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.

Key Words:

41 Retention time index, retention time interpolation scale, liquid chromatography, high
42 resolution mass spectrometry, isotopically labelled reference standards, Kovats index,
43 screening

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 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 scales (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**). 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 BEH C_{18} analytical column 2.1x100 mm with 1.7 µm particle size (Waters) and a Cortecs C_{18} 2.1x100 mm with 2.7 µm particle size were employed for chromatographic separation. Mobile phase, at a flow rate of 0.3 mL min^{-1}, consisted of water and MeOH both with 0.01% HCOOH. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10% B; 14 min. 90% B; 16 min. 90% B; 16.01 min, 10% B; 18 min, 10% B. The column was set at 40 ºC. MS data were acquired in the range of *m/z* 50 - 1000. A capillary voltage of 0.7 kV in positive mode and 2.5 kV in negative mode were used with a cone voltage of 25 V. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 650 ºC and the source temperature at 120 ºC. For automated accurate mass measurement, the lock-spray probe was used, using a lock mass solution of leucine enkephalin (2.5 mg L^{-1}) in ACN:water (1:1 v/v) at 0.1 % HCOOH pumped at 30 µL min^{-1} through the lock-spray needle. The (de)protonated molecule of leucine enkephalin at *m/z* 556.27658 in positive mode and *m/z* 554.26202 in negative mode was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

For MS^E, two acquisition functions with different collision energies were generated. The low collision energy function (LE) with a collision energy of 4 eV, and the high collision energy function (HE) with a collision energy ramping from 15 to 40 eV. MS data were acquired in continuum mode and processed with the screening platform within UNIFI v1.8 (Waters Corporation).

2.5 Retention Time Interpolation scale (RTi) calculation

Based on the equation developed by Kovats [23] and the modifications applied by Van der Dool and Kratz [24], the equation used for the calculation of RTi values is shown in **Equation 1** where *n* corresponds to the elution position of the marker eluting just before the analyte (*j*) and *n +1* corresponds to the markers eluting right after the analyte. In addition, the deviation was calculated as a relative difference between the measured value and an average RTi value (obtained with the injection of a mixture of standards in solvent). The equation used is shown in **Equation 2.**

$$RTi_j = 100 \cdot \left(n + \frac{(t'_R)_j - (t'_R)_n}{(t'_R)_{n+1} - (t'_R)_n} \right) \qquad \textbf{(Equation 1)}$$

$$Dev_j(\%) = 100 \cdot \frac{\overline{RTi}_{Solvent} - (RTi)_j}{\overline{RTi}_{Solvent}} \qquad \textbf{(Equation 2)}$$

2.6 General data processing

The impact of chromatographic parameters in RTi strategy were tested by spiking both the set of markers and RS in solvent. Standard RTi ($RTi_{Solvent}$) values were established by calculating them when injected with the conditions specified in section 2.4. Then, experimentally obtained values after forcing chromatographic parameters were compared with RTi in solvent by means of **Equation 2**.

The same strategy is applied for assessing the impact of matrix. Both markers and analytes were spiked in sample extracts and deviations of experimental RTi values were calculated by means of **Equation 2**.

The maximum allowed absolute RT deviation in several guidelines is ≤ 0.1 min, which represents 5 % of the average 2 min window between markers in RTi-P. Therefore, RTi

deviation should be below 5 % as a way of translating absolute deviation from guidelines to our RTi system.

3. Results and Discussion

3.1 Selection of markers

The first selection of potential markers to include in the study was based on their availability at our lab as well as their amenability for liquid chromatography. A set of 54 different ILRS was selected and evaluated as markers for its application in both positive and negative ionization modes. The development and optimization of RTi strategy was performed separately for each ionization mode. It was decided to use ILRS as markers because of the applicability of this strategy in screening analyses. The coupling of LC with HRMS allows the differentiation of naturally occurring compounds in a matrix from the isotopically labelled ones used as markers. Therefore, the potential reporting of false positives and negatives was avoided since compounds intentionally spiked to a sample can easily be differentiated from the naturally occurring ones by their *m/z*.

The final set of markers selected encompassed 12 markers for RTi in positive ionization mode (RTi-P) and 6 for negative ionization mode (RTi-N) (**Figure 1**). The markers showed good performance in both solvent and matrix-matched analysis, except for ecgonine-d_3 which was observed in around 80 % of the matrices as it elutes early in the chromatogram and can be more affected by matrix suppression.

Finally, markers were arbitrarily assigned a value of *n* depending on the elution order ranging from *n = 0* (ecgonine-d_3) to *n = 11* (diethylhexylphathalate-d_4) in RTi-P and *n* = 0 (ecgonine-d_3) to *n = 5* (THC-COOH-d_3) in RTi-N (**Table 1**). When applying **Equation 1**, RTi values ranged from 0 to 1100 in RTi-P and from 0 to 500 in RTi-N.

3.2 The impact of chromatographic parameters

Changes in chromatographic conditions should not affect the RTi calculation since markers should, in theory, correct for possible shifts in RT. The performance of RTi approach was evaluated forcing several chromatographic parameters i.e. variation of injection volume, gradient, column aging and type of solid support of stationary phase in chromatographic column. In-solvent mixtures of markers and reference standards (acting

as target analytes) were injected under these different conditions. Analytes of a wide range of polarity were chosen in order to cover the whole chromatogram and to have analytes within each interval between two markers. RTi values of analytes in the different conditions were compared with those obtained with the conditions explained in section 2.4 to assess the impact of the different chromatographic parameters.

Injection volumes 10, 20, 30, 40 and 50 µl (n=5) were tested resulting in little distortion of RTi. The majority of the compounds (85 % for RTi-P and 100% for RTi-N) showed a deviation in their RTi < 5 %. In theory, if the organic phase of the sample solvent is the same as the initial conditions of the gradient, a higher injection volume should not alter the eluent composition. In general, if detection of low concentrated compounds is required, injection volume can be increased without affecting the performance of RTi considerably.

Retention capability of a chromatographic column is often reduced with the *column aging*. For the development of RTi-P, the performance of a new chromatographic column was compared to the performance after roughly 800 injections. Although many injections can still be done with the column, RT varied in the range of 0-1 min with an average variation of 0.15 min. RTi values showed a deviation < 5% and > 10% in 55% and 27.5% of the cases, respectively. The high deviation observed i.e. 45% of compounds above 5% deviation remarked that the degradation of the stationary phase in the column produced different retention patterns between analytes and markers. Additionally, for RTi-N, the effect of column aging was assessed after 300 injections (from injection 700 to injection 1000). In this case, values showed a deviation of < 5% in 90% of the cases (10% for > 10% deviation). These results considerably differ from the ones obtained for RTi-P. The different percentage of compounds having a deviation value >10% also highlighted that the degradation of the column is a progressive effect.

In terms of *gradient*, the effect of 5, 10, 15 and 20 % of organic phase at the beginning of the gradient was tested (n=5). Compounds eluting at the beginning of the chromatogram were strongly affected suffering from variations in the RT of up to 2.3 min. However, RTi values showed a deviation < 5% in 72 % of the cases (RTi-P) and 71 % of the cases (RTi-N). Only 7% of the compounds showed a deviation > 10 % for RTi-P and 14% for RTi-N. Additionally, the impact of extending or shortening the chromatographic elution was assessed by the comparison of some run durations (5, 10, 15, 20, 25 and 30 min.). In

total, 73% of the compounds showed a deviation in their RTi-P < 5 % (86% of the cases in RTi-N) and only 5% of the compounds had a deviation > 10% for both RTi-P and RTi-N.

The effect of implementing the same strategy by using a different *type of column* was assessed. The utilization of a chromatographic column of a completely different stationary phase would completely change the chromatographic retention mechanisms and, therefore, makes the application of the RTi strategy not feasible. Therefore, some C_{18} columns were tested, but with different solid support for the stationary phase which, in theory, would not strongly affect the retention of compounds.

A Cortecs C_{18} and Acquity UPLC BEH C_{18} columns were compared. BEH columns are polymeric based columns with a particle size of 1.7 µm, whereas Cortecs are made with 2.7 µm solid-core silica particles. Despite both chromatographic columns were reverse phase, the distinct solid support and particle size was expected to produce small variations in absolute RT from one column to the other. When using Cortecs column, 59% of the compounds showed a deviation on their RTi-P value < 5% (73% for RTi-N). Notwithstanding, 24% of compounds showed a deviation > 10 % (18% for RTi-N). A clear trend was observed differentiating two regions in the chromatogram. From 0 to 10 min, where high RTi value deviations were observed, and from 10 to 18 min where RT correction by means of RTi was satisfactory. Those deviations could be due to the selectivity of the stationary phase *i.e.* the distinct solid support. Therefore, alternative markers were studied in order to improve performance in this type of column in the first half of the chromatographic run. However, no improvements were observed and it was decided to maintain the set of markers optimized in previous sections. These deviations suggested that the RTi strategy could not be directly implemented in a different type of column than the one used for its development without previous adaptive studies.

Table 2 shows a summary of the deviations in RTi values for analytes in both RTi-P and RTi-N when some chromatographic parameters were forced to change. As previously stated, the application of UHPLC-HRMS techniques for screening analyses usually means that resolution power is only entrusted to mass analyzer and therefore, the chromatography is rarely modified to improve compound separation. Even though, RTi application allows, as demonstrated, the variation of the some chromatographic parameters such as injection volume and gradient if better separation of compounds or

signal are necessary without affecting the interpolating performance. Additionally, the RTi strategy can be applied regardless the amount of organic compound present in the extract because any possible distortion of RT is be corrected by RTi.

3.3 The influence of matrix

The influence of different matrices on the RTi performance was evaluated by assessing the reproducibility of RTi values for RS in 47 different matrices. The selection of the matrices was based on the availability of extracts, the availability of raw matrix (in the cases that sample treatment was simple), and the coverage of different scientific fields such as food (including acidic, basic, fatty and sugary matrices), environmental (drinking, surface and ground water as well as influent and effluent wastewater), feed (bovine, poultry and rabbit feed) or biological fluids (plasma, blood and urine) to demonstrate its applicability at different research areas. The impact of the matrix on the calculation of RTi was evaluated by spiking the set of 16 markers and 67 analytical reference standards to 47 samples/extracts of different origin. A target screening was performed in order to obtain the RT and RTi for all compounds. **Table S5** (in Supplementary Information) summarizes RT and RTi for all markers and analytes in all matrices tested for both RTi-P and RTi-N. For comparison purposes, in-matrix RTi deviation from RTi in solvent were calculated resulting in average values below 5 % in the 93 % of the cases for RTi-P and only 2% of the cases analyzed showed a deviation greater than 10 % from the RTi values in solvent. For the analysis of RTi-N, the five most troublesome matrices and other five random matrices were analyzed and the same behavior was observed.

The current analytical guidelines for compound identification generally require an absolute deviation in RT from the correspondent standard less than 0.1 min [25–27]. When applying this criteria, identification would not have been possible for the 7 % of the RS spiked in matrices, since analytes differ (> 0.1min) in RT compared to its standard in solvent. **Table 3** shows some examples of potential false negatives in matrices. Nevertheless, these could all be corrected by the application of RTi-P. An illustrative example is demonstrated in **Figure 2**, where azithromycin in lettuce showed a RT deviation of 0.38 min. When strictly applying the guidelines, azithromycin would not have been reported i.e. resulting in a false negative. However, its RTi differed only 0.63 % with the RTi of the standard (well below the 5% deviation threshold) and would, therefore, not have been discarded.

As demonstrated, the application of RTi is feasible in a wide range of matrices. Moreover, its application in a real screening scenario would be much more successful since it is known that usually the majority of compounds did not elute in the first part of the chromatogram [28,29], which is the region of not optimal performance of RTi. So, the application of RTi in a real suspect screening would lead to even higher percentages of success than those obtained when the whole chromatogram was covered with analytes for RTi development.

In addition, wide-scope screening strategies are usually affected by the wide range of matrices analyzed as well as a combination of other chromatographic parameters alterations (column aging, percentage of organic phase in extract…). Oftentimes, the analyst faces cases where exact mass of (de)protonated molecule and fragment ions, and isotopic pattern fit within a candidate but RT is deviated from the standard. Consequently, further analysis by spiking the extract with the candidate compound or updating RT databases should be performed, resulting in time-consuming and costly tasks. For broader applicability of these type of strategies, the utilization of RTi will avoid these 'extra' analyses, also reducing the rate of false negatives in wide-scope screening analyses.

4. Conclusions

A robust retention time interpolation scale strategy has been developed for screening applications in UHPLC-HRMS systems in both positive and negative ionization modes by means of 16 ILRS. The impact of several chromatographic parameters on its performance has been tested resulting in satisfactory performance. Consequently, RTi strategy allows the modification of some chromatographic parameters if better resolution of compounds is required. In addition, the strategy has been tested for its application through nearly 50 matrices from different origin (i.e. food, biological, animal feeding and environmental), showing a success rate of approximately 90% in the identification of analytes in both positive and negative ionization modes. RTi developed has shown an additional value for LC-HRMS screening applications laying the basis of a harmonized retention parameter that could easily be implemented in other systems and laboratories. The substitution of any marker with another ILRS could be evaluated for further application if any ILRS used in this study is not available in the implementing laboratory. Lately, the in-lab RTi values for the reference standards of interest need to be established for specific chromatographic conditions.

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6. References

[1] F. Hernández, O.J. Pozo, J. V. Sancho, L. Bijlsma, M. Barreda, E. Pitarch, Multiresidue liquid chromatography tandem mass spectrometry determination of 52 non gas chromatography-amenable pesticides and metabolites in different food commodities, J. Chromatogr. A. 1109 (2006) 242–252. doi:10.1016/j.chroma.2006.01.032.

[2] A.L.N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. Lopez de Alda, H. Neels, E. Zuccato, D. Barcelo, A. Covaci, Illicit drug consumption estimations derived from wastewater analysis: a critical review., Sci. Total Environ. 409 (2011) 3564–77. doi:10.1016/j.scitotenv.2010.05.030.

[3] E. Beltrán, M. Ibáñez, J.V. Sancho, F. Hernández, Determination of six microcystins and nodularin in surface and drinking waters by on-line solid phase extraction-ultra high pressure liquid chromatography tandem mass spectrometry, J. Chromatogr. A. 1266 (2012) 61–68. doi:10.1016/j.chroma.2012.10.017.

[4] S.J. Lehotay, Y. Sapozhnikova, H.G.J. Mol, Current issues involving screening and identification of chemical contaminants in foods by mass spectrometry, TrAC - Trends Anal. Chem. 69 (2015) 62–75. doi:10.1016/j.trac.2015.02.012.

[5] S.L. Kaserzon, A.L. Heffernan, K. Thompson, J.F. Mueller, M.J. Gomez Ramos, Rapid screening and identification of chemical hazards in surface and drinking water using high resolution mass spectrometry and a case-control filter, Chemosphere. 182 (2017) 656–664. doi:10.1016/j.chemosphere.2017.05.071.

[6] J. Aceña, S. Stampachiacchiere, S. Pérez, D. Barceló, Advances in liquid chromatography–high-resolution mass spectrometry for quantitative and qualitative environmental analysis, Anal. Bioanal. Chem. 407 (2015) 6289–6299. doi:10.1007/s00216-015-8852-6.

[7] I. Ojanperä, M. Kolmonen, A. Pelander, Current use of high-resolution mass spectrometry in drug screening relevant to clinical and forensic toxicology and doping control, Anal. Bioanal. Chem. 403 (2012) 1203–1220. doi:10.1007/s00216-012-5726-z.

[8] M. Sundström, A. Pelander, V. Angerer, M. Hutter, S. Kneisel, I. Ojanperä, A high-

sensitivity ultra-high performance liquid chromatography/high- resolution time-of-flight mass spectrometry (UHPLC-HR-TOFMS) method for screening synthetic cannabinoids and other drugs of abuse in urine, Anal. Bioanal. Chem. 405 (2013) 8463–8474. doi:10.1007/s00216-013-7272-8.

[9] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying small molecules via high resolution mass spectrometry: Communicating confidence, Environ. Sci. Technol. 48 (2014) 2097–2098. doi:10.1021/es5002105.

[10] F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez, Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry, Anal. Chim. Acta. 684 (2011) 96–106. doi:10.1016/j.aca.2010.10.043.

[11] T.R. Croley, K.D. White, J.H. Callahan, S.M. Musser, The Chromatographic Role in High Resolution Mass Spectrometry for Non-Targeted Analysis, J. Am. Soc. Mass Spectrom. 23 (2012) 1569–1578. doi:10.1007/s13361-012-0392-0.

[12] R. Bade, L. Bijlsma, T.H. Miller, L.P. Barron, J.V. Sancho, F. Hernández, Suspect screening of large numbers of emerging contaminants in environmental waters using artificial neural networks for chromatographic retention time prediction and high resolution mass spectrometry data analysis, Sci. Total Environ. 538 (2015) 934–941. doi:10.1016/j.scitotenv.2015.08.078.

[13] I. Marginean, W.F. Rowe, I.S. Lurie, The role of ultra high performance liquid chromatography with time of flight detection for the identification of synthetic cannabinoids in seized drugs, Forensic Sci. Int. 249 (2015) 83–91. doi:10.1016/j.forsciint.2015.01.013.

[14] B. Ardrey, Liquid chromatography-mass spectrometry: an introduction, John Wiley & Sons, Inc., Huddersfield, UK, 2003.

[15] H.-J. Hübschmann, Handbook of GC/MS. Fundamentals and applications., First, Wiley-VCH, Wendelsheim, Germany, 2001.

[16] V.I. Babushok, Chromatographic retention indices in identification of chemical

compounds, TrAC - Trends Anal. Chem. 69 (2015) 98–104. doi:10.1016/j.trac.2015.04.001.

[17] N. Strehmel, J. Hummel, A. Erban, K. Strassburg, J. Kopka, Retention index thresholds for compound matching in GC-MS metabolite profiling, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 871 (2008) 182–190. doi:10.1016/j.jchromb.2008.04.042.

[18] N. Ulrich, G. Schüürmann, W. Brack, Prediction of gas chromatographic retention indices as classifier in non-target analysis of environmental samples, J. Chromatogr. A. 1285 (2013) 139–147. doi:10.1016/j.chroma.2013.02.037.

[19] N. Ulrich, G. Schüürmann, W. Brack, Linear Solvation Energy Relationships as classifiers in non-target analysis-A capillary liquid chromatography approach, J. Chromatogr. A. 1218 (2011) 8192–8196. doi:10.1016/j.chroma.2011.09.031.

[20] S. Grosse, G. Greco, T. Letzel, Retention-time-index (RTI) – a novel chromatographic parameter in the field of LC-MS (/MS) – An interlaboratory study, in: Langenauer Water Forum, 2013.

[21] I. Bobeldijk, P.G.M. Stoks, J.P.C. Vissers, E. Emke, J.A. van Leerdam, B. Muilwijk, R. Berbee, T.H.M. Noij, Surface and wastewater quality monitoring: Combination of liquid chromatography with (geno)toxicity detection, diode array detection and tandem mass spectrometry for identification of pollutants, J. Chromatogr. A. 970 (2002) 167–181. doi:10.1016/S0021-9673(02)00398-9.

[22] L.M. Hall, L.H. Hall, T.M. Kertesz, D.W. Hill, T.R. Sharp, E.Z. Oblak, Y.W. Dong, D.S. Wishart, M. Chen, D.F. Grant, Development of Ecom 50 and Retention Index Models for Nontargeted Metabolomics: Identification of 1,3-Dicyclohexylurea in Human Serum by HPLC/Mass Spectrometry, J. Chem. Inf. Model. 52 (2012) 1222–1237.

[23] E. Kováts, Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone, Helv. Chim. Acta. 41 (1958) 1915–1932. doi:10.1002/hlca.19580410703.

[24] H. van Den Dool, P. Dec. Kratz, A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography,

J. Chromatogr. A. 11 (1963) 463–471. doi:10.1016/S0021-9673(01)80947-X.

[25] European Commission. Directorate General for Health and Food Safety., Guidance document on analytical quality control and method validation procedures for pesticide residue and analysis in food and feed. SANTE/11813/2017, 2018.

[26] U. Vincent, JRC Guidelines for 1 - Selecting and/or validating analytical methods for cosmetics 2 - Recommending standardization steps for analytical methods for cosmetics, 2015. doi:10.2787/412553.

[27] Europen Medicines Agency, Guideline on bioanalytical method validation, 2012. doi:EMEA/CHMP/EWP/192217/2009.

[28] M. Ibáñez, J. V. Sancho, F. Hernández, D. McMillan, R. Rao, Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry, Trends Anal. Chem. 27 (2008) 481–489. doi:10.1016/j.trac.2008.03.007.

[29] F. Hernández, M. Ibáñez, R. Bade, L. Bijlsma, J.V. Sancho, Investigation of pharmaceuticals and illicit drugs in waters by liquid chromatography-high-resolution mass spectrometry, TrAC - Trends Anal. Chem. 63 (2014) 140–157. doi:10.1016/j.trac.2014.08.003.

Table 1. Set of markers for RTi in both positive and negative modes.

		RTi-P		RTi-N	
n	**RTi value**	**Compound**	**t_R (min)**	**Compound**	**t_R (min)**
0	0	Ecgonine-d_3	0.98	Ecgonine-d_3	0.98
1	100	Morphine-d_3	1.43	Ampicillin-d_5	4.60
2	200	Methylone-d_3	2.97	Ethylparaben-d_4	8.00
3	300	Norfloxacine-d_5	4.03	Irbesartan-d_6	10.77
4	400	MDPV-d_8 [a]	5.66	Ibuprofen-d_3	12.54
5	500	Venlafaxine-d_6	7.02	THC-COOH-d_3 [c]	14.12
6	600	Salicylic acid-d_4	7.97		
7	700	25-B-NBOMe-d_3 [b]	9.03		
8	800	Ethofumesate-d_5	10.48		
9	900	Tebuconazole-d_6	12.39		
10	1000	THC-COOH-d_3 [c]	14.12		
11	1100	DEHP-d_4 [d]	17.09		

[a] MDPV-d_8: methylendioxypyrovalerone-d_8

[b] 25-B-NBOMe-d_3: 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine-d_3

[c] THC-COOH-d_3: 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3

[d] DEHP-d_4: di(2-ethylhexyl)phthalate-d_4

Table 2. Average percentage of analytes showing RTi deviations below 2.5 % and 5 %, and above 10 % when modifying different chromatographic parameters for both RTi-P and RTi-N.

	Deviation in RTi-P			**Deviation in RTi-N**		
	< 2.5%	**< 5%**	**> 10%**	**< 2.5%**	**< 5%**	**> 10%**
Injection Volume Variation	63%	85%	2%	97%	100%	0%
Percentage of organic at the beginning of gradient	42%	72%	7%	62%	71%	14%
Chromatographic gradient modification	45%	73%	5%	64%	86%	5%
Column aging	36%	55%	28%	90%	90%	10%
Type of solid support for stationary phase	50%	59%	24%	73%	73%	18%

Table 3. Examples of potential false negatives in matrix analysis corrected by RTi-P application.

		Retention time (min)			Retention Time Interpolation scale (RTi-P)				
Compound	**Matrix**	**Std.**	**Matrix**	**Dev. (min)**	**Std.**		**Matrix**		**Dev (%)**
Amphetamine	White Bread	3.42	3.53	0.11	*n=2;* 2.97 min 3.42 min *n=3;* 4.03 min	243	*n=2;* 3.04 min 3.53 min *n=3;* 4.07 min	248	2.04 %
Azithromycin	Lettuce	6.82	7.20	0.32	*n=4;* 5.66 min 6.82 min *n=5;* 7.05 min	483	*n=4;* 5.78 min 7.20 min *n=5;* 7.42 min	487	0.63 %
Clenbuterol	Industrial Bakery	4.96	5.06	0.10	*n=3;* 4.03 min 4.96 min *n=4;* 5.66 min	357	*n=3;* 4.06 min 5.06 min *n=4;* 5.77 min	358	-0.33 %
Thiamethoxam	Coffee	3.80	3.68	-0.12	*n=2;* 2.97 min 3.80 min *n=3;* 4.03 min	279	*n=2;* 2.97 min 3.68 min *n=3;* 4.05 min	266	-4.61 %
Sarafloxacin	Influent WW	4.79	5.06	0.27	*n=3;* 4.03 min 4.79 min *n=4;* 5.66 min	346	*n=3;* 4.16 min 5.06 min *n=4;* 5.88 min	352	1.71 %

Figure Captions:

Figure 1. Marker distribution across chromatographic injection for RTi-P (top) and RTi-N (bottom).

Figure 2. *(a)* Analysis of azithromycin and markers 4 and 5 in lettuce sample and comparison with RT in solvent; *(b)* Example of RTi calculation for azithromycin.

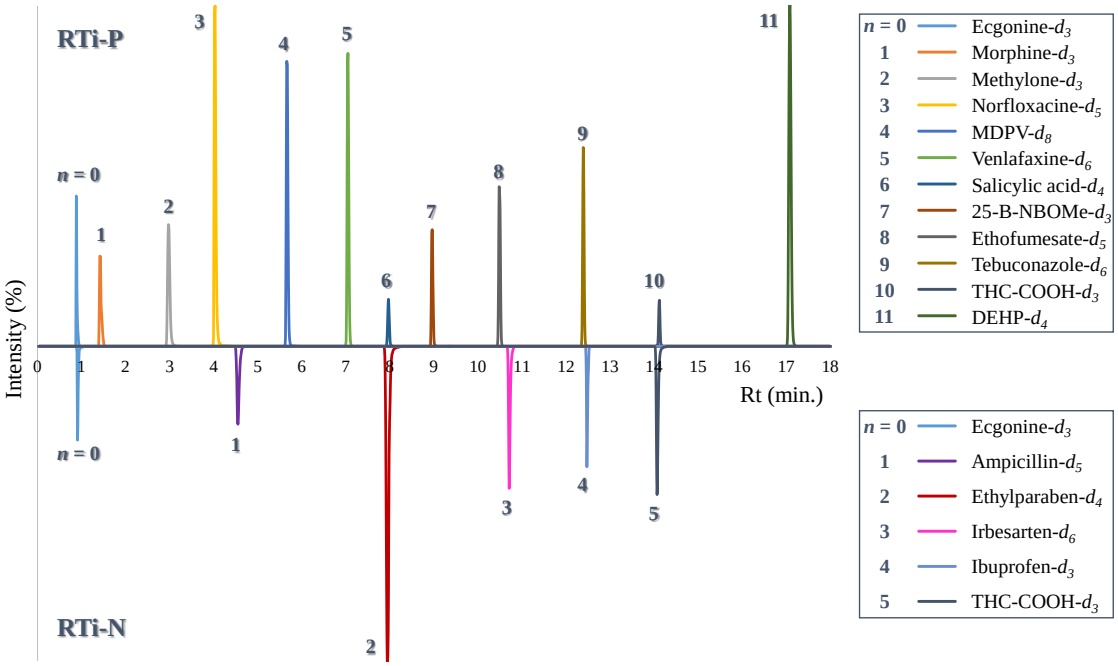

Figure 1.

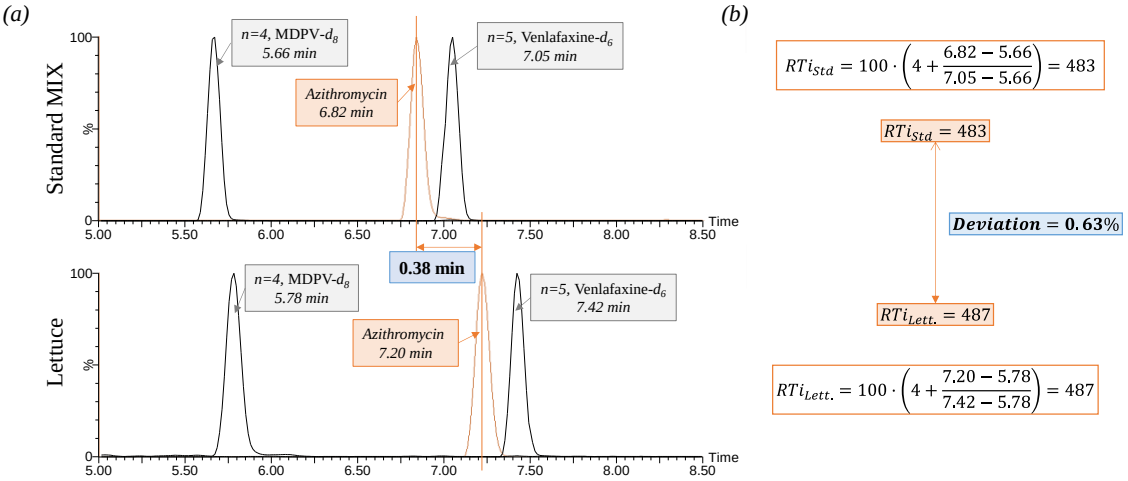

Figure 2.