White paper

Serial RPLC-HILIC coupling hyphenated with mass spectrometric detection - Polarity-extended chromatography fit for NTS?

Stefan Bieber and Thomas Letzel

Abstract

Since almost ten years the serial coupled RPLC and HILIC columns (i.e. reversed phase liquid chromatography and hydrophilic interaction liquid chromatography) are well known in their combination with high accuracy and high resolution mass spectrometric detection (HRMS). In that time 20 peer-reviewed articles and further 14 articles have been published from our group, reflecting it as innovative, flexible, as well as robust and sustainable analytical approach in various disciplines.
The polarity-extended chromatographic separation allows the simultaneous separation of non-polar, polar and very polar molecules in one run and in hyphenation with HRMS, it is perfectly fitting to analyze in a non-targeted screening type of measurement.
In disciplines like environmental analysis (like aqueous samples), food analysis (like water and beverages), process monitoring (like various oxidation techniques), plant metabolism studies and some more, the instrumental design was applied for a broad molecular view. Accompanied with data handling and data post-processing developments it is now on its way to high quality and novel non-target screening solutions.
This white paper draws a 10 years conclusion and starts a new era of polarity-extended molecule screening in non-targeted analysis. Herein, it is presented how the analytical strategy was consequently developed from a research tool into a system fit for stable non-target screening applications starting with the next level of analytical performance.

An Introduction

The increasing interest in holistic views on complex samples forces chromatographic separations to become more broaden and directly compatible with sensitive and accurate mass spectrometric detection. In recent overviews [1,2] it has exemplarily been shown that persistent, mobile and toxic (PMT) and very persistent and very mobile (vPvM) substances in waterbodies - often (very) polar (i.e. with a logD (pH) ≤ 0 and some of them ionic)- can be determined in a polarity-extended chromatographic setup [3,4]. This analytical solution combines the simultaneous separation of non-polar, polar and very polar molecules in one run.

In polarity-extended chromatographic separation techniques, the serial coupled setup of a reversed phase liquid chromatographic (RPLC) column and a hydrophilic interaction liquid chromatographic (HILIC) column is thereby one of the most prominent representatives. Although, this setup uses two chromatographic columns, it is no so-called 2-D LC approach, because compounds are only separated in one or the other columns and not in both.

A first example of such a serial coupling was published by Louw et al. for thirteen pharmaceutical compounds in 2008 [5]. And four years later, in 2012, it was part of a review describing various versions of coupled HILIC and RPLC columns [6]. Starting in 2013 our (research) group (at that time located at Technical University of Munich) developed, established and applied the serial coupling to the needs of analytical robustness and leading to non-target screening solutions. Our group consequently published 34 manuscripts on that topic so far and in all a system was applied as shown in Figure 1.

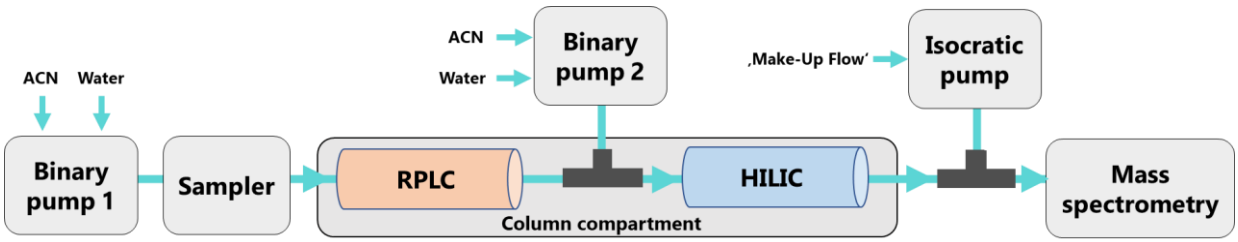

Figure 1: Scheme of the serial column coupling hyphenated with mass spectrometry. The details of the experimental setup can be found in each publication of the reference list and for generic versions in the Appendix of this 'White Paper'.

The Story of 'Applying RPLC-HILIC'

The initial method and introducing the serial coupling for complex mixtures was developed by Greco et. al for the analysis of red wine [7] and was initially investigating all the performance properties of the system and the application [8,9].

Next impressive examples using this chromatographic strategy were presented with process monitoring studies (simulating advanced wastewater treatment). Therein, the compounds (of emerging concern) diclofenac [10,11], sulfamethoxazole [12], and bisphenol A [13] were treated by electrochemical oxidation. One prominent compound, the 'EU watch list located' pharmaceutical diclofenac, was intensively be studied [10,11] and results of characterized oxidation products are shown in Figure 2. In long-exposure studies, the drug could partially be oxidized to very polar transformation products (like oxalic acid, fumaric acid (see Figure 2 top), and to inorganic by-products as well (see Figure 2 bottom)).

Besides the oxidation process monitoring with the polarity-extended strategy, various other process studies could analytically be accompanied so far. These included reduction processes, biochemical processes, chemical synthesis and others. Most of these studies were conducted in recent years at AFIN-TS under confidentiality and so have not been published. However, in all cases, the extended separation capabilities of this system were of great benefit and contributed strongly to the success of the mentioned characterization.

Furthermore, the serial coupling was successfully applied in a round-robin test on house dust [14] as well as the investigation of environmentally relevant waters in smaller [15] and in larger [16] international consortia. Therein the coupling was the exclusive and ultimate tool to monitor very polar substances, completing the observation of volatiles (with GC) and of non-polar/polar compounds (with RPLC).

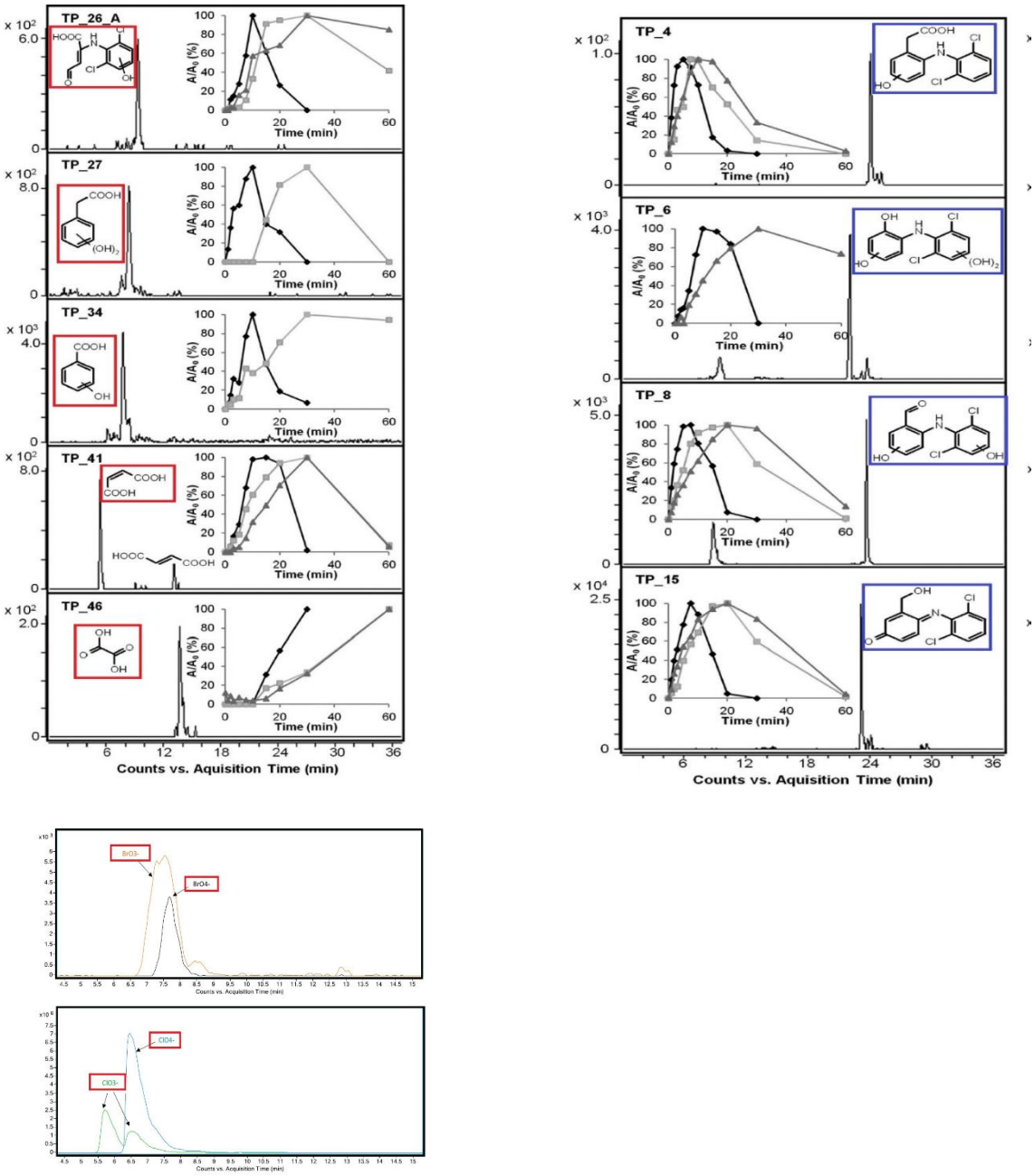

Figure 2: Extracted ion chromatograms from a transformation study of the pharmaceutical drug diclofenac oxidized with a bor-doped diamond electrode. Red marked molecules are HILIC eluting compound (i.e., before 15 minutes) and blue marked molecules are RPLC eluting compounds (i.e., later than 15 minutes). Upper part (i.e., the organic molecules) is adopted and reprinted from [10] with permission from John Wiley and Sons and lower part is from the open access supplementary section of [11].

In the years 2015-2018 we published several discussion papers presenting the advantages and bottlenecks of polarity-extended chromatography at that time (mainly used in analyzing the aqueous environment) [17-19]. Thereby the comparison with another polarity-extended setup, namely the supercritical fluid chromatography was performed several times [3, 18-20] and the serial coupling was also used in a quantitative study [21]. At this time the coupling was also contributed to a book about non-target and suspects screening in the aqueous environment [22,23]. Since that time the public interest increased regarding this strategy, documented in digitally [24] and written [25] interviews. Consequently, also an application tip to realize the coupling in own HILIC tip series [26] was published. By the way, since the beginning, our group always used zwitterionic types of HILIC columns for the serial column coupling and the generic gradients will be presented and updated for several zwitterionic phases tested so far in our lab (in the Appendix of this document).

RPLC-HILIC and NTS – a perfect match

Even the first article about red wine in 2013 [7] showed an application of RPLC-HILIC in non-target screening. Figure 3 reprints the original feature plot of the studied red wine containing a description of identification options. The 'feature' remarked there in the figure capture reflected in that days the retention time, the accurate mass and the signal intensity of a detected compound and the word 'feature' reflects it until today.

Non-target screening aims to provide a comprehensive picture of a sample by detecting all ionizable compounds. There are several limitations, which narrow the view in NTS. Among those is the challenge, that only those compounds can be detected in a sufficient way, which are well separated from matrix. As a consequence, the choice of the separation technique (RPLC or HILIC) determines the detectable compounds. With the combination of RPLC and HILIC the accessible space in NTS became significantly broader. So RPLC-HILIC and NTS match perfectly together.

As a result, starting with our first publications we also developed simultaneously data-analysis tools for the so-called suspects and the non-target screening data analysis.

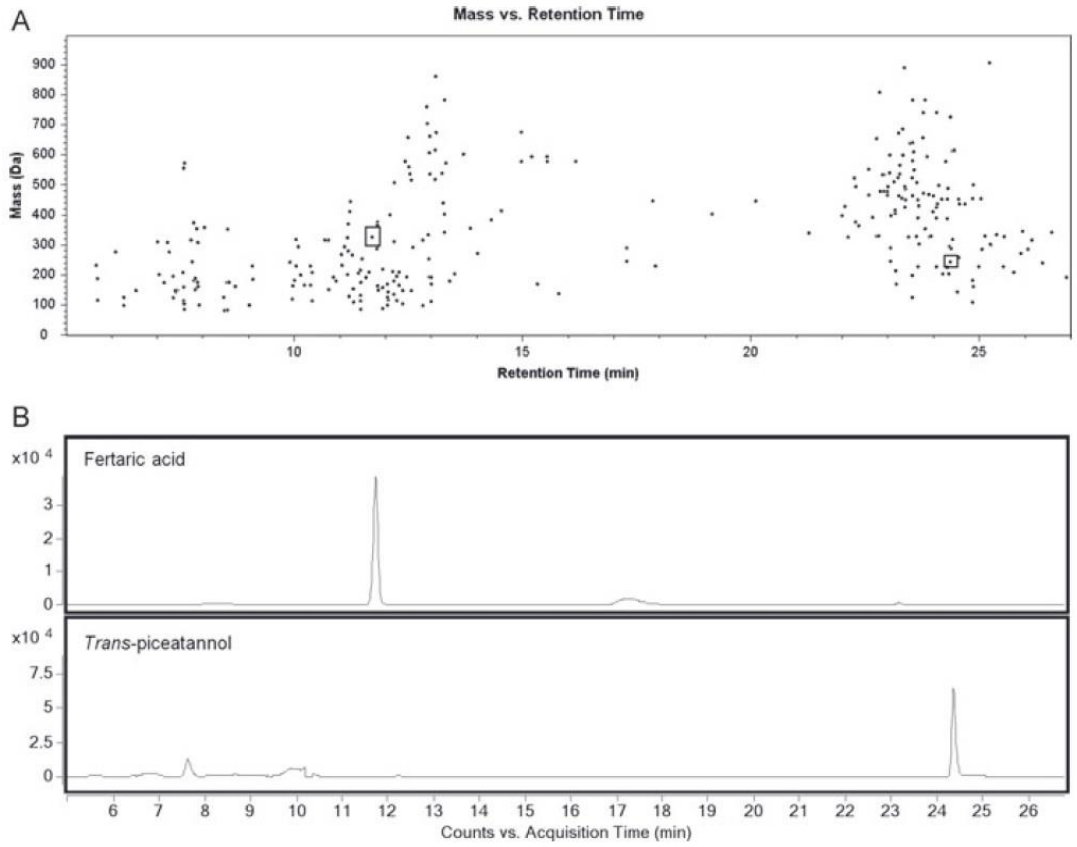

Figure 3: "(A) Mass profile of red wine. The plot displays the masses of the features detected in three out of three RPLC/ZIC-HILIC/ESI (–)-TOF-MS replicates in function of their retention times. Features corresponding to fertaric acid and trans-piceatannol are marked with squares. (B) Extracted ion chromatograms (EICs) for the masses of fertaric acid and trans-piceatannol in red wine." Original picture and original figure capture text reprinted from [7] with permission by John Wiley and Sons.

Thus, in most of the 26 publications until 2018 these strategies and data analyzing concepts were addressed and further developed. Since 2018, these data handling strategies are applied in our research in various fields, such as the aqueous environment, water analyses and in (plant) metabolomics studies. The extended perspective, which is provided by polarity-extended chromatography contributed to a better assessment of samples and applications. Besides that, the basic characteristics of the serial coupling of RPLC and HILIC can be used to elaborate advanced data evaluation workflows.

The studies of Minkus et al., introduced new analyzing processes and filters [27-30], which help to streamline data evaluation and applied that for various surface waters like the river Isar [28] and the

river Danube [29,30]. These processes can be combined in a comprehensive workflow for the determination of candidate compounds and contribute strongly to the identification of compounds. Thereby a focus was especially on characterizing and identifying suspects and unknowns in the (very) polar fraction of polarity (see especially the blue section in Figure 4). In several studies [27-30] the analytical performance, the data pre-processing and the data handling played an equal role in non-target screening approaches. This important equal status was also the core of a recent spotlight article in 2022 [31].

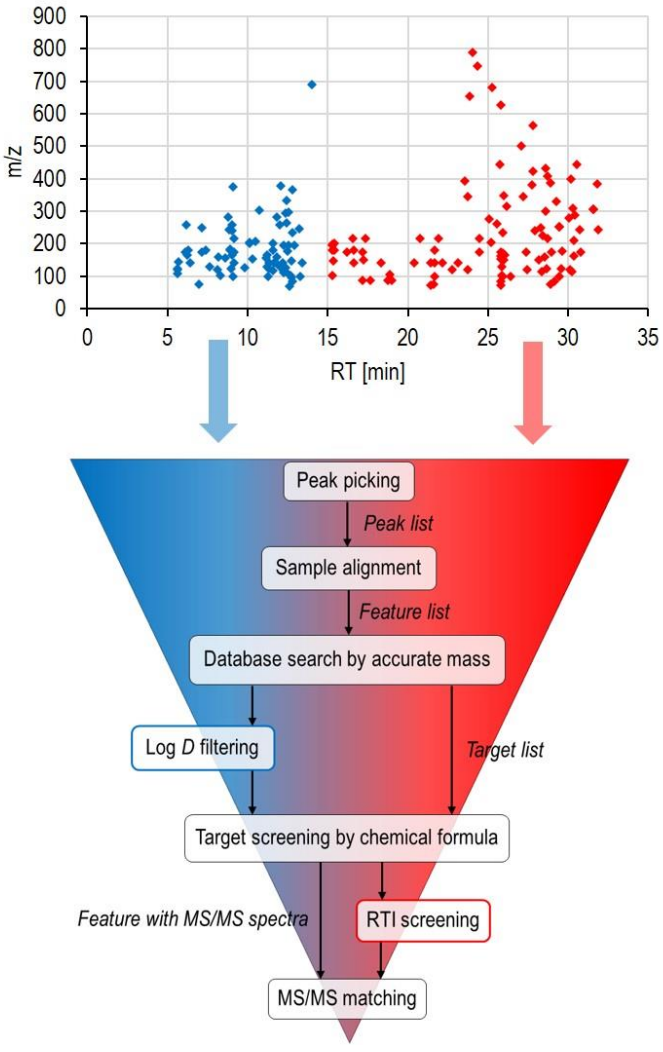

Figure 4: Data processing workflow from non-targeted raw data to a list of polar as well as nonpolar candidates present in the Danube samples. The figure is reprinted from the open access chapter [30].

Another current status is that the perfect match of the RPLC-HILIC-MS(/MS) analysis, the subsequent data pre-processing and the data handling can easily be transferred into statistical tools. The studies of Wahman et al., introduced new data handling and statistical processes [32-36], which help to understand metabolite changes in plants caused by various reasons (like sample storage [33], or incubation with (hazardous) substances [34, 36]) and using different data producing mass spectrometers [35].

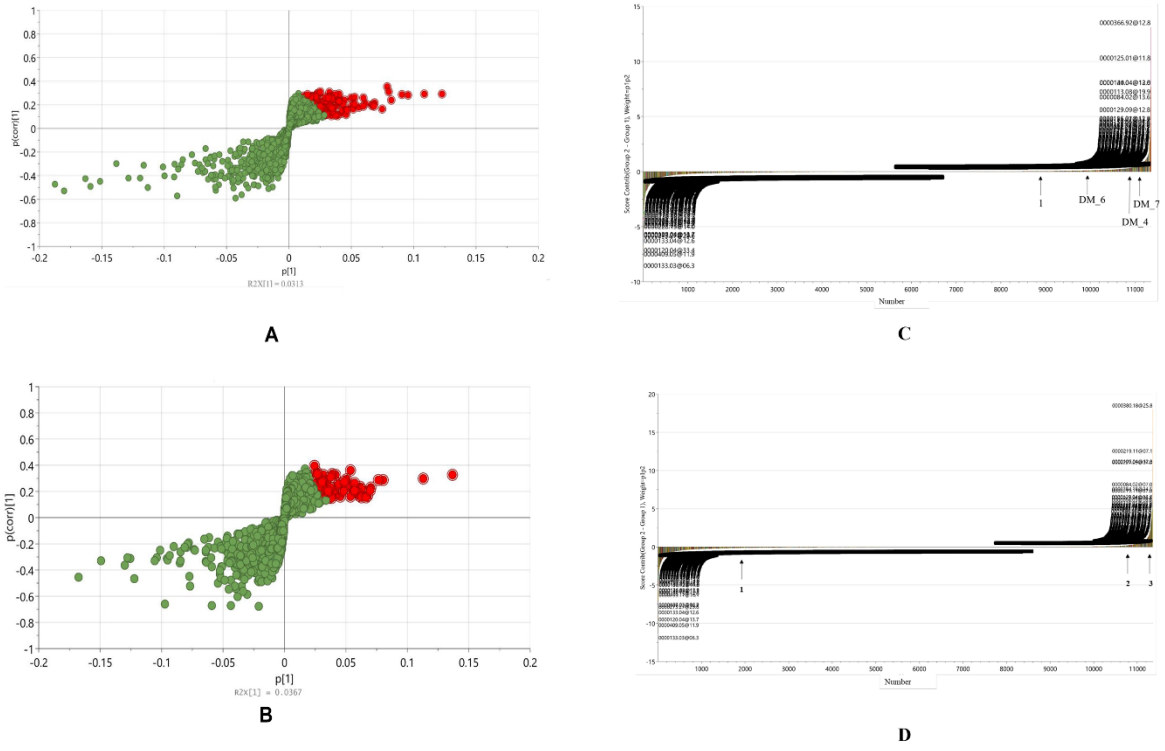

Figure 5: S-plots (A and B) and contribution plots (C and D) for statistical prioritization and performance of sample dependencies. The figure is reprinted from the open access manuscript [34].

The rich in content robust data sets can consequently be transferred into statistical tests like Analysis of Variance (ANOVA), Chi-Square Test, Student's T Test, Linear Regression, Pearson's Correlation Coefficient, Mann-Whitney U Test, Kruskal-Wallis Test, Shannon's Diversity Index, Tukey's Test, Cluster Analysis, Spearman's Rank Correlation Test and Principal Component Analysis and others. In Figure 5 such data was put into S-plots and contribution plots for statistical prioritization and performance of

sample dependencies [33] leading to completely new insights in sample properties and analytical questions. In Figure 6 an example for successful statistical usage is shown even if different mass spectrometer were applied showing the trend of interlaboratory NTS is future-proofed.

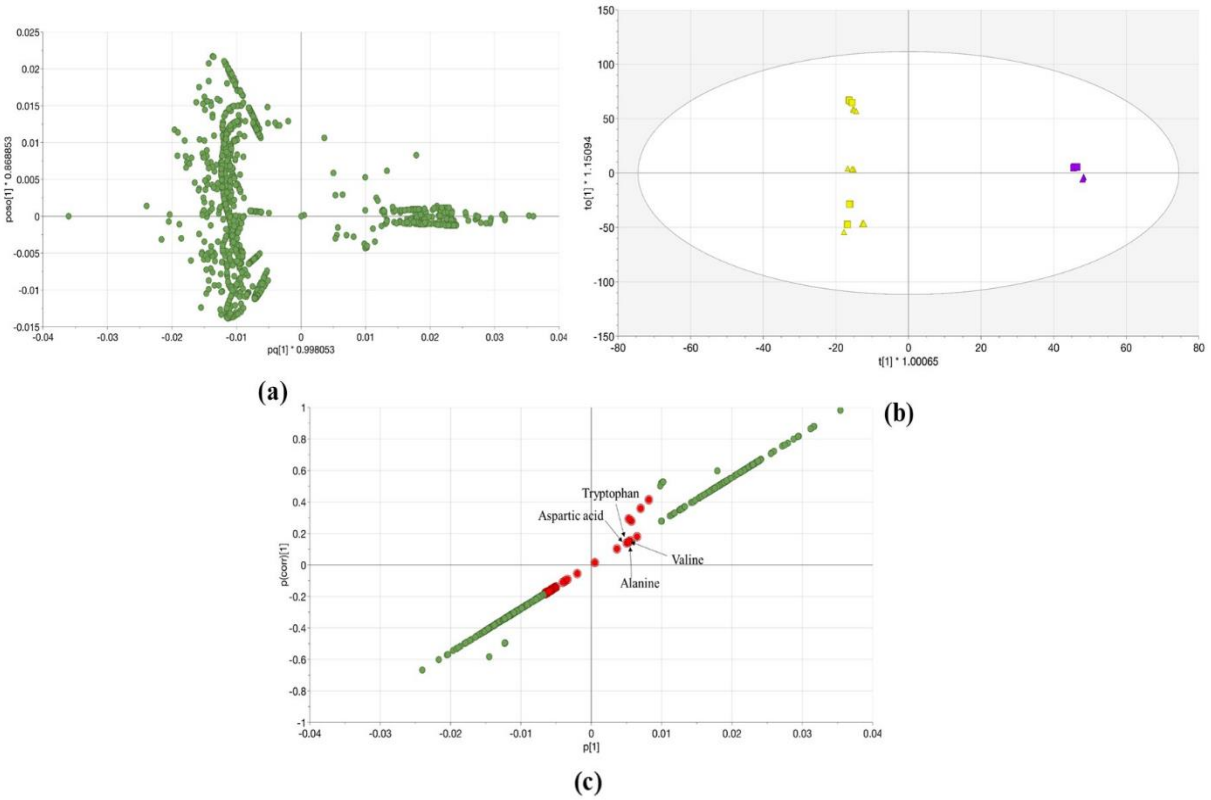

Figure 6: (a) Loading plot of different Lemna minor samples with the same RPLC-HILIC coupling but in two different mass analyzers (b) resulting OPLS-DA score scatter plot, and (c) the resulting S-plot of Lemna minor samples with red marked molecules that represent the common ones between the two mass spectrometer The figure is reprinted from the open access manuscript [35].

In current projects and solutions this (statistical relevant) data can and will be used for deep learning tools and neuronal networks (for modelling and predictions) answering todays and future questions in NTS.

The future and 'Applying RPLC-HILIC in the next decade'

One decade after introducing the RPLC-HILIC-MS(/MS) into the analytical community (and in focus on non-target screening) the technique has come of age from pure discovering applications to a more broaden use in research, commercial and public laboratories. In recent years, several laboratories have installed the serial coupling of RPLC and HILIC. Some of them were supported by AFIN-TS in realizing the general -but adapted with their needs - analytical concept for individual success in receiving new insights in complex samples. The next step is a further distribution of polarity-extended chromatography in NTS on a global stage. The time has come, the systems are ready.

Acknowledgements

We acknowledge all our colleagues and co-authors (see in the author list of the 36 references below), who worked really hard, enthusiastically and innovative to bring the serial coupling in hyphenation with mass spectrometry, the consequent data preprocessing concepts and data handling strategies to that level, so it is a really perfect match for non-target screening. We -at AFIN-TS- really appreciate this.

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APPENDIX: Generic Serial Couplings using endcapped C18 RPLC and zwitterionic HILIC coming soon

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AFIN-TS GmbH Am Mittleren Moos 48 D-86167 Augsburg Germany

www.afin-ts.de info@afin-ts.de