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The Identification and Quantification of Organic Molecules in Trace Amounts: Still a Challenge for Analytical Chemistry

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Abstract

The situation is briefly summarized, on headlines, regarding trace amount analysis of unknown molecular species, in complex samples meaning with many up to thousands of components. Whereas for molecular weights up till about 500 Da it is often possible to establish a single unique chemical formula, for higher molecular weights this still poses serious challenges. Also, structure elucidation is far from trivial, unless enough can be collected for NMR studies. Proper quantification remains what is probably the biggest challenge because of the lack of methods to collect (close to) 100% of each individual species. What we need to characterize and quantify these samples are additional approaches implemented in a workflow. One of these is the elimination approach, i.e., which potential options for structures are excluded. But as it is not to be expected that single technique will or can be developed up to the required level, combining and further integration of available tools, e.g., those mentioned in this mini review, including data management and manipulation seems the way forward.

Keywords: Trace amounts organics; Chemical analysis; Mass spectrometry; NMR; Identification; Quantification; Data processing

Featured Application

The untargeted identification and quantification of trace amounts of unkown molecular species in a range of highly relevant areas including water quality c.q. water pollution, the presence of toxic species including those resulting from metabolism in soil or otherwise, forensics, the purity and safety of products for human or animal consumption including pharmaceutical products, and finally the understanding of by-product formation in chemical processes with the aim to better process understanding and leading to process optimization with less waste. Adequate analysis can have implications for legal actions, e.g., permissions for product-sale or production units (emissions).

Introduction

The untargeted identification and quantification of trace amounts of unknown molecular species is still a serious challenge for analytical chemistry. Trace level analysis is generally understood as the analysis of very small amounts of elements or compounds at the less than 0.01 percent or 10 ppm concentration levels. In, e.g., wastewater streams by experience one can find a thousand species or more at very low concentration, difficult to identify and quantify. Because of the large number of these minority compounds individual concentration levels are expected to be really very low. Identification is, however, of importance in many highly relevant areas including water quality c.q. water pollution, the toxicity of species including those resulting from metabolism in soil or otherwise, forensics, samples with extreme number of chemical entities such as crude oil (>20,000), the purity including safety of products for human or animal consumption including pharmaceutical ingredients, and finally the understanding of by product formation in chemical processes to enable a better understanding and subsequent optimization of the processes.

Untargeted means we are looking to in essence all chemicals present, or at least a very large number of these. For targeted analysis, focusing on one or a few specific chemicals, usually dedicated analytical methods are available. For untargeted trace elemental analysis (metal atoms) one has the well-known Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique allowing for detection down to the ppt levels and an extremely large dynamic range of 12 order of magnitude [1]. For the untargeted analysis of organics, we need high mass accuracy Mass Spectrometry to identify the chemical formula. A serious problem in non-targeted analysis is the data processing, as we will

have many species in quite complex mixtures, for which recently protocols are being proposed [2]. Non-targeted methods are becoming more and more important related to the application fields named before.

The present contribution is an introduction to the theme and a brief overview on current issues and solutions, also based on the author's more than three decades of experience in both the academic and the industrial sector. Whereas it is by no means claimed to be exhaustive it will hopefully act as an incentive for others to contribute to this Special Issue and we hope to see how this problem, where there is no general approach guaranteeing success, could be approached.

The question about identification and quantification can be broken down into the following elements:

(1) Identification of the gross chemical formula

(2) Identification of the chemical structure (3) Quantification

Applying standard traditional GC-MS or LC-MS is a good approach assuming certain conditions are fulfilled. One either needs to have enough of the unknown species to characterize both structure (2) and quantity (3) by applying NMR spectroscopy, or one must be very confident about the species one expects and, e.g., known values for retention time and molecular mass are available and can be related to the structure whereas the quantity (3) can be estimated by applying a UV-detector using an available calibration curve. For instance, when degradation products are known or when characterizing certain drugs, this is a valid approach, see e.g., Zhu et al. [3]. Another tool often used is a data base comprising molecular masses and structures; for GC-MS such data bases have been around for quite a while.

However, when we do not know with certainty what species we can expect, the only ones suggested by such a tool are those present in the data base. Sasaki et al. [4] have phrased this as 'elucidation experts must proceed with caution when using a priori knowledge. While in most cases knowledge of starting materials, assumed chemistry, and related impurities generally proves to assist in the interpretation, it can sometimes bias even the most experienced elucidators, causing a mis-characterization.' So first, one has no certainty, secondly this is not a realistic toolbox for samples which contain hundreds if not thousands of different molecular species. In this context it is also to be realized that there are more than 1 billion organic molecules with 13 non-hydrogen atoms (the GDB data base, see [5]), not to think about larger species. As we will see later, modern mass spectrometer can uniquely determine the molecular formula up to a mass of around 500, and as these comprise up to more than 13 non-hydrogen atoms we are really talking about billions of possible different molecular structures.

In the first part below, we address the chemical formula and chemical structure for which, at least for quite a few species, adequate tools are available. In the second part we touch the issue of quantification where there are gaps to close. For industrial research as well as analysis related to, e.g., toxicity, quantification is of eminent importance.

Molecular Identification

Chemical formula (Atomic composition)

As indicated in the Introduction, this theme comprises two parts: determining (1) the molecular formula and determining (2) the molecular structure. These need to be discussed separately as the molecular formula can be established more easily, whereas determination of the structure is much more problematic and requires different tools. The chemical formula can be determined by applying high accuracy mass spectrometry, e.g., FT-ICR-MS (Fourier-Transform-Ion-Cyclotron-Resonance-Mass-Spectrometry) or the smaller and easier to handle Orbitrap. Both instruments can achieve a sub-ppm mass accuracy and a maximum resolution of 1,000,000 at m/z 200. An impressive demonstration of what these instruments can bring was shown by an FT-ICR-MS study reported by Krajewski et al. [6] on a volcanic asphalt sample. Data acquisition from a specified but restricted mass segment contained 170 000 peaks for which 126 264 unique elemental compositions could be assigned.

To determine the chemical formula in mixtures one may also apply direct injection MS without an initial separation step by chromatography. As Mass Spectrometry is usually highly sensitive, one can determine the chemical formula of substances often at concentrations at sub-ppb level, sufficient for almost all applications. For many organic species up till a molecular mass of approximately 500 Da it is possible to determine the atomic composition uniquely. We will illustrate this with a concrete example, in which experiment revealed a species with molecular mass 325.14278. Despite the high mass accuracy, this experimentally determined mass is not sufficiently accurate to uniquely determine the molecular formula. Setting the tolerance (maximum difference between experimentally determined value and calculated value on basis of molecular formula) at 1 ppm, there are five possible formulas when excluding metal atoms.

Formula Accuracy (ppm)

1. $C_{12}H_{25}O_6N_2S$ - 0.016
2. $C_{15}H_{22}O_2N_4Cl$ - 0.614
3. $C_1{}^1H_{24}NNaP_2$ - 0.655
4. $C_{16}H_{22}ON_4K$ - 0.797
5. $C_7H_{22}ON_{10}PS$ - 0.961

All 5 species are potential formula based on the accuracy of the FT-ICR-MS instrument employed. Therefore, we generally need to use, in addition to the gross mass, the isotope ratios. Using the natural abundance of 1.1 % of ^{13}C, the intensity ratio of the $^{13}C/^{12}C$ peaks in the mass spectrum yields the number of carbon atoms in

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the species, and when the calculated ratio cannot be determined sufficiently accurate it at least excludes several possible formulae. For chlorine, the natural abundance is 24% for ^{37}Cl versus ^{35}Cl, so the ratio of those peaks in the mass spectrum will immediately indicate whether formula 2 is either to be excluded or the correct hit. With ^{34}S having an abundance of approximately 4% compared to 32S, the five-formula presented, and the various isotope ratios revealed that the number 1, $C_{12}H_{25}O_6N_2S$, is the correct chemical formula for the species detected. This one happens to have the smallest deviation from the theoretical mass of all five potential species, -0.016 ppm, but that is just a coincidence and not the reason for the correct assignment.

When we take an example with a mass clearly larger than 600, in the present example 679.51145, we find 159 propositions for the chemical formula.

Formula	Accuracy (ppm)
1. $C_4H_3ONKNaP_7S_{10}$	0.007
2. $C_{46}H_{68}N_2P$	0.023
3. $C_{25}H_{68}ON_{16}KS$	0.026
4. $C_4H_4ONC_{19}KNaP_3S_4$	0.029
5. $C_7HONC_{17}KP_7S_2$	0.030 and 155 more within 1 ppm accuracy.

After analysis of the isotope ratios of Cl and S it could be concluded that there is no Cl atom present and no more than two S atoms are present, leading to a reduced number of 16 chemical formulae each of which is still a potential candidate. One may subsequently invoke the so-called 7 Golden Rules introduced by Kind and Fiehn [7]. These are heuristic rules which were developed as an additional tool in structure elucidation of small molecules, although it is no guarantee to find the correct formula. These rules have been implemented into an algorithm which filters molecular formula and is available as a software tool which can be downloaded for free [8]. In essence it first ranks possible formula according to isotopic patterns and then limits these formulae by their presence in large public chemical databases. For further details we refer to the original paper by Kind and Fiehn [6] which is very useful to read as it provides interesting information regarding, e.g., the explosion of the number of possible molecular formulae with increasing molecular mass. When applying all 7 Golden Rules to the 16 molecular formula, all rules were passed successfully implying no discrimination was possible. Moreover, none of the potential 16 formulae were present in the data base and consequently no unique formula could be assigned.

Another example of using a data base of chemicals after applying GC-MS reported by Orlov et al. [9] (the risks were mentioned above) gave further insight onto the possible molecular structures. However, as the number of potential formulae rises in a strong and non-linear way with increasing molecular mass, we will end up with a considerable number of potential candidates after applying the 7 Golden Rules. Moreover, the number of compounds in the public data bases decrease significantly with increasing molecular mass (see Kind and Fiehn). Thus, the most important tool we have remains a MS instrument with the highest possible mass accuracy in conjunction with an accurate determination of the various isotope ratios.

Chemical structure

Whereas methods including X-ray diffraction, IR and Raman spectroscopy may provide very useful information, the method par excellence for chemical structure determination of organics is. This methodology has been developed successively over several decades with continuous improvements. Thus, we do not need to discuss this to any extent but refer to a selection of essential references. A recent review [10] provides a good introduction and overview over recent developments, and although the title refers to metabolomics it deals with the techniques relevant to the identification of unknowns. Mikhail Elyashberg, who has worked very long in the field of NMR-related structure elucidation tools, has extensively reported on what later became known as Computer-Assisted Structure Elucidation (CASE) [11]. Whereas an expert system for the automatic atom-to-peak or multiple assignment of ^1H-NMR spectra can be invoked, in addition 2D-NMR spectra are required to unambiguously assign molecular structure. This may require a larger set of NMR spectra including 1D ^1H NMR, 1D ^{13}C NMR, 2D multiplicity-edited-(^1H–^{13}C) HSQC, 2D (^1H–^{13}C) HMBC and (^1H–^1H) COSY.

Despite all these experimental data provide a lot of information on the structural aspects of the molecule under investigation, e.g., atom-atom distances, for an unambiguous assignment computational chemistry tools are required to evaluate theoretical NMR parameters for assigning and confirmation of the correct molecular structure. These tools might be (semi-)empirical methods or ab initio quantum methods. A highly adequate and recent overview, including its historic development, was provided by Elyashberg and Argyropoulos [12]. The methodology has been turned into a software tool CASE marketed by ACD Labs. Their website also contains a series of examples of structure elucidations of non-trivial molecules using the CASE methodology [13]. In his 2015 paper Elyashberg wrote at the end 'The author believes that soon, CASE will become a routine analytical tool, which will serve as an integral part of any NMR spectrometer'. Interestingly, recently the use of this kind of software has been demonstrated for use in an undergraduate organic chemistry class [14]. This demonstrates the level of implementation for a broad use of these tools. Alternatively, the Bruker Company has developed the Complete Molecular Confidence (CMC) concept which comprises a fully integrated NMR/LC-MS based solution. This development also has a history of a decade or more, see e.g., Thiele et al. [15]. Bruker has also developed this into a software suite for complete data analysis [16].

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Whereas structure elucidation based on NMR data is meanwhile well-developed, characterization of trace amounts is hampered by a lack of sensitivity at very low concentration. The highest sensitivity is achieved using a cryoprobe, e.g., Bruker Biospin's 1.7mm TCI MicroCryoProbe™. For structure elucidation involving 2D-NMR measurements the limit of detection is of the order of 10 ppm. For trace amounts, the concentration with which a compound leaves the LC column, including the eluent, is commonly far too low to allow for NMR measurements. This problem can be circumvented by using Solid-Phase-Extraction (SPE) along with LC and NMR. This has been round for around two decades now, see e.g., Godejohann et al. and references therein [17]. There are plenty of other references that can be easily found by searching for LC-SPE-NMR.

When it is not possible to concentrate the species of interest up to the required, a possible approach to discriminate between potential structures is to invoke Ion Mobility Mass Spectrometry [18]. This technique measures the drift-time of an ion, which in turn can be used to calculate the Collisional Cross Section (CCS) as the drift time primarily depends on the cross section of a molecule. This is not a new technique, but the power to elucidate chemical structures became facilitated by applying molecular simulation enabling a comparison between experimental cross section and computed cross sections for various potential molecular structures. One may invoke classical molecular simulations to calculate this cross section by applying the freely available software tools named MOBCAL [19] or the alternative IMoS [20]. Shrivastav, et al. [21] have claimed IMoS being around two orders of magnitude faster than MOBCAL. An interesting tutorial like paper was reported by D'Atri, et al. [22]. Whereas for (larger) flexible molecules there is the need to apply classical molecular dynamics to evaluate the molecule's cross section, for small relatively rigid molecules quantum mechanical calculations can be adequate as shown by Lap thorn, et al. [23]. Finally, very many relevant publications are accessible via the Website of the Waters company [24].

When there is no both sensible and likely suggestion for the structure of the molecule of interest, molecular structure generators can be invoked, i.e., tools that generate all possible molecular structures based on the chemical formula, e.g., involving the MOLGEN program [25]. Such structures can subsequently be filtered using other knowledge and tools, e.g., Collisional Cross Section as mentioned above, or comparing chromatographic retention times with log Kow (octanol-water partition coefficient) ranges to select or eliminate candidates for further consideration. The log Kow or the dipole moment can be an appropriate choice for properties that can be linked to chromatographic retention. However, it goes without saying that we cannot expect accurate predictions of retention times based on these molecular properties, but if the predicted properties give a rough indication the results can be used to eliminate and therewith reduce the number of potential structures. A comprehensive overview with interesting examples was presented in the PhD thesis by Schymanski which is freely available [26], and the resulting peer reviewed publications can be found in this thesis.

Quantification

So as for many cases the structure can be determined by, a technique such as the LC-SPE-NMR, the difficult part is still the quantification of a trace amount. An SPE collects the molecules, but we do not know how much of it, so for true quantification this will not suffice. One could determine a lower limit as material gets lost but not more is collected than potentially present. This may suffice for certain question dealing with e.g., toxicity. Still, with a trace amount much below 1ppm this is very elaborate if practicable at all when we consider the sensitivity of NMR (see previous Section).

A potential way forward would be to increase the concentration levels in the LC-fractions. There is a variety of different potential approaches including the classical rotatory evaporation, applying what is called the Speed Vacuum machine (combined infrared light and centrifuge to evaporate the solvent), and a lyophilized also known as freeze drying. Whereas these techniques work comparatively well for organic solvents, but recoveries might vary substantially between different compounds, water-based solutions often show strongly varying recovery levels from essentially zero up to a few dozen percent. Whereas one concentration method might work relatively well for one specific molecule, a different method might work much better for another molecule. Thus, all these techniques have the disadvantage that it is not known what percentage of a molecular species is truly recovered, and this is differing largely between different molecular species and the method of recovery.

To quantify a large range of different compounds, we need what is known as a universal detector. A universal detector is defined as the one which 'can respond to every component in the column effluent except the mobile phase' [27]. In contrast, selective detectors are defined as 'detectors which respond to a related group of sample components in the column effluent'. There is a range of universal detectors including Mass Spectrometry, the traditional Flame Ionization Detector (FID), and the later developed detectors including the aerosol-based detection systems, e.g., evaporative light scattering detection (ELSD) [28], the related nano quantity analyte detector (NQAD) [29] and the corona-charged aerosol detection (CAD) [30]. Whereas FID is the most common detector in GC, FID is when one takes it strictly with the definition not a universal detector as it is not or very little sensitive to some compound classes. Still, many compounds can be detected, and the detection limit is around 0.1 ppm (0.1 ng).

What we need to quantify trace amounts of unknown chemicals, often in complex mixtures, is thus a detector which is universal. In addition, it should have a response level proportional to the quantity but independent of the type of molecule, and finally the sensitivity should be very high, preferably down to ppb

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level. Unfortunately, such a detector does not exist. The universal detectors, e.g., mass spectrometry, commonly suffer from very molecule specific response. NMR suffers from too low sensitivity. Detectors with high sensitivity are the chemiluminescence detectors including the sulfur chemiluminescence detector (SCD) [31] in combination with GC and the nitrogen specific cheminescence detector (CLND) [32] in combination with LC, even though as the names already indicate they are S- and N-specific, respectively. On the other hand, they really quantify the S-and N-atoms, respectively.

As many practically relevant organics contain a N atom the CLND can be a very useful asset in the context of the current theme. These detectors work element specific as the compound is burned and subsequently, using ozone, an excited state SO_2* or NO_2* is formed and upon relaxation to the ground state a photon is emitted. Photomultipliers can ultimately countdown to single photons, and therefore this technique is so very highly sensitive. The SCD and CLND detection limits are truly superior with reported values of 0.1pg for sulfur and <0.1 ng for nitrogen. Many compounds of practical relevance contain at least one nitrogen, including pesticides, herbicides, drugs of abuse and degradation products of all of these. Important to note is of course that the eluent used in the LC must be nitrogen free, so for instance one cannot use acetonitrile an eluent. In some cases, this implies the LC protocol for separation must be reestablished with another eluent.

In GC one could combine the highly sensitive S-detector with an FID for non-sulfur containing species even though this is a compromise as the FID lacks some of the advantages of the chemiluminescence detectors, e.g., less sensitive. For GC we can also combine with the sensitive nitrogen-phosphor detector which, however, works based on the same principle as the FID. There are meanwhile relatively old but still as valid, publications on the performance of detectors as the technique behind these has not or hardly changed, see e.g., Lane et al. [33] who report on issues including the need for calibration. For LC only NMR and CLND currently seem appropriate options. Obviously, when only few nitrogen or sulfur atoms are present in the molecule, the sensitivity towards the compound will decrease with increasing size of the molecule as the chemiluminescence only detects these specific atoms.

Discussion and Conclusion

The summary of this introduction to the theme organic trace analysis is that for lower molecular weights it is possible to establish a single unique molecular formula (in a typical example 95% of unknown compounds m/z < 500 Da could be identified uniquely), whereas for higher molecular weights (starting typically around 500) this still poses serious challenges. Also, structure elucidation is far from trivial, unless enough can be collected for NMR studies. Proper quantification remains what is probably the biggest challenge because of the lack of methods to collect (close to) 100% of each individual species.

What we need therefore are other approaches, e.g., the elimination approach. Structure generators in conjunction with Ion Mobility Mass Spectrometry and molecular simulation may exclude certain structures. Exclusion can also be practiced by collecting a series of samples from time-slices from a HPLC, and subsequently analyzing total sulfur content using traditional highly sensitive analytical methods. This allows for the exclusion of S-containing molecules in specific time-slices and the corresponding molecular formulae. This can, when considered useful based on the potential formula generated after the high mass accuracy MS experiment, be repeated for other elements. Also, though rarely applied in this context, functionalization by e.g., sulfonation or the more well-known H/D exchange can give directions for elimination or identification of structural parts. These approaches might, in specific cases, help to find upper limits of concentrations present. Alternatively, or perhaps it is more appropriate to say in addition, by combining and further integration of tools that were named progress may be expected. We have seen this being realized in the past for structure elucidation by NMR [12-17]. For multiple species to be characterized data management and manipulation, i.e., protocols such as suggested in Ref. [2], can be a valuable toolbox.

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