, a double bond equivalent of -1.5 to 30, a mass accuracy of 1 ppm and sum formula parameters of $C_{6\text{-}50}H_{4\text{-}100}N_{0\text{-}2}O_{0\text{-}5}S_{0\text{-}3}$. For positive ion mode, 0-1 Na atom can be considered due to possible adduct formation.

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion ESI measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

6. GC-APCI-FT-ICR MS for polar and semi-polar species in diesel samples and similar distillation cuts

Introduction

Application field

Polar species in diesel contribute significantly to the fuels' physical properties, such as lubricity and stability and are therefore important to analyse.

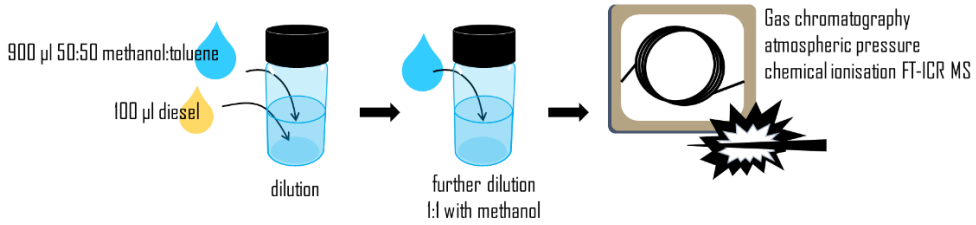

Figure 16. Graphical illustration of the preparation procedure. For GC experiments, only two dilution steps are necessary. 1) Dilution of the sample 1:10 in methanol:toluene mixture (50:50) 2)Dilution of the mixture 1:1 in methanol 3) GC-APCI-FT-ICR MS measurements.

Materials

Reagents

Chemicals

- PAH standard - EPA 525 mix A PAH (supelco, Merck, certified reference material, 500 µg/mL each component in dichloromethane)

Solvents

- Methanol (Romil-UpS Ultra Gradient for HPLC-MS,>99.9%)
- Toluene (Sigma–Aldrich Chromasolv Plus for HPLC,≥99.9%)
- Dichloromethane (LiChrosolv, Sigma Aldrich, Germany)

Gases

- Nitrogen 5.0 (for FT ICR MS)
- Helium 5.0 (carrier gas for GC)

Equipment

Instruments

- 7T FT-ICR MS and software (APEX Qe Series II with infinity cell, Bruker Daltonics GmbH and Bruker Compass Data Analysis 4.0 SP 5 software package, Bruker Daltonics GmbH)
- Gas chromatography (Model CP 3800, Varian Technologies) equipped with a programmed temperature vaporizing injector (1079 PTV injector) and ChromatoProbe sample introduction device (Agilent technologies)

Consumables

- GC column (BPX5, SGE Analytical Science, Australia)
- Glass microvials for ChromatoProbe (Agilent Technologies, 8010-0419)
- Glass vials, with sealings and screw caps (1.5 ml, BGB analytics)

Further equipment

- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)
- Glass syringe (10 µl, conical needle, Hamilton)

Equipment Setup

For the GC-APCI-FT-ICR MS measurements, a gas chromatograph (Model CP 3800, Varian) is connected via the commercial GC-APCI II source (Bruker Daltonics GmbH) to the 7 T FT-ICR MS (Bruker Daltonics GmbH). The chromatographically separated analytes are transferred into the ion source via a heated transferline (300 °C) and then ionised with atmospheric pressure chemical ionisation (APCI).

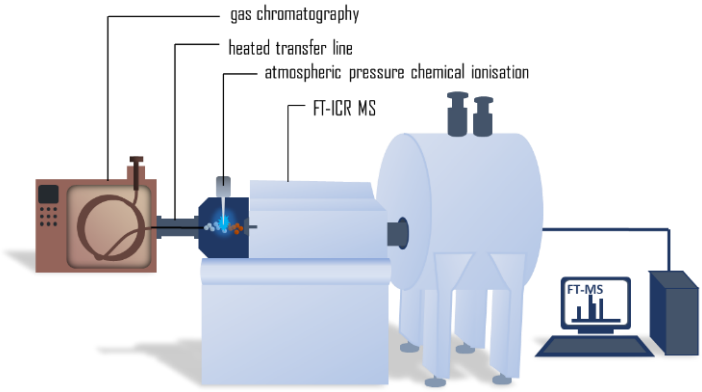

Figure 17. Schematic GC-APCI-FT-ICR MS setup.

Procedure

Dilution of the PAH standard

1) Wear gloves to avoid contamination of the samples and the equipment.
2) Take a new, clean 1.5 ml brown glass vial with cap and sealing. Fill 990 µl of dichloromethane into the vial using an adjustable Eppendorf pipette (1000 µl).
3) Add 10 µl of PAH standard mixture to the vial using an adjustable Eppendorf pipette (10 µl).
4) Close the vial with a sealing and a cap.

5) Gently shake the mixture by hand for approximately 1 min.

Dilution of the sample

6) Wear gloves to avoid contamination of the samples and the equipment.

Prepare a mixture of toluene and methanol (50:50 v/v), which is needed for the dilution of the sample.

7) Take a clean 250 ml conical flask with plug.
8) Add 100 ml of toluene and 100 ml of methanol to the flask and close the flask with the plug.
9) Gently shake the mixture by hand to mix both solvents.

Now, the sample can be diluted.

10) Take a clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 900 µl of the toluene:methanol (50:50 v/v) to the vial. Add 100 µl of the neat fuel to the vial to get a dilution of fuel:(toluene:methanol) 1:10 (v/v).
11) Close the vial with a sealing and a cap.
12) Gently shake the mixture by hand for approximately 1 min.
13) Dilute the mixture obtained in the previous step further 1:1 with methanol. Take a new, clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 500 µl of the previously diluted sample. Add 500 µl of methanol.
14) Close the vial with a sealing and a cap.
15) Gently shake the mixture by hand for approximately 1 min.

Preparation of blank sample

16) Wear gloves to avoid contamination of the samples and the equipment.

The blank is prepared with the same procedure as used for the samples. The blank measurement can be also later used for background correction in sample spectra.

17) Take a new, clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 1000 µl of the toluene:methanol (50:50 v/v) mixture prepared in steps 2) to 4) to the vial.
18) Close the vial with a sealing and a cap.
19) Gently shake the mixture by hand for approximately 1 min.
20) Take another clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 500 µl of the toluene:methanol (50:50 v/v) from steps 12) to 14) to the vial. Add 500 µl of methanol to the vial.
21) Close the vial with a sealing and a cap.
22) Gently shake the mixture by hand for approximately 1 min.

Measurement

Before the first sample measurement, an empty GC run needs to be done to clean the injector and the column from impurities, which may have been accumulated over night.

23) Check the GC parameters, especially column flow, head pressure, split ratio, and the temperature program of the column oven.

24) Set the values as given below.
25) Wait for the GC being ready.
26) Start the empty GC run.

Measure a PAH-Standard for retention time calibration and performance check of the GC.

27) Wear gloves to avoid contamination of the samples and the equipment.
28) Set the parameters given for the FT-ICR MS given below.
29) Unscrew the ChromatoProbe holder.
30) Use tweezers to insert a new, clean glass microvial in the holder.
31) Place the ChromatoProbe back.
32) Clean the 10 µl glass syringe before usage 10 times with 10 µl dichloromethane for each flush. Collect the used solvent in a waste bottle.
33) Fill the clean syringe with approximately 2 µl of the diluted standard and discard the volume to the waste.
34) Fill the syringe with 1 µl of diluted PAH standard. Make sure, that no air bubbles are left inside the syringe. You may have to repeat the filling of the syringe several times, but without discarding the sample.
35) Fill a small volume of air (1 µl) to the syringe after the PAH standard to avoid evaporation.
36) Take out the ChromatoProbe again.
37) Fill the PAH standard with the syringe into the microvial.
38) Place the ChromatoProbe back and screw it hand tight.
39) Wait for the head pressure to stabilize.
40) Start the GC measurement.
41) Start the FT-ICR MS measurement.

Measuring the sample is quite similar to measuring the PAH standard.

42) Wear gloves to avoid contamination of the samples and the equipment.
43) Set the parameters given for the FT-ICR MS given below.
44) Unscrew the ChromatoProbe holder.
45) Use tweezers to insert a new, clean glass microvial in the holder.
46) Place the ChromatoProbe back.
47) Clean the 10 µl glass syringe before usage 10 times with 10 µl toluene:methanol (50:50 v/v) for each flush. Collect the used solvent in a waste bottle.
48) Fill the clean syringe with approximately 2 µl of the diluted sample and discard the volume to the waste.
49) Fill the syringe with 1 µl of diluted sample. Make sure, that no air bubbles are left inside the syringe. You may have to repeat the filling of the syringe several times, but without discarding the sample.
50) Fill a small volume of air (1 µl) to the syringe after the sample to avoid evaporation.
51) Take out the ChromatoProbe again.
52) Fill the sample with the syringe into the microvial.
53) Place the ChromatoProbe back and screw it hand tight.
54) Wait for the head pressure to stabilize.
55) Start the GC measurement.
56) Start the FT-ICR MS measurement.

GC Parameters

The method is optimized to also address higher boiling point species.

Carrier gas	Helium 5.0
Column	BPX5, 15 m, 250 µm inner diameter, 0.10 µm film
Flow rate	10 ml/min
GC oven program	50 °C (5min) → 5 K/min → 200 °C → 10 K/min → 250 °C → 20 K/min → 330 (10 min)
Injector oven program	50 °C (1 min) → 10 K/min → 80 °C → 60 K/min → 320 °C (20 min)
Transferline	300 °C
Split ratio	1:5 – 1:10

APCI Parameters

capillary voltage	+ 3 kV
Collision induced dissociation (in-source)	30 V
Corona needle	3000 nA
dry gas flow	2-3 l/min
dry gas temperature	230-250 °C
nebulizer gas flow	2-3.5 l/min

FT-ICR Mass Spectrometry Parameters

Chromatography mode	enable
mass-to-charge ratio (m/z) range	120-1000 Da
resolution	340 000 @m/z 200 *
spectra number	1
transient length	1.15 s *
Time-of-flight	0.6-0.7 ms

*For a 7 T FT-ICR MS with infinity cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Data Handling and Anticipated Results

Calibration

For high mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.). For calculation, we recommend to limit the assignment boundaries as following:

Signal-to-noise ratio above 6, even and odd electron configuration, a double bond equivalent of -1.5 to 30, a mass accuracy of 1 ppm and sum formula parameters of $C_{6-50}H_{4-100}N_{0-2}O_{0-5}S_{0-3}$. The GC mode enables besides the analysis of the summed spectra over the analysis time also the investigation of the time resolved measurements. The peak annotation can be done in Bruker Data Analysis or with self-written scripting.

Retention index and structure assignment with data bases

The retention index (RI) and the exact mass are necessary to assign structures from data bases to the found compounds in the sample. Therefore, a retention index calibration is necessary for each spectrum. The retention index can be calibrated by an added internal standard or on sample internal species, for example, core structure poly cyclic aromatics which are normally present in petroleum-derived samples. For the calibration, assign a fixed retention times to the internal markers. Calculate the retention times for all other compounds eluting between two standards. Be careful, because the described GC method changes the heating ramp in between which causes a non-linear shape for the retention index calibration.

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion ESI measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

7. TG-APCI-FT-ICR MS for solid or highly viscous petroleum fractions, such as asphaltenes and bitumen

Introduction

Application field

Heavy petroleum fractions contain species with high molecular weight and a large number of heteroatoms such as nitrogen, oxygen or sulphur. Because of their enormous complexity, high boiling fractions are still an analytical challenge. Because of its high mass resolution and high mass accuracy, Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FT-ICR MS) enables the identification of thousands of mass peaks in one spectrum. The coupling to thermogravimetry enables the temperature resolved analysis of the desorbable and pyrolysable material of the samples without prior sample treatment.

Materials

Reagents

Standards

- Polystyrene (NIST standard with narrow molecular weight distribution, Merck)

Gases

- Nitrogen 5.0 (for FT ICR MS and for TG)

Equipment

Instruments

- 7T FT-ICR MS and software (APEX Qe Series II with infinity cell, Bruker Daltonics GmbH and Bruker Compass Data Analysis 4.0 SP 5 software package, Bruker Daltonics GmbH)
- Thermobalance (TG 209 cell, NETZSCH Gerätebau)
- Analytical balance (Sartorius AG, Göttingen, Germany)

Consumables

- Aluminium crucibles (85 µl, up to 600 °C, Thepro GbR)
- Alumina crucibles (85 µl, up to 1000 °C, Thepro GbR)
- Fuzz-free tissue (Kimberly-Clark Europe Limited/Professional Sector, Reigate, UK)
- Single-use glass pipettes

Further equipment

- Tweezers

- spatula
- Side-cutting pliers
- Laboratory gloves

Equipment Setup

For the TG-APCI-FT-ICR MS measurements, a thermobalance (Model TG 209 cell, NETZSCH Gerätebau) is connected via the commercial GC-APCI II source (Bruker Daltonics GmbH) and an additional heated transferline (300 °C) to the 7 T FT-ICR MS (Bruker Daltonics GmbH). The evaporated compounds are transferred into the ion source via a slight overpressure of 8 mbar over the heated transferline and then ionised with atmospheric pressure chemical ionisation (APCI). The TG oven atmosphere is flushed with nitrogen during the measurements for avoiding oxidation and combustion of the material. Because of the comparatively slow evaporation of the material when using heating rates of 5 °C/min to 10 °C/min, alternating MSMS experiments can be conducted to get a reflection of the core structures of the previous spectrum.

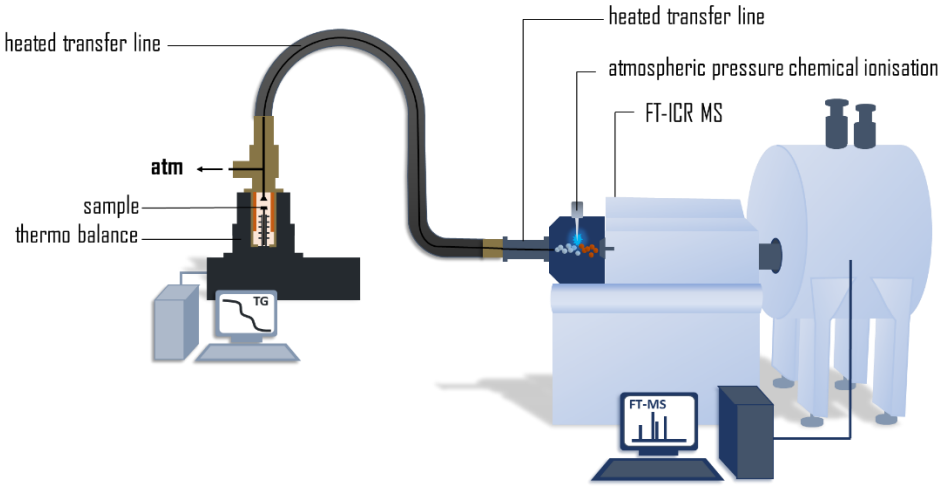

Figure 18. Schematic TG-APCI-FT-ICR MS setup.

Procedure

Sensitivity check

In the beginning of each measurement day, it is recommended to measure a polystyrene standard with the same settings as for the sample measurement to test the sensitivity of the set-up. The polystyrene granules need to be cut in smaller pieces of 0.3-0.5 mg.

1) Wear gloves to avoid contamination of the sample materials and equipment.
2) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.

3) Weigh an empty crucible. Therefore, take a new, empty aluminium crucible with tweezers and place it on the analytical balance. Write down the weight of the crucible and use “tara” for setting the value to zero again. Take off the crucible from the balance with tweezers and store it on a clean tissue.
4) Take side-cutting pliers and clean the blade with clean methanol and dichloromethane using a fuzz-free tissue.
5) Take a polystyrene granule with tweezers and cut a small piece of 0.3-0.5 mg from the granule with the cleaned side-cutting pliers.

Figure 19. Polystyrene granule sampling for sensitivity test.

6) Use the before-weighed crucible to fill in the cut polystyrene and check the weight on the analytical balance. If the weight is too high, cut the piece again into smaller pieces until it fits. Write down the exact sample mass.
7) Slowly open the thermobalance to release the slight overpressure. Caution! If the balance is opened to fast, the crucible can fall from the holder into the balance oven, which in turn can destroy the thermocouple of the oven.
8) Take the filled crucible with tweezers and gently place it on the sample holder.
9) Carefully close the thermobalance and allow the overpressure to stabilize. Check that the overpressure is set to 8 mbar. If not, adjust the pressure by gently opening or closing the outlet valve at the thermobalance.

10) Enter the heating protocol to the thermobalance software (for the herein described thermobalance, NETZSCH Proteus Analysis Software is used). For polystyrene, a heating rate of 10 K/min is recommended, starting at 20 °C for 2 min and then heating to 600 °C hold for 10 min.
11) Set the parameters as given below for the FT-ICR MS.
12) Start the measurement at the thermobalance.
13) Start the measurement at the FT-ICR MS.
14) After the measurement is finished, wait until the thermobalance has cooled down before removing the crucible.

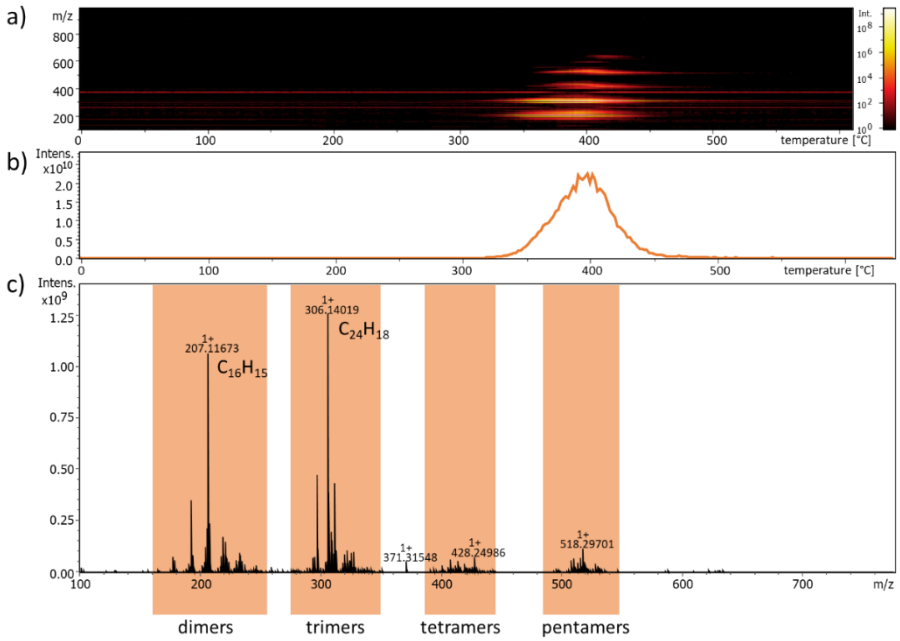

Figure 20. Typical TG-FT-ICR MS spectrum of polystyrene ionised by APCI. The heating rate is 5 K/min. The main decomposition of the polymer occurs between 350 °C and 450 °C. a) survey view of the temperature resolved mass spectrum. Intensity is color-coded. b) Total ion chromatogram (TIC). c) summed mass spectrum of polystyrene. Dimers to pentamers can be identified.

Uplift Correction

An empty crucible needs to be measured at least once for uplift correction with the same temperature program as used for the samples. Due to the heating, the viscosity of the inert gas (nitrogen) changes during the measurement also effecting the measured sample mass. However, the uplift can be adjusted by subtracting the measurement of an empty crucible from the sample measurement.

15) Wear gloves to avoid contamination of the sample materials and equipment.
16) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.

17) Weigh an empty crucible. Therefore, take a new, empty aluminium crucible with tweezers and place it on the analytical balance. Write down the weight of the crucible. Take off the crucible from the balance with tweezers and store it on a clean tissue.
18) Slowly open the thermobalance to release the slight overpressure. Caution! If the balance is opened to fast, the crucible can fall from the holder into the balance oven, which in turn can destroy the thermocouple of the oven.
19) Take the empty crucible with tweezers and gently place it on the sample holder.
20) Carefully close the thermobalance and allow the overpressure to stabilize. Check that the overpressure is set to 8 mbar. If not, adjust the pressure by gently opening or closing the outlet valve at the thermobalance.
21) Enter the desired heating protocol to the thermobalance software (for the herein described thermobalance, NETZSCH Proteus Analysis Software is used). For solid to viscous petroleum-derived

materials, a heating rate of 5-10 K/min is recommended, starting at 20 °C for 2 min and then heating to 600 °C hold for 10 min.

22) Set the parameters as given below for the FT-ICR MS.
23) Start the measurement at the thermobalance.
24) Start the measurement at the FT-ICR MS.
25) After the measurement is finished, wait until the thermobalance has cooled down before removing the crucible.

Measurement of a sample

26) Wear gloves to avoid contamination of the sample materials and equipment.
27) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.
28) Weigh an empty crucible before filling with the sample to determine the residual mass of the sample after the measurement. Take a new, empty aluminium crucible with tweezers and place it on the analytical balance. Write down the weight of the crucible and use “tara” for setting the value to zero again. Take off the crucible from the balance with tweezers and store it on a clean tissue.
29) Use a clean spatula or single-use glass pipette to fill the sample into the crucible. For bitumen, 1 mg of sample is recommended, whereas for asphaltenes 0.5 mg sample is recommended.
30) Weigh the crucible again on the analytical balance to get the exact sample mass. Adjust the amount of sample if necessary. Write down the exact sample mass.
31) Slowly open the thermobalance to release the slight overpressure. Caution! If the balance is opened to fast, the crucible can fall from the holder into the balance oven, which in turn can destroy the thermocouple of the oven.
32) Take the crucible with the sample with tweezers and gently place it on the sample holder.

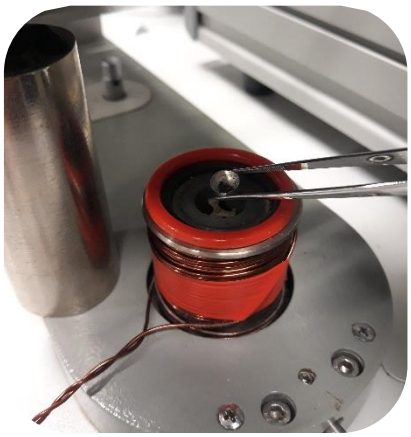

Figure 21. Introduction of the crucible in the thermobalance sample holder.

33) Carefully close the thermobalance and allow the overpressure to stabilize. Check that the overpressure is set to 8 mbar. If not, adjust the pressure by gently opening or closing the outlet valve at the thermobalance.
34) Enter the desired heating protocol to the thermobalance software (for the herein described thermobalance, NETZSCH Proteus Analysis Software is used). For solid to viscous petroleum-derived

materials, a heating rate of 5-10 K/min is recommended, starting at 20 °C for 2 min and then heating to 600 °C hold for 10 min.

35) Set the parameters as given below for the FT-ICR MS.
36) Start the measurement at the thermobalance.
37) Start the measurement at the FT-ICR MS.
38) After the measurement is finished, wait until the thermobalance has cooled down before removing the crucible.

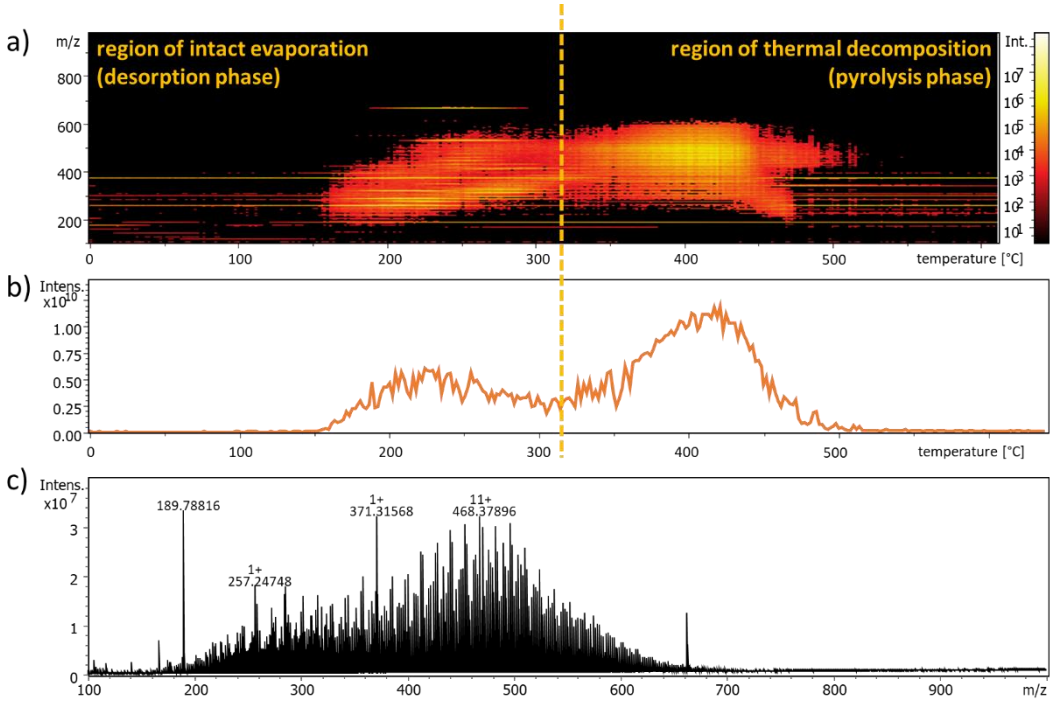

Figure 22: TG-FT-ICR MS spectrum of a heavy petroleum sample ionised by APCI. The heating rate is 5 K/min. The spectrum can be divided into a region of intact evaporation (desorption phase) and a region of thermal decomposition (pyrolysis phase). a) survey view of the temperature resolved mass spectrum. Intensity is color-coded. The dotted line indicates the separation temperature of desorption and pyrolysis phase. b) Total ion chromatogram (TIC). c) summed mass spectrum of polystyrene. Dimers to pentamers can be identified.

TG Parameters

Carrier gas	Nitrogen 5.0
Overpressure	8 mbar
Temperature Program	for Bitumen: 20 °C (2 min) → 10 K/min → 600 °C (10 min) for Asphaltenes: 20 °C (2 min) → 5 K/min → 600 °C (10 min)
Transferline	deactivated fused silica capillary, 300 °C

APCI Parameters

capillary voltage	- 3.5 kV
Corona needle	3000 nA
dry gas flow	2 l/min
dry gas temperature	220 °C
nebulizer gas flow	3 l/min

FT-ICR Mass Spectrometry Parameters

Accumulation time	0.1 s (MS), 1 s (MSMS)
Chromatography mode	enabled
CID voltage (MSMS)	30 V
mass-to-charge ratio (m/z) range	100-1000 Da
resolution	290 000 @m/z 400 *
spectra number	5
transient length	2 s *
Time-of-flight	0.7 ms

*For a 7 T FT-ICR MS with infinity cell; for other magnetic field strength or ICR cells, these parameters have to be adjusted.

Data Handling and Anticipated Results

Calibration

For high mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm. For first calibration in DataAnalysis, the spectrum can be summed over the time frame, in which sample signals occur. Calibrate the summed spectrum and recalculate each line spectrum. Each line spectrum can be further recalculated after export with self-written routines.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.). For calculation, we recommend to limit the assignment boundaries as following: Signal-to-noise ratio above 6, even and odd electron configuration, a double bond equivalent of 0 to 30, a mass accuracy of 1 ppm and sum formula parameters of $C_{4\text{-}100}H_{4\text{-}200}N_{0\text{-}2}O_{0\text{-}5}S_{0\text{-}3}$. The TG mode enables besides the analysis of the summed spectra over the analysis time also the investigation of the temperature resolved measurements. The peak annotation can be done in Bruker Data Analysis or with self-written scripting.

TG curves

The TG curves show the mass loss of the sample with increasing temperature. Different stages of evaporation or pyrolysis can be extracted from the data. An uplift correction is necessary due to the changing viscosity properties of the nitrogen carrier gas with increasing temperature.

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion ESI measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

8. Taxonomy and classification of Upper Pleistocene bones by ultrahigh resolution MALDI-FT ICR

Introduction

Application field

The archaeological bones of extinct species contain ancient molecules (DNA, proteins) which allow us to understand the biological past of extinct species and their evolutionary process. However, during excavations, it is common to find fragmented bones due to the presence of carnivores and human activity. This leads some difficulties to make identification of these bones for the community of archaeology. To solve this, the development of a high throughput proteomics method based on the analysis of bones collagen by MALDI-FT ICR allows characterization of biological classification rank of upper Pleistocene mammalian bones.

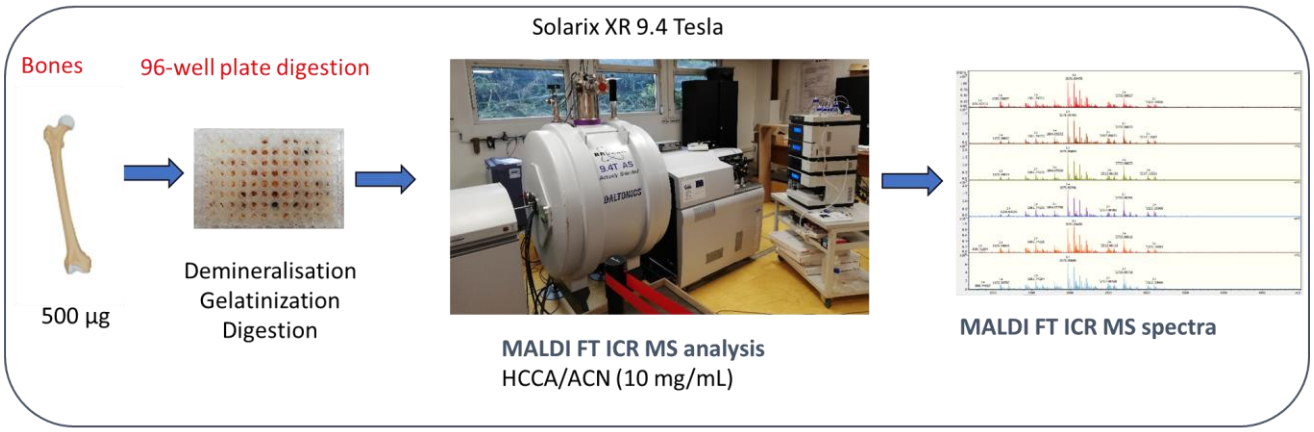

Figure 23. Workflow of ancient bones analysis by MALDI-FT ICR. At first, a small amount of bone powder is collected from ancient bone and introduced into a 96-well plate 0.45 µm PVDF. Here, we work on 500 µg. Then, the sample preparation is performed by demineralization of bone powder, collagen gelatinization by heat and collagen digestion with trypsin/LysC. Finally, Samples are analysed by mass spectrometry MALDI-FT ICR.

Materials

Reagents

Chemicals

- Trifluoroacetic acid (Sigma Aldrich, for HPLC-MS, > 99.9%) used at 5% in water

- Acetic acid 98-100 % (Sigma Aldrich, for HPLC-MS, > 99.9%) used at 0.5% in water; and 0.5% in 80% acetonitrile
- Formic acid 98-100 % (Sigma Aldrich, for HPLC-MS, > 99.9%) used at 0.1% in water Caution! Formic acid is corrosive and toxic if inhaled

Solvents

- Water (LC/MS grade)
- Ammonium bicarbonate 50 mM, pH 8.8
- Methanol (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Methanol is highly flammable and toxic
- Ethanol (Sigma Aldrich, HPLC-MS, > 99.9%) used at 70%
- Acetonitrile (Sigma Aldrich, HPLC-MS, > 99.9%) Caution! Acetonitrile is highly flammable and toxic
- Trypsin/LysC MS grade recomposed with ammonium bicarbonate 50 mM, pH 8.8 (20 µg enzyme for 400 µL of ammonium bicarbonate solution)
- HCCA (a-Cyano-4-hydroxycinnamic acid) used at 10 mg/mL in 80% acetonitrile

Gases

- Nitrogen 5.0 (for FT ICR MS)
- Helium

Equipment

Instruments

- Shaker & mixer (Heidolph Instruments Gmbh&Co.)
- TurboVap 96 evaporator (Caliperr)
- PlatePrep 96-well Vacuum Manifold Manifold (Sigma Aldrich)
- FT-ICR MS Bruker SolariX XR (9.4T with harmonised cell, Bruker Daltonics GmbH; Bruker Compass Data Analysis 5.0, Bruker Daltonics GmbH)

Consumables

- 96-well Filtration Plate MultiScreen HTS IP Sterile Plate 0.45 µm Hydrophobic High Protein Binding Immobilion-P Membrane
- AttractSPE DISKS 96 plate C18 Affinisep
- 1.5 mL tube (SARSTEDT)
- 15 mL and 50 mL tube (SARSTEDT)
- 10 µL/200 µL/1000 µL tips (SARSTEDT)
- 96-well plate (SARSTEDT)

Equipment Setup

For the MALDI measurements, FT-ICR MS SolariX 9.4T (Bruker Daltonics GmbH) is used. The general set-up can be abstracted from **Erreur ! Source du renvoi introuvable.**.

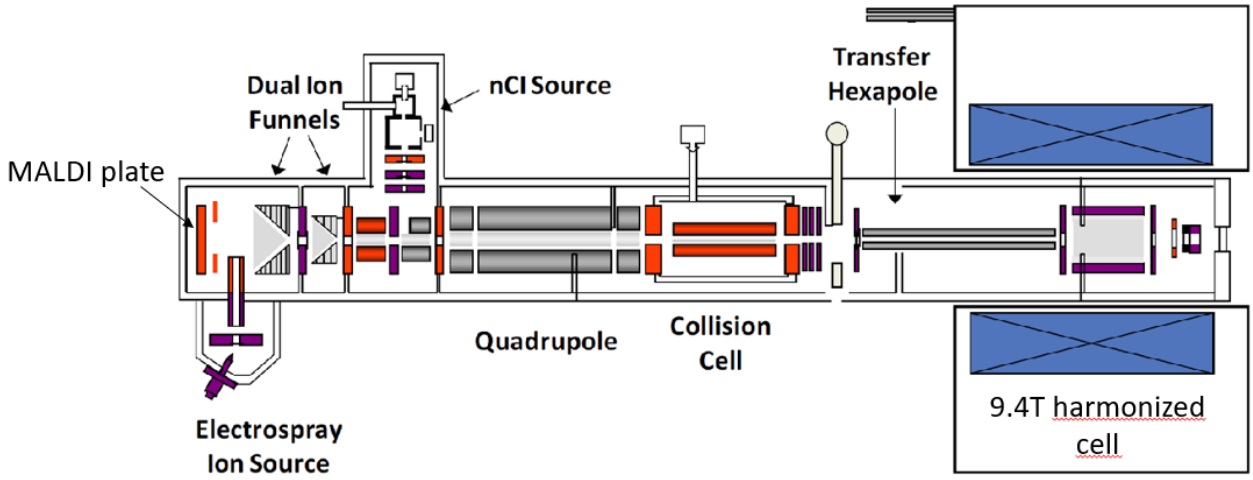

Figure 24. Schematics MALDI FT-ICR MS setup.

Laser Nd:Yag shots are fired on samples in order to ionizes peptides. The matrix co-crystallized with the sample will absorb energy from the laser and transfer it to the sample. The ionized peptides will be injected into the mass spectrometer and will be trapped in the harmonized cell of the FT-ICR. Once trapped, the ions will be excited by radio frequency (RF), which will induce their rotation under the action of the 9.4T magnetic field. Then, thanks to Fourier transform, the FT-ICR will measure this oscillation and deduce their *m/z. This mass spectrometer has a very high measurement precision of m/z at very high resolution.*

Procedure

Sampling

Ancient bones are collected on archaeological site by palaeontologist. Bone powder is collected from fragmented bones and well-conserved bones.
IMPORTANT: wear gloves during all manipulations to avoid keratin contamination.

Sample Preparation

1) 1) Crush the bone to a powder with mortar or by using scraping the bone with a scalpel.
2) Hydration of the 96-well plate 0.45 µm PVDF with 100 µL of 70% ethanol, filter by aspiration
3) Washing with 200 µL of 50 mM pH 8.8 Ammonium bicarbonate solution (ABC) filter by aspiration
4) Introduce 1 mg of bone powder and add 200 µL of 2% TFA for bone demineralization, incubation for 4 hours at room temperature, cover the plate with an adhesive plastic film
5) Remove the demineralization solution by aspiration with PlatePrep 96-well Vacuum Manifold.
6) Washing the demineralized bone powder with 200 µL of 50 mM pH 8.8 ABC solution, filter by aspiration. Repeat this step two time.
7) Add 200 µL of 50 mM pH 8.8 ABC solution, incubation 1 hour at 60°C in the oven and cover with an adhesive plastic film for bone collagen gelatinization
8) Add 10 µL of Trypsin/LysC, cover the plate with an adhesive plastic film and overnight incubation at 37°C, 400 rpm agitation for collagen digestion
9) the supernatant containing the peptides are recovered in a 96-well plate after aspiration with the system PlatePrep 96-well Vacuum Manifold.
10) Add 50 µL of 50 mM pH 8.8 ABC solution in PVDF plate and filter by aspiration to be sure to recover all the collagenic peptides
11) The classical 96-well plate is placed into a speed vacuum to dry peptides

Sample Purification

Dried peptides are dissolved in 200 µL of acidified water with 0.5% acetic acid and purified in C18 Affinisep 96-well plate on PlatePrep 96-well Vacuum Manifold.

1) Hydration of C18 96 plates with 200 µL of methanol.
2) Conditioning with 200 µL of 0.5% acetic acid in 80% acetonitrile.
3) Washing with acidified water with 200 µL of 0.5% acetic acid 2 times.
4) Loading samples in the plate.
5) Sample washing with 200 µL of acidified water with 0.5% acetic acid.
6) Repeat this step 5 times.
7) Purified samples elution in a new classical 96-well plate with 100 µL acetonitrile/water 50/50 v/v and 100 µL 100% acetonitrile
8) Dry the eluted peptides into a speed vacuum.
9) Dried peptides are dissolved in 10 µL of acidified water with 0.1% formic acid

Mass spectrometry analysis

Samples are analysed by FT-ICR MS Bruker SolariX XR 9.4T. Sample crystallization is made with HCCA matrix at 10 mg/ml, 0.5 µL of sample and 0.5 µL of HCCA matrix are spotted on the MALDI 384 ground steel plate and air dry.

FT-ICR Mass Spectrometry Parameters

mass-to-charge ratio (m/z) range	693-5000 Da
resolution	910 000 @*m/z* 400
spectra number	2000 scans/spectra
acquisition	2M
free induction decay (FID)	5,0332 s
run time	1,30 min

Data Handling and Results

Peak assignment

Spectra was generated with FT MS Control (Bruker) and then analysed with DataAnalysis 4.2 with snap algorithm. This algorithm was used with the following parameter: quality factor 0.6; S/N 1; absolute intensity threshold 0; relative intensity threshold 0.001% maximum charge state 1.
For the analysis, biomarker peptide species specific are compared to a homemade DataBank in order to identify the bone sample.

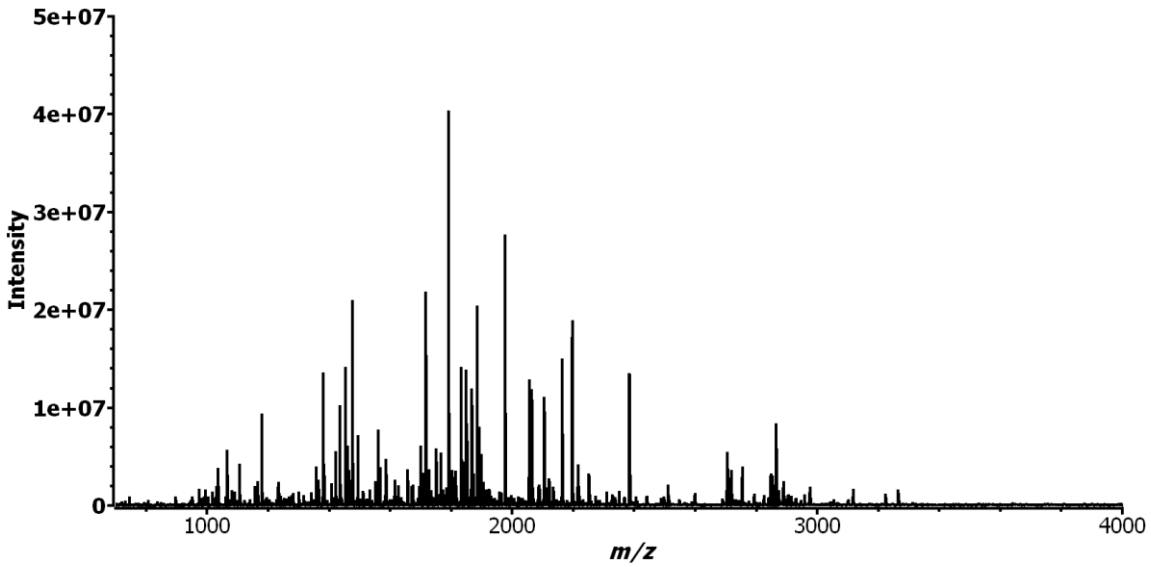

Figure 25. Example of beaver bone mass spectrum from Waziers archaeological site, Hauts-de-France, France. This spectrum was obtained with 500 µg pf bone powder.

The spectra are calibrated on trypsin digestion peptides (Table 1).

Table 1. m/z, start and end of tryptic peptide, sequence of peptide

m/z	[Start-End]	Peptide sequence
842.51	[100-107]	(R)VATVSLPR(S)
1045.5642	[90-99]	(K)LSSPATLNSR(V)
2211.1046	[50-69]	(R)LGEHNIDVLEGNEQFINAAK(I)

Deamidation study

Deamidation is a collagen post translational modification. It is a conversion of asparagine in aspartic acid and glutamine in glutamic acid. This modification gives information about sample conservation because rise over time.

To study this, a highly interspecies collagen peak is used. The quantification of the deamidation percentage is carried out by rationing the intensity of the non-deamidated peak (110.5590) and the deamidated peak (1106.5781). The FT-ICR is able to differentiate the first isotope of the non deamidated peptide and the monoisotopic peak of the deamidated peptide. The difference between the two peaks is 0.019 Da (Figure 4).

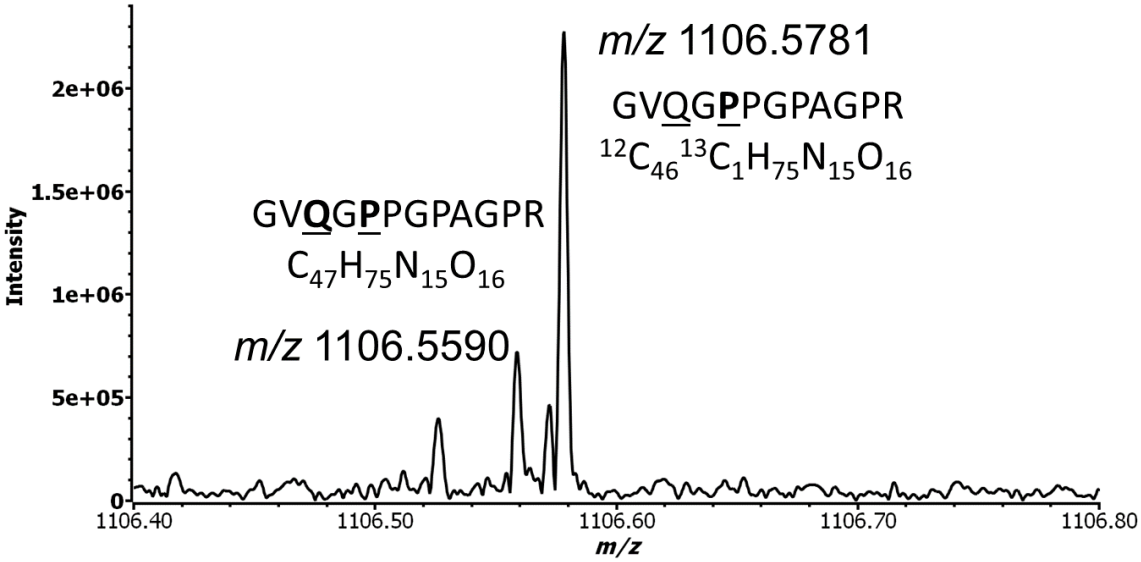

Figure 26. Zoom of the first isotope of the non deamidated peptide and the monoisotopic peak of the deamidated peptide .

Table 2 show the experimental and theoretical mass of native and deamidated peptide. The rate of deamidation is calculated by the ratio of intensity of deamidated peptide over intensity of deamidated and native peptide multiplied by 100.

Table 2. Comparison of calculated theoretical masses for the monoisotopic and the first isotope of GVQGPPGPAGPR peptide with one hydroxyproline and glutamine deamidation to experimental masses.

			Theoretical		Experimental	
Sequence	**PTMs**	**Formula**	**$[M+H]^+$ $^{12}C_{47}$**	**$[M+H]^+$ $^{12}C_{46}$, $^{13}C_1$**	**$[M+H]^+$ $^{12}C_{47}$**	**$[M+H]^+$ $^{12}C_{46}$, $^{13}C_1$**
GVQGPPGPAGPR		$C_{47}H_{76}N_{16}O_{14}$	1089.5799			
GVQG**P**PGPAGPR	1 oxydation	$C_{47}H_{76}N_{16}O_{15}$	1105.5748	1106.5776	1105.57501	1106.57811
GV**Q**G**P**PGPAGPR	1 oxydation, 1 deamidation	$C_{47}H_{75}N_{15}O_{16}$	1106.5580	1107.5617	1106.55902	1107.56207

Statistical analysis

The principal component analysis (PCA) allows to see the difference and similarity between samples regarding to *m/z* and intensity. This allows to see the dispersion and aggregation of samples. The list of mass obtain after SNAP algorithm are treated by metaboanalyst 5.0 There are two components: PC1 which is the most important, it gives information about difference between two samples; and PC2 gives information about replicates variability in a sample. PCA is a graphic representation that allows to quickly discriminate modern bone and ancient bone (Figure 5).

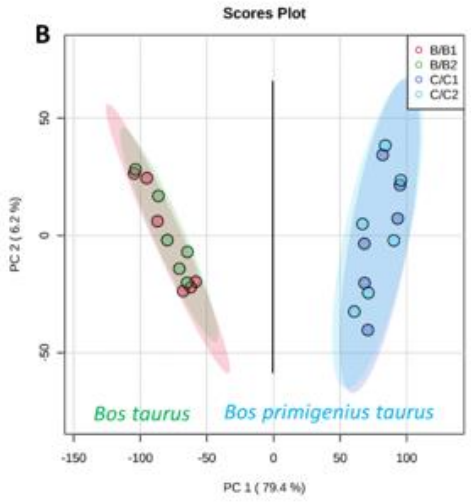

Figure 27. Example of PCA of a Bos Taurus (modern bone) and Bos primigenus taurus (ancient bone).

Supplementary information may be found in the paper:
Robust High-Throughput Proteomics Identification and Deamidation Quantitation of Extinct Species up to Pleistocene with Ultrahigh-Resolution MALDI-FTICR Mass Spectrometry
Fabrice Bray*, Isabelle Fabrizi, Stéphanie Flament, Jean-Luc Locht, Pierre Antoine, Patrick Auguste, and Christian Rolando
Analytical Chemistry, 2023, vol. **95**, no 19, p. 7422-7432.

9. Direct infusion (+)-nano-ESI-FT-ICR MS for analysis of insoluble polydienes

Introduction

Application field

Polydienes constitute an extremely important group of polymers. This group includes natural polyisoprene and synthetic polybutadiene rubbers. The structural characterization of these insoluble polymers raises important environmental, economic and scientific applications. Their analysis by direct infusion nano-ESI-FT-ICR MS requires to solubilize them in compatible solvents by developing new methods of chemical cleavage by cross-metathesis using new generation organometallic catalysts such as the Hoveyda-Grubbs second generation catalyst based on Ruthenium and an acrylate ester as a cross-metathesis chain transfer agent. The obtained complex mixtures issued from the depolymerization reaction can then be analysed by FT-ICR MS to afford a comprehensive, rich and detailed description of their molecular structure and identify their crosslinking motifs.

Materials

Reagents

Chemicals

- Polybutadiene (1,4-PB; 98% Z; Mw ~200,000– 300,000 g·mol^{-1}), polybutadiene (1,4-PB; 36% Z; 55% E; Mw ~200,000 g·mol^{-1}), polyisoprene (1,4-PI; 98% Z; Mw ~38,000 g·mol^{-1}), polyisoprene (1,4-PI; 99% E; Mw ~35,000 g·mol^{-1}),
- (Z)-1,4-Diacetoxy-2- butene, 2,6-dichloro-1,4-benzoquinone, Lithium-7 Li chloride
- (1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene) dichloro(o-isopropoxyphenylmethylene) ruthenium (Hoveyda–Grubbs second-generation catalyst; 97%; Mw ~626.62 g· mol^{-1}

Solvents

- Methanol (Sigma–Aldrich, suitable for HPLC, ≥99.9%)
- Dichloromethane (Sigma–Aldrich, anhydrous, ≥99.8%
- Acetonitrile (Sigma–Aldrich, suitable for HPLC, ≥99.9%)

Gases

- Nitrogen 5.0 (for FT ICR MS)

Equipment

Instruments

- FT-ICR MS (9.4T Solarix XR with para cell, Bruker Daltonics GmbH)
- Varian Nano-electrospray online source (Varian Nano ESI ON, Bruker Daltonics, Bremen, Germany)
- Consumables

- Glass vials, with screw caps (1.5 ml, Supelco)
- Single-use pipette tips

Further equipment

- Eppendorf pipettes, adjustable (1000 µl, 100 µl, 10 µl)
- 250 µl Hamilton glass syringe (51mm, Gastight)
- Cole-Parmer single-syringe infusion pump (Cole-Parmer®, Vernon Hills, USA)
- SilicaTipTM needle (10±1 µm, PicoTipTM Emitter, New Objective, Woburn, MA, USA)

Equipment Setup

For the nano-ESI measurements, a commercial nano-ESI ion source (Bruker Daltonics GmbH) is used.

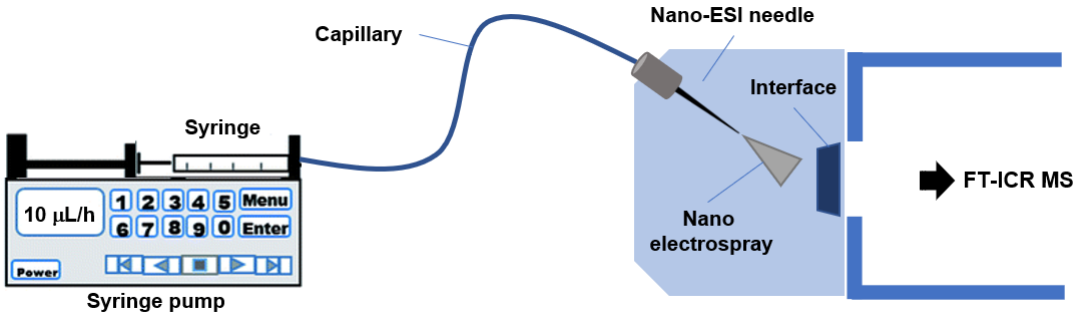

Figure 21: Schematic nano-ESI setup for direct infusion measurements.

Procedure

Start by recording a blank spectrum (methanol or acetonitrile) to make sure all the equipment is clean. Repeat prior to each sample introduction.

Dilution of the sample

1) Wear gloves to avoid contamination of the samples and the equipment.
2) Take a new, clean 1.5 ml glass vial with cap and sealing. Fill 999 µl of acetonitrile into the vial using an adjustable Eppendorf pipette (1000 µl).
3) Add 1 µl of the depolymerized polybutadiene or polyisoprene solution to the vial using an adjustable Eppendorf pipette (10 µl).
4) Close the vial with a sealing and a cap.
5) Shake the mixture using an agitator for approximately 20 seconds.
6) Take another new, clean 1.5 ml glass vial with cap and sealing. Fill 999 µl of acetonitrile into the vial using an adjustable Eppendorf pipette (1000 µl).
7) Add 1 µl of the diluted solution from step 2-5 to the vial using an adjustable Eppendorf pipette (10 µl).
8) Close the vial with a sealing and a cap.

9) Shake the mixture using an agitator for approximately 20 seconds.
10) Prepare a solution of Lithium-7 Li chloride in methanol (10 mg/mL)
11) Add 0.1 volume-% (1 µL) of the prepared solution in 10 to solutions in 7.
12) Shake the mixture using an agitator for approximately 20 seconds.
13) The sample is ready for analysis by direct infusion.

Preparation of blank sample

14) Wear gloves to avoid contamination of the samples and the equipment.
15) Take a new, clean 1.5 ml glass vial with cap and sealing. Use an Eppendorf pipette to add 1000 µl of acetonitrile to the vial.
16) Close the vial with a sealing and a cap.
17) Take another clean 1.5 ml glass vials with caps and sealings. Use an Eppendorf pipette to add 1000 µl of methanol.
18) Close the vials with sealings and caps.
19) For positive nano-ESI measurements, add 0.1 volume-% of formic acid for acidification to the blank sample. Therefore, add 1 µl formic acid with a pipette to 1 ml of the blank mixture.

Measurement

Before each sample, a solvent blank has to be acquired.

20) Wear gloves to avoid contamination of the samples and the equipment.
21) Clean the syringe by flushing the whole volume with acetonitrile for 3 times.
22) Clean the syringe by flushing the whole volume with methanol for 3 times.
23) Fill the syringe with approximately 200 µl of the blank sample prepared in step 19. Make sure that no air bubbles are left inside the syringe.
24) Set the parameters for the FT-ICR MS as given below.
25) Place the syringe into the syringe pump and connect the capillary.
26) Start tune modus at the FT-ICR MS.
27) Press “fast forward” until a stable signal is obtained.
28) Start the measurement.

Measurement of the sample.

29) Wear gloves to avoid contamination of the samples and the equipment.
30) Clean the syringe by flushing the whole volume with acetonitrile for 3 times.
31) Clean the syringe by flushing the whole volume with methanol for 3 times.
32) Fill the syringe with approximately 200 µl of the sample prepared in step 12. Make sure that no air bubbles are left inside the syringe.
33) Set the parameters for the FT-ICR MS as given below.
34) Place the syringe into the syringe pump and connect the capillary.
35) Start tune modus at the FT-ICR MS.
36) Press “fast forward” until a stable signal is obtained.
37) Start the measurement.

Cleaning

After each sample measurement, it is necessary to clean the syringe and capillary.

38) Wear gloves to avoid contamination of the equipment.
39) Clean the syringe by flushing the whole volume with acetonitrile for 3 times.
40) Clean the syringe by flushing the whole volume with methanol for 3 times.
41) Switch on “tune” modus at the FT-ICR MS.
42) Keep flushing the capillary until the capillary is clean, which can be checked online in the tune mode.
43) The instrument is now ready for the next measurement.

ESI Injection Parameters

Positive mode

capillary voltage	1300 V
dry gas flow	4 l/min
flow rate (syringe)	10 µl/h
source temperature	200 °C

FT-ICR Mass Spectrometry Parameters

Positive mode

accumulation time	0.05 s
mass-to-charge ratio (m/z) range	144-1500 Da
mode	broadband
octopole energy	350 V_{pp}
quadrupole collision energy	1200 V_{pp}
quadrupole lower cut-off	100
resolution	1,000,000 @m/z400
Number of scans	300
transient length	4.2 s
Time-of-flight	0.8 ms

Data Handling and Anticipated Results

Calibration

Instrument calibration was achieved using an external calibrant (Sodium trifluoroacetate TFA, 0.01 mg/mL in H_2O/MeOH 50/50 v/v) with a linear calibration. Following acquisition, spectra were internally calibrated in the Data Analysis software version 5.0 (Bruker Daltonics, Bremen, Germany) using a calibration list of assigned signals limiting the mass error to under 100 ppb.

Peak assignment

Peak assignment was done using *i*-van Krevelen python script composed of three different parts

Formula generator: this script will generate lists of possible ion formulas according to strict rules and predefined elemental limits. The list is generated between a well-defined m/z region (for example 100 and 1000 m/z) which can be adjusted according to the user's needs. Elements present in the analyzed compounds can be chosen such as C, H, O, N and many others as well as adducts such as K, Na or Li. The list generated by this script represents the maximum number of each element present in the molecules in the defined m/z range.

Formula assignment: this script will automatically assign input peak lists to molecular formulas. It is based on the Kendrick mass defect (KMD) and z* approach.

Static plotter: This tool generates a number of van Krevelen, DBE vs C# and other plots to simplify data visualization and interpretation.

H/C ratio and van Krevelen plots

The formulas generated from each sample are transposed to two-dimensional van Krevelen diagrams, known as elemental ratio analysis, constructed by plotting the molar hydrogen to carbon ratio (H/C) vs. the molar ratio of oxygen to carbon (O/C) for each data point. According to their own characteristic H/C and O/C ratios, main classes of compounds are specifically localized as areas in the plot, thus allowing a depiction of a sample's composition.

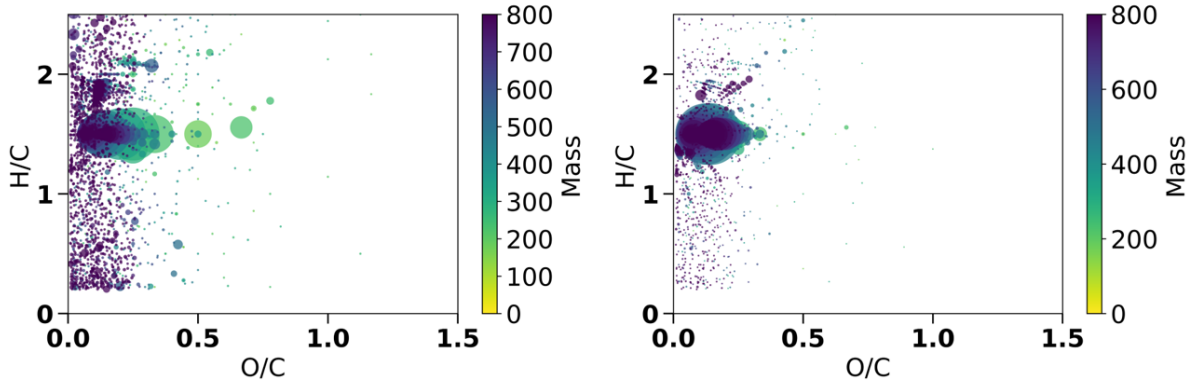

Figure 28. Van Krevelen diagram highlighting the identified species based on the hydrogen to carbon (H/C) versus the oxygen-to-carbon ratios (O/C) in function of the mass for Z-PB (left) and E-PB (right)

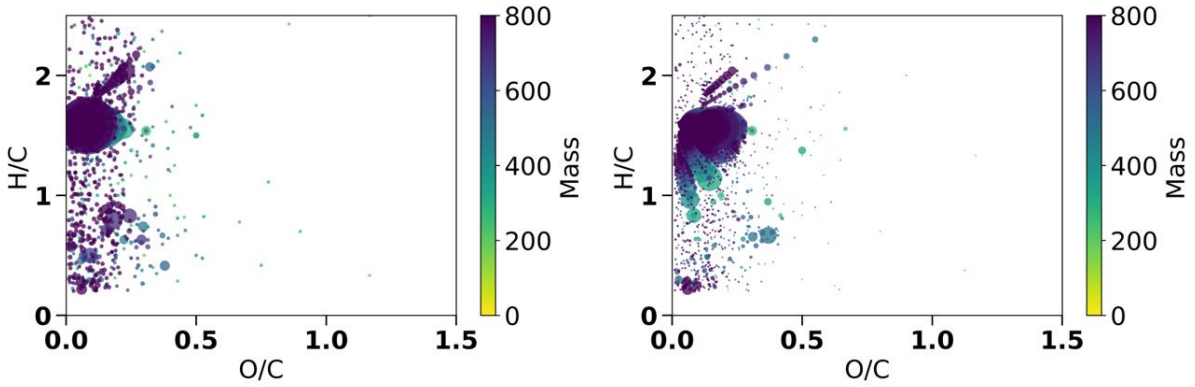

Figure 29. Van Krevelen diagram highlighting the identified species based on the hydrogen to carbon (H/C) versus the oxygen-to-carbon ratios (O/C) in function of the mass for Z-PI (left) and E-PI (right)

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

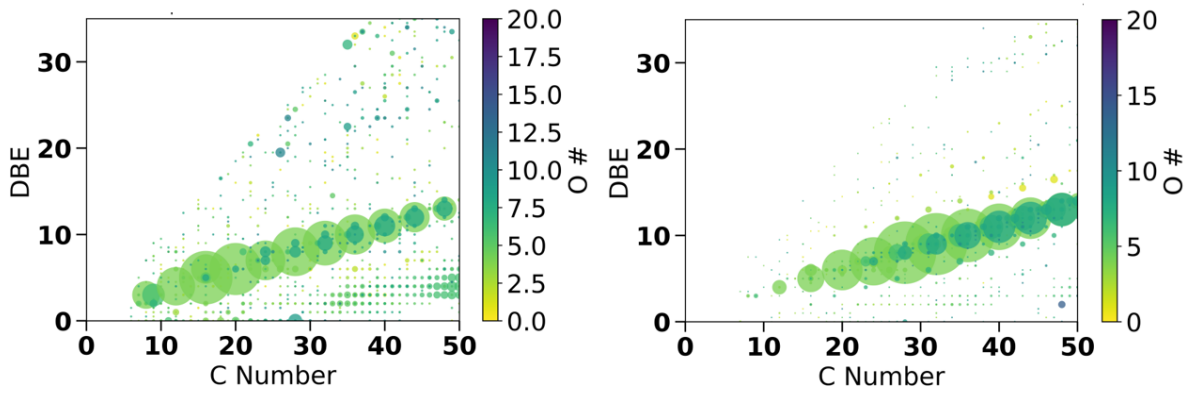

Figure 30. Plot DBE versus carbon number in function of oxygen number for Z-PB (left) and E-PB (right)

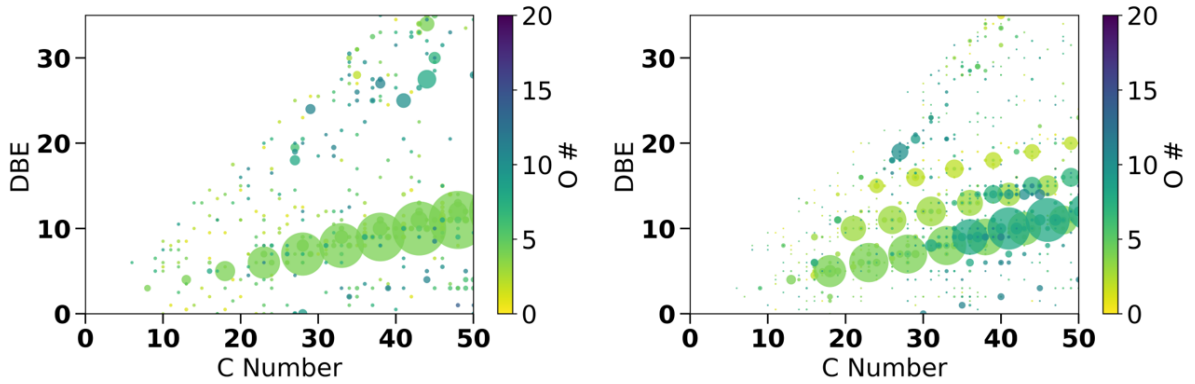

Figure 31. Plot DBE versus carbon number in function of oxygen number for Z-PI (left) and E-PI (right)

Data Handling and Anticipated Results

Calibration

The mass spectra can be externally m/z calibrated by standards from quality measurements. For higher mass accuracy, the mass spectra can be calibrated on internal homologues rows of the measured samples. Therefore, more than one homologues row should be used and the whole mass range should be covered. The spectra should be calibrated with a mass accuracy of 1 ppm.

Peak assignment

High mass accuracy and ultra-high resolving power enable the possibility to calculate sum formulae from the measured m/z values due to mass defect, but also other chemical-based validation rules (H/C ratio, homologues rows, etc.).

Kendrick mass defect

The Kendrick mass defect is a retransformation of the mass scale, typical based on CH_2. It is applied in the fields of environmental science, proteomics, petroleomics, metabolomics, polymer science, etc. The transformation enables the alignment of homologues rows as a horizontal line. The Kendrick mass for CH_2 and the Kendrick mass defect (KMD) are calculated as follows:

$$Kedrick\ mass = IUPAC\ mass\ \frac{14.00000}{14.01565}$$

$$Kendrick\ mass\ defect = nominal\ mass - Kendrick\ mass$$

A Kendrick Plot (KMD vs. nominal mass) allows an estimation of the alkylation degree of the present species.

Calculated sum formulae

Direct infusion measurements result in a large number of calculated sum formulae, which are in the field of Petroleomics most often too complex to be investigated manually. A variety of data visualisation techniques may help to investigate differences between different sample sets. Different compound classes can be grouped as bar plots and compared regarding the summed intensity.

H/C ratio and van Krevelen plots

Van Krevelen plots are plotted H/C vs. a heteroatom/C value. For Petroleomics, mostly O, S and N are interesting as heteroatom/C ratios. The plots give an evidence for aromaticity, alkylation and separates different heteroatom-containing classes. For example, the H/C value of the aromatic benzene is 1, whereas linear alkanes have a H/C value above 2.

Double bond equivalent

The double bond (DBE) equivalent gives the number of rings and double bonds present in a molecule and can be seen as a measure for the level of unsaturation and aromaticity. The DBE is calculated as follows:

$$DBE = C + 1 - \frac{H}{2} - \frac{X}{2} + \frac{N}{2}$$

C is the number of Carbon atoms present, *H* is the number of Hydrogen atoms, *X* is the number of halogen atoms (Cl, Br, I, F), N is the number of Nitrogen atoms present. For example, benzene has a DBE value of 4, consisting of three double bonds and one ring. DBE vs. #C plots can give evidence on the aromatic distribution and alkylation/size of the molecules present.

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Supplementary information may be found in the paper:

Regio- and Stereo-Specific Chemical Depolymerization of High Molecular Weight Polybutadiene and Polyisoprene for Their Analysis by High-Resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry: Comparison with Pyrolysis-Comprehensive Two-Dimensional Gas Chromatography/Mass Spectrometry, Atmospheric Solid Analysis Probe, Direct Inlet Probe-Atmospheric Pressure Chemical Ionization Mass Spectrometry, and Ion Mobility Spectrometry-Mass Spectrometry

Ziad Mahmoud, Fabrice Bray, Marie Hubert-Roux, Michel Sablier, Carlos Afonso, and Christian Rolando*

Analytical Chemistry, 2020, vol. 92, no 24, p. 15736-15744.

10. MALDI-FT-ICR MSI analysis of mouse brain tissues slices

1 Introduction

2 Application field

Observing the spatial distribution of ions is information of choice for pharmaceutical and toxicological fields. Matrix Assisted Laser Desorption/Ionization-Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry Imaging (MALDI-FT-ICR MSI) offers a label free localisation method of tissue slices with a high spectral resolution and mass accuracy measurements. Such performances allow the discerning of many isobaric ions often encountered during the analysis of complex biological samples.

3 Materials

4 Reagents

Powders

- Calibrant : Red Phosphorus (≥97%, Merck)
- MALDI matrix : α-cyano-4-hydroxycinnamic acid (>97%, Merck)

Solvants

- milliQ water
- Acetonitrile (HPLC grade, Biosolve)
- TriFluoroacetic Acid (99%, Merck)
- Acetone (HPLC grade, Merck)
- Ethanol (70%, Biosolve)
- Methanol (technical grade, Biosolve)

5 Equipment

Instruments

- FT-ICR : solariX XR 9.4T with Paracell cell (Bruker Daltonics)
- Cryo-microtome : CryoStar NX70 (ThermoScientific)
- Automatic Sprayer : SunCollect (SunChrom)
- Analytical balance
- Vacuum desiccator

Software

- ftms control 2.2.0 (Bruker Daltonics GmbH)
- Bruker Compass Data Analysis 5.0 (Bruker Daltonics GmbH)
- FlexImaging 5.0 (Bruker Daltonics GmbH)
- SCiLS Lab 2016b or above (SCiLS)
- SunCollect Spray Control v2.6

Consumables

- ITO glass slides (Bruker Daltonics)
- Disposable spatulas (VWR)
- 2mL Eppendorf (Fisher)
- SEC 35e low profile blades (Fisher Scientific)
- Tissue-Tek NEG50 (Fisher Scientific)

Further equipment

- MTP slide adaptor
- Scanner
- Laboratory gloves
- Utrasonic bath
- 30mm specimen chuck
- Forceps
- Brushes

6 Sample Cutting Procedure

Cut process of a mouse brain tissue destined to mass spectrometry imaging.

7 Preparation

1) Wear gloves to avoid contamination of the sample and equipment.
2) Place the frozen tissue in the Cryostar NX70 enclosure to come up to the instrument temperature for 10 to 15 minutes.
3) Set the sample holder temperature to-20°C and the blade temperature to-12°C.
4) Place a pair of forceps inside the enclosure to cool down.
5) Clean an ITO glass slide with ethanol 70% then place in the Cryostar NX70 enclosure to come down to the instrument temperature.
6) Place with caution a SEC 35e low profile blade in the razor blade holder.
7) Put a Tissue-Tek NEG50 on a 30mm specimen chuck and place the chuck inside the Cryostar NX70 enclosure.
8) When the Tissue-Tek NEG50 starts to solidify, quickly dispose the sample on the Tissue-Tek NEG 50 with the cooled pair of forceps.
9) When the Tissue-TEK NEG50 has completely solidified place the specimen chuck on the cryostat sample holder.
10) If needed, correct the cutting incidence in the x and y axis using top and side knobs of the sample holder.
11) Set the sample cutting size to 12µm and bring the blade stage close to the sample using the left control know or the right crank.

12) If needed, trim the sample until a complete sample slice can be obtained or until the region of interest is reached. During the trimming process a bigger cutting size can be set to accelerate the process.
13) Perform a slice at 12µm by rotating the crank 360° while the anti-roll glass slide is down on the razor blade.
14) Lock the crank for safety and move the anti-roll glass slide to the side gently. Use a brush to gently remove the slice from the anti-roll glass slide if stick together.
15) Using brushes relocate the tissue slice on the sample plate for easier collection.
16) Warm up for two seconds a portion of an ITO glass slide by placing our thumb of the back of the slide (thumb on the non-coated side).
17) Press the heated up ITO coated side of the slide in contact with the sample to collect it. If a tissue sample is already on the ITO glass slide, avoid making a second contact of already collected samples with the sample plate.
18) Use your thumb again on the back of the ITO glass slide, while keeping it in the cryostat enclosure, to make the tissue slice completely adheres to the ITO glass slide.
19) Repeat step 13 to 18 until enough tissues slices have been collected.
20) Clean the cryostat by throwing unused slices in an adequate manner and clean the cryostat with ethanol 70%.
21) Keep ITO glass slides with sample at-80°C for storage or continue with the MALDI-Matrix Spray Procedure for mass spectrometry imaging acquisition.

8 MALDI-Matrix Spray Procedure

9 Spray process to cover an ITO glass slide with a coating of approximatively 5nm/mm² of α-cyano-4-hydroxycinnamic acid (HCCA).

10 Preparation

1) Wear gloves to avoid contamination of the sample and equipment.
2) Place ITO glass slides with samples in a desiccator for 10 minutes or until complete dryness.
3) Switch on the analytical balance and allow it to stabilize. Set the initial value to zero.
4) Weight an empty 2mL Eppendorf and set value to zero.
5) With a disposable spatula add around 8mg of HCCA in the Eppendorf.
6) Depending of the quantity of HCCA calculate the required amount of solvent to obtain a concentration of 5mg/mL.
7) Prepare excess amount of Acetonitrile / milliQ water / TriFluoroacetic Acid mixture in proportion 70 / 29.9 / 0.1.
8) Add calculated volume of the mixture to the Eppendorf containing the matrix.
9) Agitate the Eppendorf until the matrix is completely dissolved. To accelerate this process and ensure that no particles are left place the Eppendorf 5 minutes in an Ultrasonic.

II MALDI-Matrix coating

1) Switch on the compressor linked to the SunCollect on position “II” and set the pressure to 2 bars.
2) Switch on the SunCollect and the connected computer.
3) Check the remaining amount of wash/waste solution and refill/dispose bottles if necessary.
4) Place the Eppendorf with MALDI matrix in the SunCollect.
5) Insert the tubing connected to inlet “F” inside the Eppendorf.
   Place ITO glass slide in one of the SunCollect slide adapter or sample plate

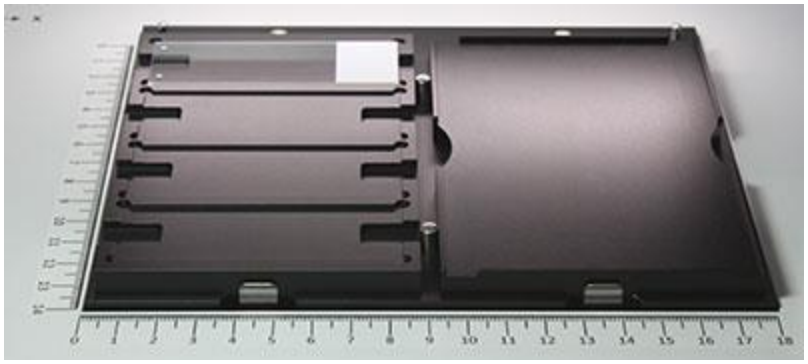

Figure 32. SunCollect slide adapter (right) and sample plate (left)

6) Open SunCollect Spray Control v2.6.
7) Select an existing spray method by clicking on “Load Method” or create a new method using the software “Method” menu. Used spray parameters are available in Table 3.

Table 3. Spray Method parameters corresponding to a coating around 5nM/mm².

z	(mm)	25
Line distance	(mm)	2
Speed while spraying	(mm/min)	600
Inlet Port		F(F)
# Layer		7
Drying time	(s)	0
Flow Rate		Variable
Flow Rate #1	(µL/min)	5
Flow Rate #2	(µL/min)	10
Flow Rate #3	(µL/min)	20
Flow Rate #4 to 7	(µL/min)	40

8) Select the region to spray on with “Load format 1” and a preconfigured existing format of the SunCollect slide adapter or by entering x1;y1 and x2;y2 coordinates while keeping a 0.5cm margin (**Erreur ! Source du renvoi introuvable.**) in the “Format” menu.

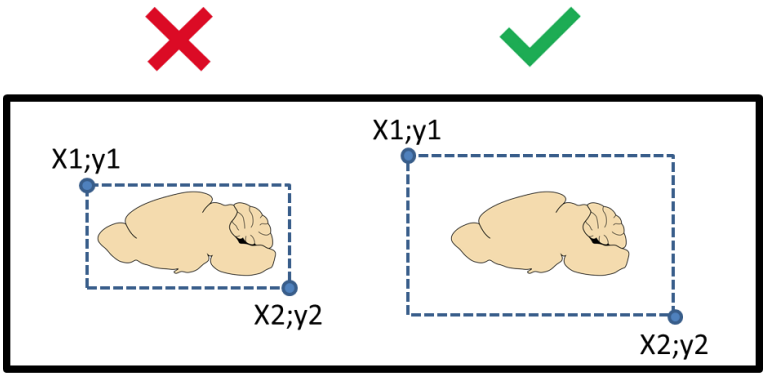

Figure 33. Schematic representation on how to properly select the spray area on the SunCollect

9) When the method and the spray area are selected press on “Start” to launch the spray process.
10) When the spray process is finished, unless another coating is planned is the coming minute, launch the “cleaning system” process and follows software instruction.
11) Clean the SunCollect with the wash solution or methanol technical grade.
12) Shut down the compressor, SunCollect and computer.
13) Place the coated ITO glass slide in an MTP slide adaptor II for MS acquisition or store at -20°C if immediate analysis is not required.

See the ad hoc paper describing parameters to get optimum resolution and minimum mass shift in draft paper

FT-ICR Mass Spectrometry Imaging at Extreme Mass Resolving Power Using a Dynamically Harmonized ICR Cell with 1ω or 2ω Detection

Mathieu Tiquet, Raphaël La Rocca, Stefan Kirnbauer, Samuele Zoratto, Daan Van Kruining, Loïc Quinton, Gauthier Eppe, Pilar Martinez-Martinez, Martina Marchetti-Deschmann, Edwin De Pauw, and Johann Far- *Analytical Chemistry*, 2022, vol. **94**, no 26, p. 9316-9326.