Customs Laboratories European Network

Recording of High-Resolution Mass Spectra of Organic Substances such as New Psychoactive Substances, or Designer Drugs

Introduction

The identification of the chemical structure of an unknown substance is required for purposes of control by enforcement authorities whether for verification of correct classification by Customs, detection of illicit substance, or reporting of new designer drugs to the competent national and international authorities. High-resolution time-of-flight (TOF) electrospray (ESI) mass spectroscopy is a powerful and flexible spectroscopic technique that provides information pertaining to the molecular mass, elemental composition (raw formula), and molecular structure of a compound. Accurate mass measurement (TOF MS) is used to determine the exact molecular mass and elemental composition a known or unknown molecule. Tandem mass spectrometry experiments (TOF MS/MS) are used to assist in the structure determination of unknown molecules.

1. Scope

The purpose of this document is to establish a standard operating procedure for the high resolution time-of-flight electrospray mass spectrometry approach for the characterization of unknown substances. This will allow other laboratories to trust the data knowing the instrument is performing accordingly. Each laboratory should have their own minimum requirement performance criteria (MRPC) set in place before they run an analysis.
This procedure is suitable for tuning and calibrating, checking the minimum performances of the instrument, setting up an experiment and recording MS spectra after preparation of the unknown substance in an appropriate solvent. Depending on the substance, additional MS/MS fragmentation experiments may be needed on the same preparation providing complementary information for chemical groups and substructures present in the molecule.

2. Normative references

Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013) published in Pure Appl. Chem. 2013, Vol. 85, No. 7, pp. 1515-1609; http://dx.doi.org/10.1351/PAC-REC-06-04-06

3. Principle

The unknown organic substance is dissolved in an appropriate solvent for performing mass spectrometry experiments. Samples of chemical compounds are generally prepared in a mixture of CH_3OH: H_2O, 50:50 to a final concentration of 10 µg/ml.
The electrospray ionization (ESI) mode is used for sample introduction in mass spectrometry to produce ions using a spray to which a high voltage is applied to create an aerosol.

ESI is also called 'soft ionization' technique, since there is very little fragmentation in the source. By default the positive ionization mode is used (ESI+); the negative mode in used when needed (e.g. labile molecules). For positive-ion mode, 0.1% HCOOH or acetic acid is usually added into the analyte solution to enhance protonation and increase sensitivity. For negative-ion mode, 0.1% NH_4OH is added into the analyte solution to help de-protonation and increase sensitivity.
The ions observed by mass spectrometry may be quasi-molecular ions created by the addition of a hydrogen cation and denoted [M + H]+ or of another cation such as sodium ion [M + Na]+ (possible formation of adducts in the source) (ESI+ mode), or the removal of a hydrogen nucleus, [M − H]− (ESI- mode).
MS spectra are recorded for each substance in the TOF MS mode to determine the accurate mass of the molecule and its elemental composition. Depending on the substance, additional TOF MS/MS fragmentation experiments may be required to give information on the fragmentation pattern and elucidate the structure of the molecule.
By default, the infusion mode is used. Infusion is for direct infusion meaning that the compound is injected from a syringe pump directly into ion source of the mass spectrometer.
A liquid chromatography (LC) separation might be necessary in case of complex mixtures or in the presence of a matrix that may affect the ionization of the compound. The analysis is performed online, by feeding the liquid eluting from the LC column directly to the ESI.

4. Reagents and materials

4.1. Methanol (CH_3OH), LC-MS grade, ≥99.9%, CAS Number 67-56-1 , EC Number 200-659-6

4.2. Formic acid (HCOOH), LC-MS grade, >98%, CAS Number 64-18-6, EC Number 200-579-1

4.3. Ammonium hydroxide (NH_4OH), ACS reagent, CAS Number 1336-21-6

4.4. Milli-Q water (Millipore)

4.5. Any other analytical grade solvents required to dissolve and analyse the chemical compound in question

4.6. Leucine-Enkephalin, CAS Number 58822-25-6

4.7. Val-Tyr-Val, , CAS Number 17355-22-5

4.8. Erythromycin, CAS Number 114-07-8

4.9. Sulfadimetoxine, CAS Number 122-11-2

4.10. Solvent: 50% CH_3OH, 49.9% H_2O, 0.1% HCOOH solution (ESI+ mode) or in 50% CH_3OH, 50% H_2O solution (ESI- mode)

4.11. Calibrant: 2ng/mL Leucine/Enkephalin in 50% CH_3OH, 49.9% H_2O, 0.1% HCOOH solution (ESI+ mode) or in 50% CH_3OH, 50% H_2O solution (ESI- mode)

4.12. Sample mixture: 2µg/mL Val-Tyr-Val, Leucine-Enkephalin, Erythromycin and Sulfadimetoxine in 50% CH_3OH, 49.9% H_2O, 0.1% HCOOH solution (ESI+ mode) or in 50% CH_3OH, 50% H_2O solution (ESI- mode)

4.13. Pipette tips and vials for preparation of chemicals

5. Apparatus and equipment

5.1. Common laboratory equipment such as micropipettes

5.2. A high-resolution mass instrument, mass spectrometer instrument that fulfil the following criteria:

- a quadrupole for ion selection and a time-of-flight as a mass analyser.

- a minimum instrument resolution (FWHM: 10,000). **Mass resolution** measures of the ability to distinguish two peaks of slightly different **m/z** ratios. The resolution of a peak is the ratio of its parent ion mass (m/z) to its peak width measured at half height.

- a maximum accepted mass accuracy or mass error (Error = 10 ppm). The mass accuracy is the ratio of the m/z measurement error (Error = measured mass –accurate mass) to the true m/z value (expressed in ppm).

Typically quadrupole time-of-light (q-TOF) hybrid instruments are convenient for obtaining spectra with adequate resolution, mass accuracy and signal-to-noise ratio fit for the purpose presented in the above introduction and scope. The mass instrument can be interfaced with a liquid chromatograph (LC) to separate the compounds present in a mixture or in case of a complex matrix.

On installation the manufacturer proceeds with the adjustment of the mass spectrometer. This includes the instrument tuning, calibration and performance check. The tuning and calibration are then performed by the instrument's operator using the calibrant (4.11).

Instrument's performance is checked by recording the spectra of the sample mixture (4.12) before running an experiment. This is achieved following the procedure defined by the manufacturer to check the performance of the instrument against its technical specifications. When needed, the operator can control the performance of the instrument with the same procedure.

6. Sampling and samples

On receipt, samples are given a unique sample number and are stored at room temperature prior to analysis (unless otherwise communicated in the instructions accompanying the sample).

7. Procedure

7.1. Sample preparation

Normal laboratory conditions are sufficient for the preparation and analysis of the compounds. In order to reduce any risk of exposure, usual precautions for manipulation of chemicals should be observed. Moreover it is recommended to manipulate limited amounts of material sufficient for the preparation of the solutions.

7.1.1. Weigh ca. 1 mg of the sample in a vial.

7.1.2. Add 1mL of solvent (4.10). Samples of chemical compounds are generally prepared in a mixture of CH_3OH: H_2O, 50:50 (with 0.1% HCOOH (ESI+) or 0.1%NH_4OH (ESI-)

7.1.3. Dilute the sample 1:100 for mass spectrometry analysis (final concentration 10µg/mL)

7.1.4. Eventually use a vortex mixer to ensure complete dissolution.

7.1.5. Samples can be stored at 4°C for a maximum of 1 week.

7.1.6. Calibration solution (tuning and calibration) and sample mixture (performance check) are prepared in accordance with manufacturer's instructions.

7.2. Mass experiments

7.2.1. The q-TOF high-resolution mass spectrometry instrument must be tuned and calibrated before running an instrument. Tuning is necessary to optimize sensitivity, mass accuracy and resolution. Minimum performances are specified by the instrument's manufacturer (5.3). Calibration is essential for accurate mass determination in a defined m/z range.

7.2.2. The calibrant (Leucin-Enkephalin solution (4.11)) is introduced directly in the spectrometer by infusion with a syringe pump to have a stable ion beam and a sufficient intensity. The following procedure is followed: 1) Nominal mass set-up, 2) optimisation of resolution settings, 3) optimisation of instrument sensitivity, and 4) mass calibration.

7.2.3. Instrument's performances are checked by infusing the sample mixture (4.12). MS spectra are checked for mass the accuracy, resolution and sensitivity.

7.2.4. Check the isotopic profiles. The isotopic profiles found experimentally shall match the theoretical ones mentioned in the following table (qualitative peak analysis).

	Monoisotopic mass		M+1 (%M)	M+2 (%M)	M+3 (%M)	M+4 (%M)
	ESI + mode	ESI - mode				
sulfadimethoxine	311,0814	309.0658	15,8	6,37	0,84	0
Val-Tyr-Val	380,2185	378.202	22,6	3,44	0,39	0
Leucine-Enkephalin	556,2771	554.2615	33,4	6,83	1,03	0
Erythromycin-H_2O	716,4585	----------	- 42,5	11,2	2,19	0,35
Erythromycin	734,4691	----------	- 42,5	11,4	2,2	0,37

7.2.5. The unknown sample is then introduced directly in the spectrometer by infusion with a syringe pump or eventually placed at the appropriate position of autosampler of the LC system when a chromatographic separation is needed.

7.2.6. Launch the sequences for acquisition of the MS spectra.

7.3. Examination of the spectra

Since the chemical structure and/or composition of the material under analysis is a-priori unknown, it is not possible to set-up specific criteria to evaluate the quality of the acquired spectra.
The spectroscopist can therefore consider the following criteria to judge of the quality of spectra:

7.3.1. Abundance of largest peak(s) does not saturate detector (peaks are not 'off-scale')

7.3.2. Spectrum abundance is normalised to the most abundant ion

7.3.3. Significant peaks mass-labelled to an appropriate number of decimal places (i.e. appropriate to the mass accuracy of the instrument, maximum error of 10 ppm)

7.3.4. Mass peaks appear as resolved isotope clusters

7.3.5. Continuum data show adequate resolution (for singly charged ions [M + H]+ or [M − H]−, minimum Resolution FWHM 10,000)

It can also be reminded that, in certain cases, the product under examination can be a mixture of several organic compounds and/or eventually contains mineral components. This may affect both the ionisation (ion suppression effect) ion and the signal to noise ratio especially of the MS spectra. A dilution or separation can be recommended. Typical features expected for the general criteria described above could be previously established from the spectra obtained for a known 'control' compound measured using similar instrument's parameters and similar conditions of concentration and solvent,. The 'control' may be a substance belonging to the same chemical family (specific function group(s)) as cathinones or cannabinoids.

8. Results

Depending on the further use of the mass spectrometry data, the results can consist of:

8.1. Electronic MS files (e.g. in raw manufacturer format or open format NetCDF or mzXML format) for possible further processing and storage in electronic libraries.

8.2. MS spectra: while small molecules (MW 100-1000) in most instances ionize quite well in electrospray they routinely display only a single charge state [M+H]+ (ESI-) or [M-H]- making interpretation fairly straight forward. Fragmentation may occur in the ESI ion source (in-source fragmentation) where it is not a desired effect and result in the presence of fragment ions.

8.3. Candidate raw formulas: Modern high-accuracy instruments allows for calculation of molecular formula from a single measured accurate mass and isotopic pattern. Mass to formula tools generate possible molecular formulae corresponding to a given mass, tolerance and composition limits. Result can be searched within several public databases such as PubChem, METLIN or ChemSpider.

8.4. Isotopic profiles: ratio of signals of each isotope relative to signal of the monoisotopic mass. In MS, the largest peak (corresponds to 12C) is called the base peak and is labelled [M+H]+ (ESI-) or [M-H]-. The intensity of every other peak is reported in comparison to the base peak. The 13C peak, is labelled [M+H+1]+ (ESI-) or [M-H+1]-.

The relative peak heights can also be used to assist in the deduction of the empirical formula of the molecule being analysed (qualitative peak analysis).

Element	Most Abundant Isotope	Secondary Isotope
Carbon	^{12}C (98.89%)	^{13}C (1.11%)
Nitrogen	^{14}N (99.63%)	^{15}N (0.36%)
Sulfur	^{32}S (94.93%)	^{34}S (4.29%)
Chlorine	^{35}Cl (75.78%)	^{37}Cl (24.22%)

8.5. Observations about presence of other substances or contaminants in the MS spectrum.

8.6. Hypotheses of possible chemical structure recorded in electronic format such as Molfiles and/or SMILES (simplified molecular-input line-entry system).

8.7. Generated identifier InChI (IUPAC International Chemical Identifier) and the derived InChIKey.

8.8. Collision induced dissociation (CID) MS/MS spectra: desired fragmentation of the parent ion is made in the collision cell of the mass spectrometer and fragment ions are measured by the time-of-flight analyser. The result is a table of masses and intensities of fragments ions. It is a phenomenon used in mass spectrometry to find the structural formula of a molecule through MS/MS data analysis, process called structural elucidation. Information is extracted from the MS/MS spectra and experimental fragmentation data are confronted to predicted fragmentation patterns (theoretical) for structure elucidation.