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Development of a Retention Time Interpolation scale (RTi) for liquid chromatography coupled to mass spectrometry in both positive and negative ionization modes

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ABSTRACT

The accuracy and sensitivity of high resolution mass spectrometry (HRMS) enables the identification of candidate compounds with the use of mass spectrometric databases among other tools. However, retention time (RT) data in identification workflows has been sparingly used since it could be strongly affected by matrix or chromatographic performance. Retention Time Interpolation scaling (RTi) strategies can provide a more robust and valuable information than RT, gaining more confidence in the identification of candidate compounds in comparison to an analytical standard. Up to our knowledge, no RTi has been developed for LC-HRMS systems providing information when acquiring in either positive or negative ionization modes.

In this work, an RTi strategy was developed by means of the use of 16 isotopically labelled reference standards, which can be spiked into a real sample without resulting in possible false positives or negatives. For testing the RTi performance, a mixture of several reference standards, emulating suspect analytes, were used. RTi values for these compounds were calculated both in solvent and spiked in a real matrix to assess the effect of either chromatographic parameters or matrix in different scenarios. It has been demonstrated that the variation of injection volume, chromatographic gradient and initial percentage of organic solvent injected does not considerably affect RTi calculation. Column aging and solid support of the stationary phase of the column, however, showed strong effects on the elution of several test compounds. Yet, RTi permitted the correction of elution shifts of most compounds. Furthermore, RTi was tested in 47 different matrices from food, biological, animal feeding and environmental origin. The application of RTi in both positive and negative ionization modes showed in general satisfactory results for most matrices studied.

The RTi developed can be used in future LC-HRMS screening analysis giving an additional parameter, which facilitates tedious processing tasks and gain more confidence in the identification of (non)-suspect analytes.

1. Introduction

Mass spectrometry (MS) has revolutionized analytical chemistry. Tandem (MS/MS) instruments are nowadays the most powerful analytical tool widely applied for the qualitative and quantitative determination of organic compounds in complex matrices [1–3], whereas the high quality data obtained by hybrid instruments involving high-resolution mass spectrometry (HRMS) allows rapid, sensitive and selective screening of hundreds of contaminants in for example food [4], environmental [5,6] and forensic samples [7,8], even for compounds for which reference standards are less accessible.

Screening strategies, based on hybrid systems, rely on the high mass resolving power and mass accuracy attainable by HRMS. The data obtained provide relevant information on both (de)protonated molecules and fragment ions, without the need of selecting precursor ions. Positive findings are tentatively identified by comparing their measured exact mass, isotopic pattern and fragmentation pattern to either those of an analytical standard, from scientific literature or theoretically calculated based on the chemical structure [9]. The number of potential candidates is, however, often not limited to one, which makes this identification process generally

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more complicated and time consuming. Furthermore, reporting false negatives cannot be excluded.

Liquid chromatography (LC) separation, i.e. retention time (RT), has not been as routinely incorporated into identification workflows as other in silico identification tools and mass spectrometric databases [9,10]. However, chromatographic resolution not only has influence on ion suppression and mass measurement accuracy [11], but RT can also be used as an additional parameter in the identification process and gain more confidence to the obtained results [12,13]. However, RT strongly depends on the type of stationary phase and affinity of compounds with the mobile phase. Other parameters such as flow rate, gradient, column temperature, length and aging, and sample matrix may also strongly affect the retention of compounds [14].

The use of Retention Time Interpolation scale (RTi) evades these variables as it is, in theory, inter-system transferable. The calculation of RTi by measuring RT relative to co-injected standards (named as markers) can help to overcome the shifting in RT across different situations. Markers are assigned to a fixed RTi value whereas the analyte is interpolated in relation to the markers eluting before and after it. Any shift is expected to affect the analyte and markers in the same manner so that the RTi remains constant [15]. With identification purposes, as it is usually done with RT, experimentally found RTi values can then be compared with known values from analytical reference standards. It is, therefore, more suitable as an identification parameter for wide scope screening strategies. Additionally, RTi could allow extrapolating screening techniques from one laboratory to another.

In gas chromatography (GC), the Kovats retention index, where RT is normalized to the RT of adjacently eluting n-alkanes, is well established and often applied [16–18]. The development of a universal RTi in liquid chromatography (LC), however, is more complicated and has presented many pitfalls [16]. LC is inherently more complex than GC as the mobile phase plays a key role in the chemical interactions with the stationary phase. This influences the selectivity and thus the retention of a compound exceedingly. Some approaches for the calculation of RTi in LC systems have been described in the literature based on the required percentage of organic modifier to elute a certain analyte in a linear gradient (Chromatographic Hydrophobicity Index) [19] or setting the index by means of the partition coefficient (logP) of 10 compounds, mainly pesticides [20]. Other approaches consider the normalization of RT using co-injected standards, either pesticides (KRetI) [21] or a series of n-nitroalkanes [22]. KRetI was applied in a non-target analysis for an inter-lab comparison of candidates by means of interpolating a retention index between two co-injected pesticides, chloroxuron and fenuron [21]. However, the series of n-nitroalkanes retention index, was developed by injecting the series of compounds before and after the samples and interpolating retention indices using Kovats-like logarithmic equation [22]. In addition, this retention index was based on an isocratic elution only, which is scarcely applied in multi-residue LC methods. As it has been previously explained, the co-injection of standards used for interpolating is preferred. In addition, these standards should easily be differentiated from compounds naturally occurring in the samples to avoid the reporting of false positives or negatives. Despite that some strategies were applied to LC–MS systems, none of them considered the approach of setting an RTi by means of isotopically labelled reference standards (ILRS). Hence, the aim of this work is to develop an RTi based on ILRS, which (i) is robust under different chromatographic conditions, (ii) can be applied to any sample matrix, (iii) provides an additional identification parameter for screening by LC-ESI-HRMS in both positive and negative ionization modes and (iv) is easy to implement in other systems and laboratories.

2. Experimental

2.1. Chemicals and materials

For this study, 121 compounds were used, consisting of 54 isotopically labelled reference standards (ILRS) and 67 analytical reference standards (RS). The complete list of compounds used can be consulted in the Supplementary information (SI) Table S1. ILRS and RS were purchased from Across Organics (Geel, Belgium), Aventis Pharma (Madrid, Spain), Bayer Hispania (Barcelona, Spain), Cayman Chemicals (Ann Arbor, MI, USA), CDN Isotopes (Quebec, Canada), Cerilliant (Round Rock, TX, USA), Dr. Ehrenstorfer (Augsburg, Germany), Fluka (Buchs, Switzerland), Fort Dodge Veterinaria (Gerona, Spain), National Measurement Institute (Pymble, Australia), Riedel-de Haën (Seelze, Germany), Sigma–Aldrich (St Louis, MO, USA), Toronto Research Chemicals (Ontario, Canada), Vetoquinol Industrial (Madrid, Spain) and Witega (Berlina, Germany). All reference standards had purities higher than 98% (*w/w*). Leucine enkephalin, used for mass correction, was purchased from Sigma–Aldrich (St. Louis, MO, USA).

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN) and formic acid (HCOOH, >98%) were supplied by Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q plus system from Millipore (Bedpore, MA, USA). A standard stock solution of each compound was prepared at a concentration level between 1000 μg L^{-1} and 10 g L^{-1} in MeOH or ACN.

2.2. Selection of markers

A preliminary study was performed with each of the ILRS included in the study to establish RT, peak intensity and in-matrix reproducibility. The final selection of markers for RTi calculation was based on different criteria. First of all, the RTi strategy needs to be extended to the whole chromatographic run and marker distribution should cover from the very first compound to the last one in an arrangement as equally distributed as possible. The proportionality in the distribution of markers across the chromatogram is important in terms of RTi values comparison. Second, regulated compounds such as drugs of abuse or new psychoactive substances were whenever possible avoided as well as those of higher cost. Third, compound ionization efficiency was considered for the establishment of an estimated concentration required for good peak intensity in complex matrices. In summary, full proportional spectrum coverage, in-matrix reproducibility, peak intensity, compound family and costs were considered for the selection of appropriate compounds.

Additional information regarding both markers and analytes can be found in the Supplementary information Table S2.

2.3. Testing matrices

A complete list of matrices used in the study (food, environmental samples, animal feed and biological fluids) and the sample treatment applied are available in SI (Table S3 and Table S4 in Supplementary information). The corresponding extracts were spiked with both the set of markers (ILRS) and reference standards (used as target analytes). A suspect screening analysis was performed in order to obtain RTi values in real samples.

2.4. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was coupled to a quadrupole TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), with a Z- spray- ESI interface operating in positive and negative ion mode. An Acquity UPLC

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