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On-line 2D-RPLC x RPLC – HRMS to assess wastewater treatment in a pharmaceutical plant

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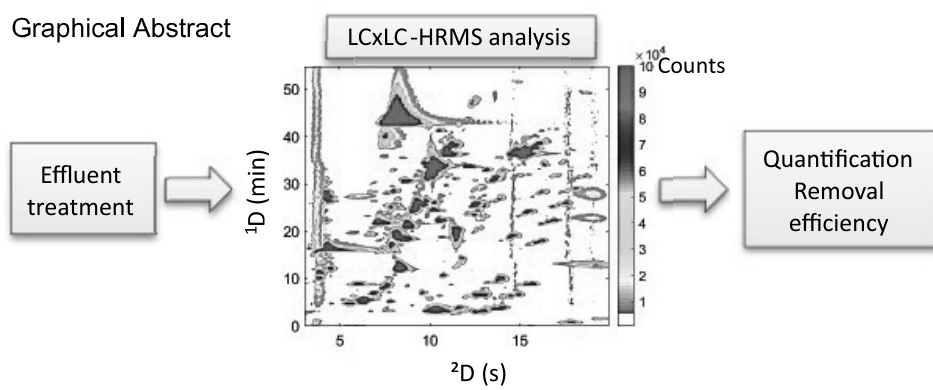
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Abstract

Pharmaceutical effluents are complex media containing hundreds of compounds including active ingredients, intermediate products and unknown impurities. Bringing an industrial wastewater treatment plant (WWTP) into compliance with European directives requires a thorough analysis of the effluent. In this study, we demonstrate how online comprehensive two-dimensional liquid chromatography (on-line LC × LC) hyphenated to high resolution mass spectrometry (HRMS) can be a powerful analytical methodology to monitoring the outlet water, by analysing the content of known molecules while characterising unknown compounds. Reversed phase liquid chromatography (RPLC) was used in both dimensions, with a penta-fluoro-phenyl silica-based column at neutral pH in the first dimension (¹D) and a C18 column at acidic pH in the second one (²D). The conditions were optimized for a total analysis time of 60 min. The variability of both retention times and peak areas was evaluated. The average standard deviation on retention times was found to be less than 0.1 s in ²D. The relative standard deviation on peak area was about 7 % for run-to-run analysis. This analytical approach, applied to the pharmaceutical effluents before (inlet) and after (outlet) wastewater treatment permitted to detect 240 compounds. These included 27 priority pharmaceutical products, 8 of which were of very high priority and their concentrations could be compared to target values. The comparison of 2D-LC and 1D-LC approaches clearly highlights the power of on-line RPLC x RPLC technique, which allows both targeted quantitative analysis and non-targeted qualitative analysis of pharmaceutical effluents.

Graphical Abstract



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Abstract

Pharmaceutical effluents are complex media containing hundreds of compounds including active ingredients, intermediate products and unknown impurities. Bringing an industrial wastewater treatment plant (WWTP) into compliance with European directives requires a thorough analysis of the effluent. In this study, we demonstrate how online comprehensive two-dimensional liquid chromatography (on-line LC × LC) hyphenated to high resolution mass spectrometry (HRMS) can be a powerful analytical methodology to monitoring the outlet water, by analysing the content of known molecules while characterising unknown compounds. Reversed phase liquid chromatography (RPLC) was used in both dimensions, with a penta-fluoro-phenyl silica-based column at neutral pH in the first dimension (¹D) and a C18 column at acidic pH in the second one (²D). The conditions were optimized for a total analysis time of 60 min. The variability of both retention times and peak areas was evaluated. The average standard deviation on retention times was found to be less than 0.1 s in ²D. The relative standard deviation on peak area was about 7 % for run-to-run analysis. This analytical approach, applied to the pharmaceutical effluents before (inlet) and after (outlet) wastewater treatment permitted to detect 240 compounds. These included 27 priority pharmaceutical products, 8 of which were of very high priority and their concentrations could be compared to target values. The comparison of 2D-LC and 1D-LC approaches clearly highlights the power of on-line RPLC x RPLC technique, which allows both targeted quantitative analysis and non-targeted qualitative analysis of pharmaceutical effluents.

Keywords:

Comprehensive 2D-LC, pharmaceuticals, industrial wastewater analysis, treatment plant efficiency.

1. Introduction

The pharmaceutical industry is a strictly regulated industrial sector. As a result of environmental awareness, much more attention is now being paid to wastewater management, which requires a specific internal policy to meet European directives [1,2]. The content of wastewater effluent from industrial plants depends on the manufactured products and may vary from batch to batch. Therefore, industrial operators promote on-site treatment plants to better adapt the treatment to the physico-chemical properties of their products. The process effluents usually consist of leftover chemical media and their rinsing water. They therefore contain hundreds of molecules including known active pharmaceutical ingredients (API), known intermediate products (IP), unknown impurities and degradation or transformation products. The degradation strategy used in wastewater treatment plant (WWTP) usually involves the application of multiple treatments to water, physical, chemical and/or biological in order to reduce the number of organics they contain [3]. The effluent can then be either reused in the industrial cycle or safely released [4]. De facto, the outlet water should contain far fewer, if any, API or IP. However, it may contain new unknowns, such as degradation products.

In order to comply with European recommendations [5] but also to maintain its technological lead, a pharmaceutical manufacturer needs to continuously improve every step of its on-site wastewater treatment infrastructure. To this end, analytical sciences must provide fast and reliable results, by monitoring existing infrastructures or evaluating new treatment processes. Focusing on a list of targets, usually consisting of API, IP and suspected impurities, the industrial objectives are then multiple:

- 1) In order to tune the treatment, it is crucial to unambiguously identify the molecules entering the treatment step.
- 2) The treatment step efficiency has to be evaluated in a timely manner, especially if upgrades are performed. For that purpose, removal efficiency of targeted products must be assessed.

3) In order to control the effluent and its fate, quantification of the outgoing molecules is essential. If regulated molecules are of concern, this can trigger an alert, while information on unregulated molecules can help industrial operators to prepare for future regulatory changes.

4) Finally, with a view to continuous improvement, it is interesting to obtain informative data on unknown molecules in outlet water, these molecules being either compounds that have passed directly through the treatment or those produced by the treatment itself (emerging substances).

The current analytical strategy for effluent monitoring is based on a set of specific LC-MS methods. The need to use multiple methods increases the time required to rule on the compliance of a given effluent and ultimately places the responsibility on the experience of the analyst.. LC-triple quadrupole with selected reaction monitoring (SRM) is currently the key technique for quantification analysis [6,7]. On the other hand, high resolution mass spectrometry (HRMS) with time of flight (ToF) analyzers can help to distinguish the numerous closely related compounds generated during pharmaceutical production. TOF analyzers operate in full scan mode, and thus detect a very large number of compounds, enabling retrospective interpretation of the data if new targets are suspected. Furthermore, HRMS can help the screening of unknown transformation products that may derive from the water treatment itself [8]. Beyond their primary identification purpose, recent QToF analyzers with improved sensitivity were found to be well suited for quantification [9].

Increasing both the selectivity and the peak capacity is an absolute requirement to avoid isobar co-elution and matrix effects in complex samples such as industrial wastewater. The potential of comprehensive multidimensional separation techniques hyphenated to HRMS was therefore explored for wastewater analysis. These techniques involved the combination of either two GC dimensions (GC x GC) [10,11], liquid chromatography with ion mobility (LC x IM) [12], or two LC dimensions (LC x LC), which was applied to the analysis of urban wastewater [13] or industrial wastewater [14]. GC x GC is limited by the need for derivatization in the case of non-volatile compounds such as most pharmaceuticals. IM is potentially attractive for the separation of isobars but its high correlation with mass spectrometry reduces its interest. Unlike the preceding combinations, the high potential of LC x LC for pharmaceuticals has often been reported [15–18]. Furthermore, LC x LC hyphenated to MS

equipped with electrospray ionization (LC x LC – ESI-MS) was found to be much more efficient than 1D-LC – ESI-MS to reduce ion suppression in wastewater analysis [19].

In light of the above, LC x LC - HRMS is expected to take up the challenge of a comprehensive characterization of wastewater from the pharmaceutical industry. In the present work, we propose a comprehensive analytical strategy for unequivocal detection and quantification of targeted pharmaceutical compounds in complex wastewater samples. This strategy is based on an optimized RPLC x RPLC – HRMS technique associated to a decision tree for data processing. To illustrate our overall strategy, a list of 27 targeted compounds under continuous monitoring, subsequently referred to as "priority products", has been established. Of these, 8 were of very high priority because of their proven toxicity above known concentrations. An in-depth analysis of these compounds was carried out in the inlet and outlet effluents of the pharmaceutical WWTP. The comparison of RPLC x RPLC-HRMS and RPLC-HRMS analyses shows that the two-dimensional method clearly outperforms the one-dimensional method for the characterization of pharmaceutical effluents. Initial attempts to identify unknowns are also discussed.

2. Material and methods

2.1. Chemical and sample preparation

Acetonitrile (ACN), and methanol (MeOH) were LC-MS grade from Sigma-Aldrich (Steinheim, Germany). Water was obtained from an Elga Purelab Classic UV purification system (Veolia water STI, Le Plessis Robinson, France). Formic acid and ammonium acetate were obtained from Sigma-Aldrich (Steinheim, Germany). All eluents were prepared in dedicated glassware to avoid contamination.

Process effluent samples as well as the 27 priority standard products (12 intermediate products and 15 active pharmaceutical ingredients) were provided by ORIL Industrie (Servier laboratories, Bolbec, France). However, for confidentiality reasons, their molecular structures are not shown. Molecular weights were between 100 and 800 Da.

Sample A contained 0.5 mg/L of each 27 priority standard products in water-MeOH (90:10, v/v). Samples B and C contained the 8 high priority products in water-MeOH (90:10, v/v) with

various concentrations ranging from 1 to 1000 µg/L. Samples A, B and C were used for a 3-point calibration.

Real samples were obtained from the inlet (sample I) and the outlet (sample O) effluents of a wastewater process treatment. The treatment was biological. Filtered aliquots were stored at -25 °C. After thawing and homogenization, reference molecule P24 was added at 0.1 mg/L and the samples were filtered on 0.2 µm filter.

2.2. Instruments

The LCxLC-HRMS system consists in an Infinity 1290 2D-LC system hyphenated to an Agilent 6560 Ion-Mobility Quadrupole Time-of-Flight LC/MS equipped with a JetStream electrospray ionization (ESI) source, both from Agilent Technologies (Waldbronn, Germany). The 2D-LC instrument includes two high-pressure binary solvent delivery pumps, an autosampler with a flow-through needle of 20 µL and an extension loop of 80 µL, two column ovens with a maximum temperature of 100 °C. A 2-position/4-port duo-valve was used as interface between the two dimensions, and was equipped with two 20 µL sampling loops. The dwell volumes were 140 µL and 80 µL for the first and the second dimensions, respectively, while the measured extra-column volumes were 22 µL and 8.5 µL for the first and the second dimensions. The extra-column variance estimations were 12 µL² and 4.5 µL², respectively. Flow splitting was applied, after the ¹D column to reduce the injection volume in ²D and after the ²D column to reduce the flow-rate entering MS.

1D-LC experiments were carried out on the first dimension of the Infinity 1290 2D-LC system, with a diode array detector (1290 DAD FS), equipped with a 0.6 µL flow-cell, set at 254 nm with an acquisition rate of 40 Hz. Experiments in 1D-LC were performed without flow-splitting.

Instrument controlling and data acquisition were performed using OpenLab software for LCxLC and MassHunter software for MS, both from Agilent Technologies (Waldbronn, Germany). 2D-data were exported to house-made Matlab program (R2019a version) designed to construct 2D-contour plots.

2.3. Chromatographic and MS conditions

Two columns were used in LC x LC, both from Waters Corporation (Milford, MA, USA): Acquity CSH PFP (50 x 2.1 mm, 1.7 µm) in ¹D and an Acquity CSH 18 (30 x 2.1 mm, 1.7 µm) in ²D. The final optimised LC x LC conditions are listed in Table 1. LC-UV experiments for injection

optimization were carried out in the same conditions as in ¹D, except that the flow-rate was increased to 0.5 mL/min, while maintaining the same normalized gradient slope (i.e. 1 %). 1D-LC-QToF experiments were performed with an Acquity CSH-C18 (150 x 2.1 mm, 1.7μm) column at 0.7 mL/min (maximum pressure). All other conditions were the same as in ²D (normalized gradient slope maintained at 0.7 %).

The ESI source settings were as follows: drying gas temperature, 300 °C; drying gas flow rate, 11 L/min; nebulizer pressure, 40 psig; sheath gas temperature, 350 °C; sheath gas flow rate, 11 L/min; fragmentor 185 V; capillary voltage, 3500 V; positive mode. Mass spectra data were collected in full scan mode with a mass range of 100-1700 m/z (with 18920 FWHM resolution at $m/z = 322$) and an acquisition rate of 14 spectra/s. No collision energy was employed during the MS data collection. Accurate mass of the precursor ion $[M+H]^+$ was recorded with a mass window of ± 20 ppm in LC x LC and of ± 40 ppm in 1D-LC.

2.4. Calculations

The gradient conditions were optimized according to the Linear Solvent Strength theory [20]. The following equations were used.

For a given dimension, the normalized gradient slope s was defined as:

$$s = \frac{C_f - C_i}{t_g} \times t_0 \quad (1)$$

C_i and C_f are the initial and final eluent compositions, t_g the gradient time and t_0 the column dead time.

The composition at elution, C_e , was given by:

$$C_e = C_i + \frac{C_f - C_i}{t_g} \times (t_R - t_0 - t_D) \quad (2)$$

With t_R the compound retention time and t_D the instrument dwell time.

The peak capacity, n_C was given by:

$$n_C = 1 + \frac{t_n - t_1}{1.7 \times w_{0.5}} \quad (3)$$

With t_n and t_1 the retention times of the most and least retained compound, and $w_{0.5}$ the peak width measured at half-peak height.

A 2D-chromatogram is constituted of a succession of ²D fraction separations. The ¹D retention time, ¹t_R of a given solute was assigned to the peak apex of its most intense peak while the ²D absolute retention time, ²t_R was calculated from both ¹t_R and the sampling time, t_s via the modulo operation:

$${}^2t_R = \text{mod}({}^1t_R, {}^1t_s) \quad (4)$$

Due to a slight time difference between the first valve switch and the start of MS acquisition, a relative retention time was considered in ²D (²t'_R). It was calculated from the retention time of a non-retained reference product (²t_{reference}), added in known concentration to all analyzed samples:

$${}^2t'_R = {}^2t_R - {}^2t_{\text{reference}} \quad (5)$$

The compound P24 was chosen as the reference product.

3. Results and discussion

3.1. Selection of chromatographic conditions

A large set of comprehensive LC x LC conditions was recently screened with a large variety of pharmaceuticals. 190 different LC x LC combinations were compared considering their peak capacity and their degree of orthogonality [17]. It was concluded that the best results should be obtained with highly efficient reversed phase columns in both dimensions operating at neutral pH in ¹D and acidic pH in ²D with different organic modifiers in the two dimensions. The combination of a PFP and a C18 stationary phases was found to be the most relevant for those compounds. An earlier study also reported that combining a C18 and a PFP stationary phase could be attractive for pharmaceuticals in sewage water [13]. As a result, RPLC x RPLC conditions with ammonium acetate 5 mM in ¹D and formic acid 0.1 % in ²D were considered in the present study. An Acquity CSH PFP column was used at 30 °C in ¹D with methanol as organic modifier. In order to benefit from fast efficient separations in ²D, a short Acquity CSH C18 column was used at 80 °C with acetonitrile as organic modifier. Gradient conditions were optimized in both dimensions so that the analysis time did not exceed one hour. That was achieved according to an optimization strategy, based on a pareto-optimality approach which was developed in our laboratory. This one aims at maximizing the peak capacity while

minimizing the dilution factor [21,22] for a given analysis time (here 60 min). The optimized parameters were the flow-rates, the gradient time in ²D, the column dimensions, the sampling rate and the split ratio between the first dimension and the valve. Resulting optimized conditions are listed in Table 1.

Some compounds were expected to be present at very low concentrations in both the inlet and outlet water. It was therefore important to set the highest possible injection volume in ¹D to increase the sensitivity (decrease the dilution factor) without compromising the separation quality due to excessive band broadening. The effluent samples being in pure water, on-column focusing was expected to be important and in favor of large injected volumes in ¹D. Increasing volumes (from 20 to 100 µL) of the inlet sample (sample I) were injected in the ¹D conditions (UV detection at 254 nm) in order to assess the maximum volume that could be injected. For each injected volume, the peak capacity (Eq. 3) was calculated for three isolated peaks well distributed in the first third of the separation. Fig. 1a shows the chromatograms obtained by injecting 20 µL (red) and 80 µL (black), which represents 19 % and 77 % of the column dead volume, respectively. The three considered peaks are indicated by arrows. As can be observed the peak intensity increases for the three peaks. As expected, it is multiplied by a factor of about four for the two most retained peaks. However, it is multiplied by a factor of three only for the least retained one. The variation of the calculated peak capacity with the injected volume is shown in Fig. 1b for the three peaks. While the least retained peak #1 ($C_e = 8\%$, Eq.2) clearly exhibits continuous decrease in peak capacity (down to less than 60% of the initial value with 100 µL injected), more retained peaks #2 ($C_e = 19\%$, Eq.2) and #3 ($C_e = 39\%$, Eq.2) globally maintain their initial peak capacity with up to 80 µL injected (loss of 13 % and 8 % with 80 µL injected). With the objective of losing less than 30% of the peak capacity, an injected volume of 80 µL was considered as a good trade-off between peak intensity and peak capacity.

Online RPLC x RPLC - HRMS separation was carried out using a synthetic mixture containing all targeted analytes that could be released in the effluents, considered as priority products and denoted here Sample A. The full spectrum of each analyte made it possible to select the most abundant ion for identification and quantification process. That corresponded to the molecular ion for 25 out of 27 priority products (see Table 2). Two compounds (P11 and P15) were suspected to be fragmented in the ionization source, leading to the use of the mass-to-

charge ratio (m/z) of their major fragment. The extracted ion chromatogram (EIC) of each of the 27 priority products (listed by increasing monoisotopic mass in Table 2) was processed with a maximum mass deviation of 20 ppm. The sum of EICs is represented by a 2D contour plot as shown in Fig. 2, where the Y-axis represents the first dimension separation and the X-axis the second one. The color-scale (Z-axis) represents the peak intensity as MS counts. It has to be noted that very intense peaks may appear larger depending on the chosen Z-scale focus. Retention data are expected to be diagonalized in RPLC x RPLC [17]. In Fig. 2, a fairly large repartition of the peaks across the separation space and around a diagonal can be observed. A retention surface coverage of 0.71 was evaluated using a method previously described [23], which is based on the ratio of the confidence area in which solutes are evenly distributed throughout the separation area. This value of 0.71 is of the same order of magnitude as that previously determined with a similar 2D-configuration applied to drug samples [17], thus confirming its relevance for the present study. It is interesting to note in Fig. 2 that many compounds that were not separated in 1D were separated in 2D and vice versa.

3.2. Creation of a reference table in RPLC x RPLC - HRMS for the priority products

The signal-to-noise ratio (S/N) must be above 3 to detect a compound. In LC x LC, the most intense peak among those obtained from the 2D -separations of successive fractions, was considered for this purpose. The noise level was evaluated at 200 counts (white zones in Fig. 2). As can be observed in Fig. 2, the number of relevant spots exceeds the number of priority products, suggesting the presence of isobars (circled in black in Fig. 2). They may result either from another product present in the sample or from the product itself. Out of the 27 EICs, 22 resulted in only one spot, so that 22 priority products could be unambiguously identified and related to three features, their two retention times (in first and second dimensions) and their m/z value. The standards of the other five priority products (P9, P14, P15, P21 and P24) for which the EIC of sample A presented multiple spots were individually injected to definitely ascertain their retention times (in both 1D and 2D). Their identification was also confirmed by in-house spectrum database of pharmaceuticals and finally, the code name of each target compound could be duly informed in Fig. 2.

During the course of method development, it appeared a slight time difference between the first valve switch (start of the 2D analysis) and the start of MS acquisition, which could lead to retention time errors in 2D . This time lag was in the range 1.8 s to 2.2 s and had, of course, no impact on the determination of the retention time in 1D . However, since the peaks widths in 2D were of about 1 s only, an error of 2 s could lead to false identification. Consequently, absolute retention times were considered in 1D (1t_R , peak apex of the most intense fraction) while relative ones in 2D ($^2t'_R$ determined by Eq. 5). The compound P24, retained in 1D but not retained in 2D , was chosen as the reference compound. It was added in known concentration (0.1 mg/L) to all samples to ensure its presence.

Table 2 lists the codes for the priority products, the retention times in both dimensions (1t_R , $^2t'_R$) obtained from the analysis shown in Fig. 2 and the mass data. For confidentiality reasons, the m/z values cannot be given and only the considered extracted ions are reported. Such a table was used to unambiguously identify the 27 priority products in any real samples analyzed in a sequence.

3.3. Establishing a mass window and a time window for the identification of priority products

As indicated above, the identification of priority products in a given sample is entirely based on three data (m/z, 1t_R , $^2t'_R$), as listed in Table 2. The reliability of these measurements is hence of prime importance to ensure proper identification of the compounds. A variability study was therefore carried out, to find out the repeatability of these measurements (run-to-run) as well as their intermediate precision (day-to-day).

The variability of mass accuracy (expressed in ppm) was evaluated from the analysis of sample A. The accurate masses of the 27 priority products were calculated from their chemical formulas considering the isotopic mass distribution. For the 27 products, analyzed by the proposed RPLC x RPLC – HRMS method, the mean mass error value was found to be 2.0 ppm which is quite satisfactory according to both the instrument specifications (i.e. 1 ppm) and the literature [24]. The maximum deviation was observed for the less retained compounds, P1 and P2, with a value of 4.7 and 8.6 ppm, respectively. However, we had to be aware of the possible existence of matrix effects, interfering components or highly concentrated products, all of which could shift the measured mass and lead to false negatives [25]. Mass extraction windows of 10 and 20 ppm were therefore assessed. It was found, for some products such as

P19, which have a very good MS response and therefore a risk of detector saturation, that the 10 ppm window could be insufficient at high concentrations. In order to analyze highly concentrated inlet samples, a mass extraction window of 20 ppm was definitely chosen.

Both retention time repeatability (3 consecutive injections of sample A) and retention time intermediate precision (3 inter-day injections of sample A) were assessed in both dimensions. The resulting retention time standard deviations are shown in Fig. S1 for the 27 priority products. For a given solute in ¹D, the considered retention time was that of the most intense peak among those obtained from the two or three analyzed fractions. This peak could easily be shifted by one sampling time (i.e. 0.4 min) between two analyses. Despite this, the standard deviation of three consecutive measurements was always lower than 0.25 min (Fig. S1a) with an average value over all compounds of 0.15 min. In light of these results and taking into account the possible difference in time that could be up to one sampling time, a detection window of ± 0.40 min was defined for run-to-run analyses in ¹D. As can be observed in Fig. S1a, the retention times usually varied with a greater extent from day-to-day analyses, with much larger standard deviations for five products. At a closer look, the related peaks presented a large tailing, making probably the time at the peak apex more subject to variations.

In ²D (Fig. S1b), the retention time standard deviations were similar for run-to-run and for day-to-day analyses with an average standard deviation of 0.07 s and 0.05 s respectively. For the large majority of compounds, the standard deviations were lower than 0.05 s, which is remarkable considering that the average peak width at half-peak height was ten times higher (i.e. 0.5 s). This highlights the high degree of reliability of the second dimension separation. For run-to-run analyses, the retention time window was set at twice the average standard deviation (i.e. ± 0.15 s). The relative standard deviations (RSD) were informed in Table S1.

Due to the large variability in ¹D retention times for some products from day to day (Fig. S1a), it was decided to systematically include sample A in the analysis sequence in order to have a more reliable comparison between the retention times of suspected compounds in real samples with those of priority products unambiguously identified in sample A. In that respect, a reference table similar to Table 2 (with updated retention times) was constructed for each sequence. As said before, there was an identification ambiguity for 5 out of 27 compounds in sample A. While not separated from their isobars in ¹D, they were well separated in ²D, making

the corresponding retention times and time windows (listed in Table 2), the references for definite identification.

3.4. Quantitative analysis in RPLC x RPLC – HRMS

Once a priority product has been unambiguously identified in the effluent, it should be quantified to monitor its fate in the treatment plant.

3.4.1. Reliability study

LC x LC – HRMS is well designed for qualitative analysis as it often permits unambiguous identification of suspected compounds. However, it is quite rare to use QToF analyzers for quantitative analysis. Two main issues have indeed to be addressed in LC x LC - HRMS: (i) how to determine the peak volume in LC x LC and (ii) the variability of MS-response.

In LC x LC, each peak in ¹D is displayed in several peaks in ²D. Summing the consecutive peak areas was found to be the most reliable method in LC x LC – UV for quantitative analysis [15,26,27]. This method was therefore used in the present study. The same approach as for retention times was followed for the sum of peak areas in order to assess the variability of the measures and hence the reliability of quantitative analysis in RPLC x RPLC - HRMS. This was achieved with sample A from three non-consecutive runs in the same sequence (repeatability) and over three days (intermediate precision). With an acquisition rate of 14 Hz, each peak could be described by about 12 points. As illustrative example, the peaks obtained for three consecutive fractions of P13 are shown in Fig. S2. Increasing the acquisition rate up to 20 Hz improved the precision of peak area with 17 data points but decreased the number of accumulated mass spectra per point and hence the sensitivity.

In terms of repeatability, the average relative standard deviation (RSD) on the sum of peak areas was found to be 6.9 % with QToF detection while only 1.4 % with UV detection. Such difference between these two detection modes was ascribed to both variations in ionization efficiency between runs and/or insufficient description of MS-peaks as discussed above. In terms of intermediate precision, the obtained results were unsatisfactory with an average RSD value of 49 %, ranging from 17 % for P4 to an impressive 135 % for low-responding P11. This is not surprising since ionization conditions are expected to significantly vary from day to day, thereby requiring external calibration standards, which was not an easy task in view of the large number of compounds that needed to be quantified. In the light of these results,

quantitative analysis was performed by injecting the calibration solutions and the samples to be analyzed within the same sequence.

3.4.2. General strategy

Quantitative analysis of effluents was completed in about six hours with a RPLC x RPLC - HRMS analysis sequence that included in order: a blank, sample A (highest concentrations), sample I (inlet sample), sample B (intermediate concentrations), sample O (outlet sample) and sample C (lowest concentrations). Among the 27 priority products to look for in effluent samples, 8 were considered as high priorities for quantitative analysis. Four of them were already regulated (P6, P7, P9 and P16) while the other four were monitored to anticipate future regulatory changes (P4, P10, P19 and P20). In sample C, the concentrations of the high priority products were set at the maximum permitted value in the treatment plant outlet, except P10 and P19 for which the concentrations were set at a lower value. The maximum permitted concentrations (listed in Table 3) were either those defined by regulatory authorities or our own target concentrations designed to improve treatment efficiency.

Fig. 3 shows the decision flowchart to process the analytical data. It was designed firstly to detect the presence or absence of priority products in the effluents and then to quantify them if necessary. The first step (Fig. 3a) was intended to create the reference table (similar to Table 2) for the analysis sequence. ICH guidelines recommend signal-to-noise ratio values (S/N) above 3 for detection and above 10 for quantification [28]. Given the concentrations in sample A, all compounds were expected to be detected unless there was an instrumental problem. In this case, the compound could no longer be considered in the current analysis.

In the second step (Fig. 3b), the processing data continued with samples I, B, O and C. Each EIC was scrutinized. If S/N was higher than 3, a product was detected. If the retention data matched those of the reference table, the priority product identity was confirmed.

The third and fourth steps (Figs. 3c and 3d) were related to the characterization of the outlet sample, including the calculation of the removal efficiency for all detected priority products and the quantitative analysis of the eight high priority products when they were detected in sample O. The quantification was done with a 3-point calibration. It is important to underline the assumption that matrix effects did not hinder this process.

The removal efficiency, RE , for a given product, can be defined as the amount of product treated compared to the starting amount:

$$RE (\%) = \frac{m_{inlet} - m_{outlet}}{m_{inlet}} \times 100 \quad (6)$$

where m_{inlet} and m_{outlet} are the amounts of product in the inlet and outlet samples respectively.

RE was calculated either from the sum of peak areas if $S/N > 10$ for at least one of the two samples (inlet or outlet) or from the main peak intensity if $3 < S/N < 10$ for both samples. In this case, the noise was subtracted from the main peak intensity for a fairer calculation. In the case where the product was not detected in the outlet sample while detected with $S/N > 10$ in the inlet sample, the removal efficiency was considered to be 100 %.

In case of a high priority product, its concentration in the outlet sample was compared to that in sample C in order to check the compliance of the treatment plant. Quantitative analysis was possible provided that a calibration curve could be constructed from the sum of peak areas, that means if S/N values were above 10 for the three calibration samples (A, B and C).

3.4.3. Application to industrial process effluents

The general strategy described above was applied to process effluents from ORIL Industrie (Bolbec, France). They were collected before (sample I) and after (sample O) the WWTP. The samples were collected over two months. Monitoring the continuous process of an industrial WWTP is a difficult task, as the inlet effluent varies from one production cycle to another (usually over a period of a couple of months). The sampling process (inlet and outlet water) was performed on the same day while the residence time of the analyzed products in the WWTP was estimated to seven days. The collected outlet effluent was therefore not directly a consequence of the treatment of the collected inlet effluent, but of the inlet effluent entering the WWTP seven days before. Nevertheless, as most products are continuously produced, the two samples were expected to be representative of the issues the environmental engineers may encounter in monitoring the WWTP.

Figures 4a and 4b show the obtained 2D-contour plots (sum of 27 EICs) of the inlet and outlet samples respectively. It is obvious that the number of peaks detected in these 2D-plots far exceeds the number of priority products revealed in Fig. 2. This can be ascribed to the presence of numerous isobars in the effluents as exemplified at the bottom of the figure,

which displays the EICs (m/z of P15) for samples A, I and O. Two spots can be observed for sample A (Fig. 4c). One corresponds to P15 (identified via individual injection) while the other one (circled in black solid line) resulted from a fragmentation of P20 in the MS-source. These two spots are also observed with samples I (Fig. 4d) and O (Fig. 4e), revealing the presence of P15 and P20 in both effluents. The additional observed spots are likely to be due to reaction impurities and/or product degradation. According to their retention times, some are common to both inlet and outlet water (circled spots in the black dotted line) while others are specific to the outlet effluent. These 2D-contour plots clearly point out the separation power of the RPLC x RPLC - HRMS analysis, which effectively separated the isobars of P15 when they would not have been separated in either 1D-RPLC methods. Furthermore, the reference retention times in both dimensions were decisive for the unambiguous identification of P15 in both effluents without any bias due to the presence of isobars.

Based on the decision-making flowchart shown in Fig. 3 with a limit of detection set at 600 MS-counts, 21 out of 27 priority products were detected in the inlet sample (Fig. 4a). The number of products and their concentrations depends on the production scheme of the pharmaceutical plant and hence may greatly vary from one production to the next. For example, P19 was highly concentrated in sample I (Fig. 4a). In outlet sample (Fig. 4b), 20 out of 21 products were detected in much smaller amounts as reflected by the corresponding spot intensities. P11 and P22, detected in sample O, were not detected in sample I, possibly due to the production timeline or to the residence time of the products in the treatment plant of several days as discussed above.

In Fig. 5, the removal efficiency of the 20 priority products that were detected both in inlet and outlet effluents, is plotted against the ratio of the signal of the most intense peak (inlet or outlet sample) to the noise (S/N). The data dot is blue or orange depending on whether the inlet or outlet sample provides the most intense signal. The orange dots were obviously not expected and can in no way reflect removal efficiency. They can be explained by the fact, as mentioned above, that although both samples were collected at the same time, there was necessarily a time lag between a possible change in production and the end of the treatment. Intermediate precision on removal efficiency was assessed from three analysis sequence performed on three different days. Resulting relative standard deviations were found to be in the range 0.2 to 6 %, which underlines the good reliability of the method. For 12 out of 20

products, the treatment process reduced the amount present in the effluent by 80 %. For 3 products (P13, P21 and P24), the removal efficiency was found to be lower than 50 %.

Quantitative analysis of high priority products in sample O was intended to reassure or alert to their concentration level, and thus draw attention to a possible problem in the operation of the WWTP. Table 3 summarizes the obtained quantitative results. The determination coefficients (R^2) were all above 0.99 (with calibration lines forced to 0) despite the very low concentrations in sample C, thereby providing a fair estimation of the concentrations in the sample O, with an error estimated at about 7.0 %. For one product (P16), the concentration was well above the maximum concentration (56 vs 1 $\mu\text{g/L}$), showing a lack of efficiency of the WWTP for this product. In contrast, the concentrations of other high priority products were in line with expectations.

3.5. Advantages of RPLC x RPLC-HRMS analysis over RPLC-HRMS analysis

Compared to one-dimensional separations, on-line LC x LC separations are often perceived as being more difficult to implement, due to complex optimization and complex data processing. We therefore attempted to establish an objective comparison between these two techniques, both coupled with high-resolution mass spectrometry. A RPLC gradient method was developed with the same gradient time (60 min), injection volumes as in ^1D and stationary phase (Acquity CSH C18) as in ^2D . Finally, in order to obtain the same MS response, we used the same mobile phase (water-acetonitrile-formic acid) and the same column temperature (80 $^{\circ}\text{C}$) as in ^2D . A 15 cm column was selected so that the peak capacity per time unit was maximized in such RPLC-conditions [22,29]. Samples I and O are two examples of very complex media in which a tremendous number of molecules are present, including pharmaceutical products (reactants, active ingredients, intermediate products, impurities) but also products derived from the WWTP process (enzyme substrate, degradation products). The same analytical strategy as described above (Fig. 3) was applied in RPLC-HRMS to the inlet and outlet effluents.

Base peak chromatograms (BPC) of the outlet sample in RPLC x RPLC and RPLC are shown in Figs. 6a and 6b respectively. The main points of comparison for these two methods are given in Table 4. The peak capacities were calculated according to Eq. 3. In RPLC x RPLC, the product of peak capacities was corrected by taking into account both the under-sampling [30] and the occupation of the retention space as delimited by the red and black dotted lines (86 % space

occupation) [23]. The calculated peak capacity was found to be 900 in RPLC x RPLC while 430 in RPLC. The higher separation power in RPLC x RPLC allowed the detection of 240 peaks while only 130 could be detected in RPLC.

The great advantage of RPLC x RPLC - HRMS is highlighted in Fig.6 by the mass spectra of the two peaks shown on the chromatograms (labels 1 and 2). The first peak corresponds to P19 while the second peak is an unknown compound. For both peaks, the mass spectra are much more complex in RPLC, revealing the presence of various masses in addition to the main one. In addition, the signal of the most intense mass for peak 2 is about six times higher in RPLC x RPLC.

Peak identification was sometimes biased in RPLC for inlet and outlet samples as illustrated in Fig. S3a, showing overlaid EICs of P10 for samples A, C and I. As can be observed, both the time at the peak apex and the peak shape changed for this poorly retained compound depending on the sample. One explanation could be a change in the surface state of the stationary phase by the matrix when a large amount of sample I is injected (here 80 μ L). This hypothesis was confirmed by injecting smaller volumes of sample I (Fig. S4). In this case, the peaks became similar to those obtained by injecting samples A or sample C (both without matrix). In contrast, no retention shift was observed in RPLC x RPLC for sample I compared to samples A and C (Fig. S3b), as the upstream separation greatly simplifies the sample injected in the second dimension. Due to such a retention shift, identification of the corresponding peaks in RPLC was made impossible unless a sufficiently large retention window was used (i.e. as large as several peak widths in this case). This did not suit our purpose.

A quantitative analysis problem, resulting from the presence of numerous isobars in the effluents, was also specifically highlighted in RPLC-HRMS. In addition to a higher effective peak capacity, the second dimension provides another retention mechanism, which increases the chances of separating new isobars. Whereas all standard products could be unambiguously identified by their time windows in RPLC as in RPLC x RPLC, we often observed far fewer peaks in the EICs of inlet and outlet samples in RPLC than in RPLC x RPLC. As a result, in some cases, isobars were found to co-elute with the standard product in RPLC, thus distorting the quantitative results. An example is given in Figure S5 where many isobars of P1 were separated in 1 D and not in 2 D, suggesting that they were not separated in 1D-LC either, the conditions being the same as in 2 D.

Finally, by improving the separation of the priority products with the interfering matrix components, RPLC x RPLC is expected to reduce matrix effects in the ionization source compared to RPLC. In case of industrial wastewater coming from WWTP, it was reported that matrix effects were more important for the inlet sample than for the outlet one and also in the first part of the RPLC separation [31]. An in-depth comparison between RPLC and RPLC x RPLC in terms of matrix effects was beyond the scope of this study. However, we compared the mass error obtained for the priority products, which can increase with high concentrated compounds present in the matrix [25]. In RPLC, the mass error attained up to 20 ppm with an average value of 10 ppm (calculated for the detected priority products) while only 3.5 ppm in RPLC x RPLC. This probably highlights more important matrix effects in RPLC and forced us to consider a larger mass error in RPLC (i.e 40 ppm vs 20 ppm in RPLC x RPLC) for EICs. In addition to an increase in the mass error, we observed an increase in noise in RPLC (300 vs 200 counts), hence increasing the limits of both detection and quantification. This is probably the reason why (i) less products were detected in sample O (i.e. 10 in RPLC vs 12 in RPLC x RPLC) and (ii) their quantification was expected to be less accurate considering the R^2 value much lower than 0.99 while higher in RPLC x RPLC.

All the above results allowed us to confirm that RPLC x RPLC far outperforms RPLC when it comes to analysing such complex matrices as industrial effluents.

3.6. Characterization of unknown compounds in process effluents

The high potential of both RPLC x RPLC in term of separation power and HRMS in term of mass accuracy permitted to gain valuable information on the various molecules that are either transported through or produced during the WWTP process. While knowledge of the molecules sent to the WWTP helps to reduce sources of pollution, knowledge of the molecules released by the WWTP helps to monitor emerging contaminants, which can sometimes represent a higher risk to the environment or health than their parent products [8].

The 2D-contour plots (BPC) obtained from the separations of the inlet and outlet samples are shown in Figs. 7a and 7b respectively. In both separations, some spots seem to be highly correlated, suggesting the presence of homologous series. The different correlation lines are plotted on graphs that reconstruct the presence of these spots. Each line is labelled with a number and a letter. The spots located on the same line differ from one spot to another by the same mass value (44.026, 58.042 and 14.015 Da for lines 1, 2 and 3 respectively).

According to the literature, a mass of 44.026 Da might be related to ethoxylate [32], 58.042 Da to propylene oxide [32] and 14.015 Da to methylene. Surprisingly, similar differences in mass were observed from one line to another as highlighted by coloured arrows in Fig. 7, with colour depending on the mass value (14.015 Da in black, 0.036 Da in blue, 15.995 Da in purple). As said above, 14.015 is most probably related to a methylene group, 0.036 could correspond to a difference in mass between O and [CH₃ + H], 15.995 is the monoisotopic mass of an oxygen atom. Finally, a last correlation can be noticed between lines 1a and 1c (in red). These are compounds with the same m/z, probably isobars. Overall, these correlations and repetitive mass differences most certainly reflect the presence of polymeric structures that have yet to be identified. Further studies are being carried out in our laboratory on this subject. However, it is interesting to note that the compounds in question are mostly different in the two samples.

4. Conclusions

Effluent from wastewater treatment plants requires a specific internal policy to meet official guidelines. The performance of RPLC × RPLC - HRMS was explored with respect to its potential for the analysis of WWTP effluents. The analysis of the samples was carried out without extraction and/or preconcentration, which limits the bias for quantification. HRMS allowed to perform full-scan acquisition with detection limits in the ng/L range and high mass accuracy (mass errors lower than 5 ppm).

An excellent retention surface coverage was achieved for these samples with the PFP x C18 column combination, allowing, together with the MS data, the unambiguous identification of all suspected contaminants in the inlet and outlet samples of the treatment step.

In addition to the removal efficiency for the 27 priority products, the concentration in the outlet sample could be assessed for 8 high priority products and compared to target values.

The RPLC x RPLC method was compared to the RPLC method performed under optimized 1D-LC conditions and was proved to give much more reliable results. The peak capacity was more than doubled between RPLC and RPLC x RPLC. Matrix effects, identification and quantification problems were clearly highlighted in RPLC-HRMS unlike RPLC x RPLC.

The proposed RPLC x RPLC - HRMS method should therefore make it possible to qualify both known and unknown products of a wastewater treatment plant and to accurately quantify the known compounds. Work is underway to complete the identification of unknown compounds whose retentions were found to be fully correlated in both dimensions.

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Figure captions

Figure 1: Effect of the injection volume on the RPLC separation of an inlet sample of WWTP; (a) Overlaid chromatograms with injection volumes of 20 μ L (red) and 80 μ L (black). (b) Peak capacity loss as a function of injection volume for three different peaks (shown in the top figure). Peak width measured at half peak height. Flow rate: 0.5 mL/min; gradient time: 18.69 min. UV-detection at 254 nm. Other conditions given in Table 1 (¹D conditions).

Figure 2: On-line RPLC x RPLC separation of the 27 priority standard products (sum of EICs). Products are numbered close to their corresponding spots (P_n). Isobars are circled in black and numbered (ISO-P_n). Experimental conditions are given in Table 1.

Figure 3: Decision making flowchart for qualitative and quantitative analysis of a given suspected priority standard product in treatment plant samples: (a) Creation of the reference table (purple dotted frame); (b) Detection of priority products in samples B, C, I and O (green dotted frame); (c) Removal efficiency for priority products according to Eq.6 (red dotted frame) and (d) quantitative analysis for high priority products (blue dotted frame). “I”, “O” and “C” stand for samples I, O and C respectively. See text for explanations.

Figure 4: On-line RPLC x RPLC separations of (a) sample I (sum of 27 EICs), (b) sample O (sum of 27 EICs), (c) sample A (EIC P15), (d) sample I (EIC P15) and sample O (EIC P15). Product numbers are located close to the corresponding spots. The spot circled in solid black line corresponds to a fragment of P20. The spots circled in dotted black line are common to samples I and O. Experimental conditions are given in Table 1.

Figure 5: Removal efficiencies against the most intense S/N for the priority products detected in both inlet and outlet samples. RPLC x RPLC – HRMS analyses shown in Figure 5. Blue and orange data dots correspond to products that were more concentrated in the inlet and outlet samples respectively. The vertical dotted lines indicate the limits of detection (LoD) and of quantification (LoQ).

Figure 6: Analysis of sample O (Base Peak Chromatogram) in (a) on-line RPLC x RPLC – HRMS (2D and 3D-plots) and (b) RPLC-HRMS. The given mass spectra correspond to peaks 1 and 2 as indicated on the chromatograms. Black and red dotted lines delimit the available separation space and the occupied separation space respectively. Experimental conditions are given in the experimental section.

Figure 7: Unknown compound correlations in (a) the inlet sample and (b) the outlet sample after an on-line RPLC x RPLC – HRMS separation. The arrows highlight the differences in mass (values shown with the same colour on the figure) between two spots. Experimental conditions are given in the experimental section.

Tables

Table 1: Experimental conditions in RPLC x RPLC.

	First dimension (¹ D)	Second dimension (² D)
Injection volume	10 µL (samples A and B) and 80 µL (samples C, I and O)	15 µL
Column parameters	Acquity CSH PFP (50 x 2.1 mm, 1.7 µm)	Acquity CSH C18 (30 x 2.1 mm, 1.7 µm)
Temperature	30 °C	80 °C
Flow rate	0.15 mL/min	1.8 mL/min
Mobile phase A	¹ A: water-ammonium acetate (5 mM)	² A: water-formic acid (0.1 %)
Mobile phase B	¹ B: MeOH	² B: acetonitrile-formic acid (0.1 %)
Gradient conditions	1-90 % ¹ B in 61.41 min, 90-1 % ¹ B in 0.8 min, 1 % ¹ B from 62.2 to 66 min	1-86 % ² B in 0.24 min, 86-1 % ² B in 0.04 min, 1 % ² B from 0.28 to 0.4 min
Split ratio	1:3 interface:waste	1:2 MS:waste
Sampling time	0.4 min	

Table 2: Main characteristics, reference retention times and retention time windows for the 27 priority products for the RPLC x RPLC separation shown in Fig. 2. $^2t'_R$ is the relative retention time in 2D (calculated according to Eqs. 4 and 5). High priority products are indicated by an asterisk (*). These retention times are used as reference for the identification of the 27 products in any sample A. They are further updated for each analysis sequence. The conditions are given in Table 1.

Product number	Function (pKa)	Extracted ion	1t_R (min)	$^2t'_R$ (s)	1t_R window (min)	$^2t'_R$ window (s)	
					Run-to-run	Run-to-run	Day-to-day
P1	Basic	$[M_1+H]^+$	6.10	0.48	± 0.4	± 0.15	-
P2	Basic	$[M_2+H]^+$	8.50	0.24	± 0.4	± 0.15	-
P3	Acidic	$[M_3+H]^+$	3.76	4.02	± 0.4	± 0.15	-
P4*	Basic	$[M_4+H]^+$	14.57	4.74	± 0.4	± 0.15	-
P5	Basic	$[M_5+H]^+$	31.79	5.82	± 0.4	± 0.15	-
P6*	Neutral	$[M_6+H]^+$	15.05	9.60	± 0.4	± 0.15	-
P7*	Acidic/Basic	$[M_7+H]^+$	4.59	5.52	± 0.4	± 0.15	-
P8	Basic	$[M_8+H]^+$	35.87	10.50	± 0.4	± 0.15	-
P9*	Basic (9.0)	$[M_9+H]^+$	22.55	3.48	± 0.4	± 0.15	± 0.15
P10*	Basic (4.5/9.2)	$[M_{10}+H]^+$	22.15	3.60	± 0.4	± 0.15	-
P11	Basic	$[M_{11}-C_4H_{10}O_3P]^+$	30.59	6.06	± 0.4	± 0.15	-
P12	Basic	$[M_{12}+H]^+$	35.79	5.64	± 0.4	± 0.15	-
P13	Acidic/Basic (14.1/9.8)	$[M_{13}+H]^+$	37.81	6.78	± 0.4	± 0.15	-
P14	Basic (7.0)	$[M_{14}+H]^+$	39.78	4.98	± 0.4	± 0.15	± 0.15
P15	Basic	$[M_{15}-NH_2]^+$	31.79	6.06	± 0.4	± 0.15	± 0.15
P16*	Neutral	$[M_{16}+H]^+$	33.46	9.78	± 0.4	± 0.15	-
P17	Acidic/Basic (4.1/1.4)	$[M_{17}+H]^+$	19.09	11.82	± 0.4	± 0.15	-
P18	Acidic (8.9)	$[M_{18}+H]^+$	25.86	9.96	± 0.4	± 0.15	-
P19*	Basic (3.8/5.5)	$[M_{19}+H]^+$	21.03	8.10	± 0.4	± 0.15	-
P20*	Acidic/Basic (4.2/8.1)	$[M_{20}+H]^+$	32.22	7.62	± 0.4	± 0.15	-
P21	Basic (9.4)	$[M_{21}+H]^+$	42.60	6.48	± 0.4	± 0.15	± 0.15
P22	Acidic/Basic (14.3/7.5)	$[M_{22}+H]^+$	51.89	11.52	± 0.4	± 0.15	-
P23	Acidic (4.3)	$[M_{23}+H]^+$	33.91	12.90	± 0.4	± 0.15	-
P24 ⁽¹⁾	Basic	$[M_{24}+H]^+$	27.31	0.00	± 0.4	± 0.15	± 0.15
P25	Acidic (8.5)	$[M_{25}+H]^+$	35.41	7.26	± 0.4	± 0.15	-
P26	Acidic (9.5)	$[M_{26}+H]^+$	28.62	7.32	± 0.4	± 0.15	-
P27	Basic (6.6)	$[M_{27}+H]^+$	42.20	6.18	± 0.4	± 0.15	-

⁽¹⁾Reference product

Table 3: Quantitative results for the high priority products in the outlet sample.

Pharmaceutical code	Allowable concentration (µg/L)	Calibration range (µg/L)	Slope (counts.min.ng ⁻¹)	Determination coefficient, R ²	Estimated concentration (µg/L)
P4	40	40 – 500	4298	0.990	< 40 ^b
P6	100	100 – 500	3047	0.999	6 ^a
P7	100	100 – 500	4083	0.990	31 ^a
P9	10	10 - 500	37505	0.995	< 10 ^b
P10	1000	500 - 1000	10090	1	818
P16	1	1 - 500	15764	1	56
P19	200	100 - 300	68961	0.991	114
P20	20	20 - 500	24241	0.990	11 ^a

a: extrapolated value

b: not detected in the outlet sample

Table 4 : Comparison of the results obtained for the analyses of the outlet sample in RPLC - HRMS and RPLC x RPLC – HRMS (see Fig. 6)

	RPLC -HRMS	RPLC x RPLC -HRMS
Occupation of the retention space (γ)	-	86 %
Effective peak capacity	430	900
Number of peaks detected	130	240
Average mass error (27 priority products)	10.0 ppm	3.5 ppm
Analysis time	60 min	60 min

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

FIG1

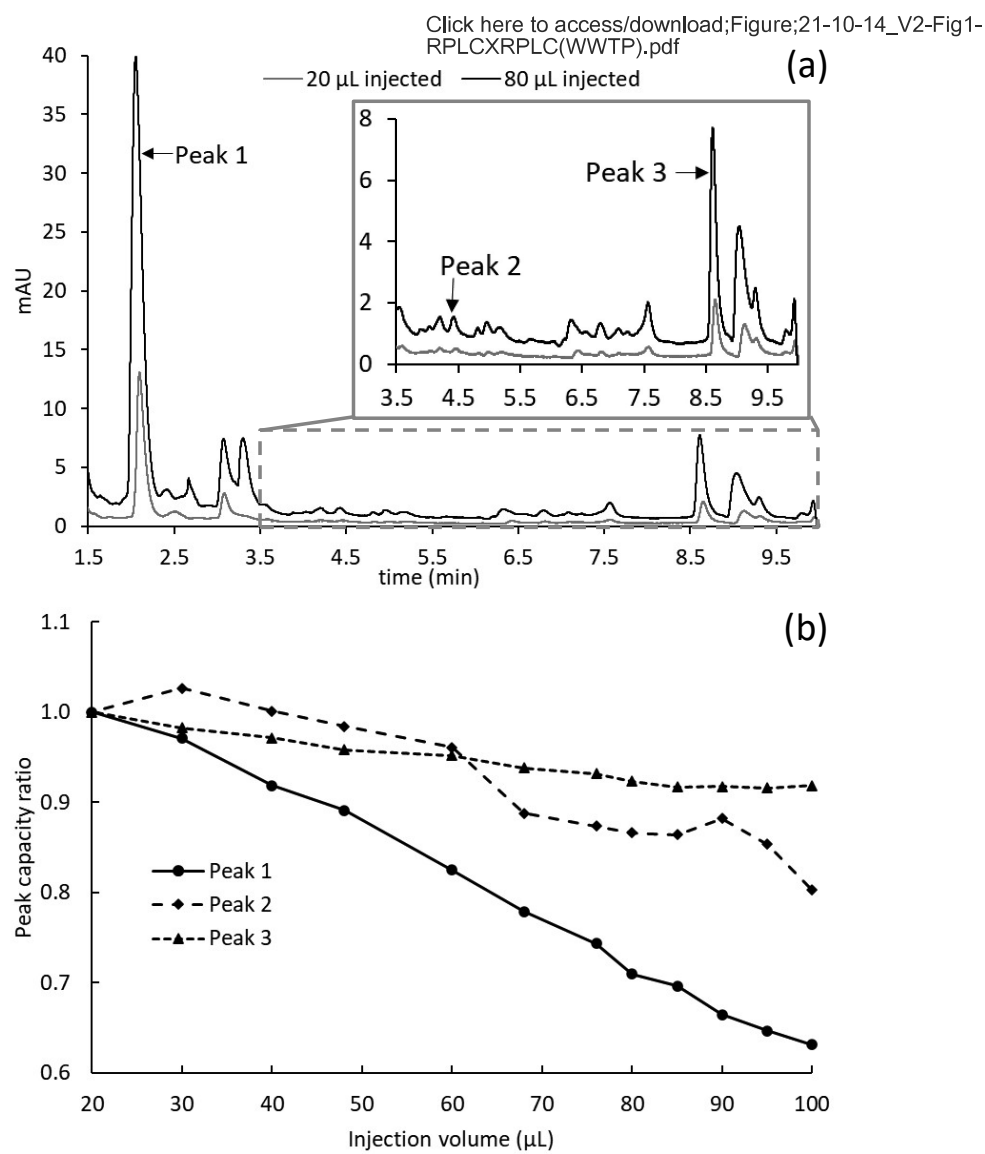


FIG2

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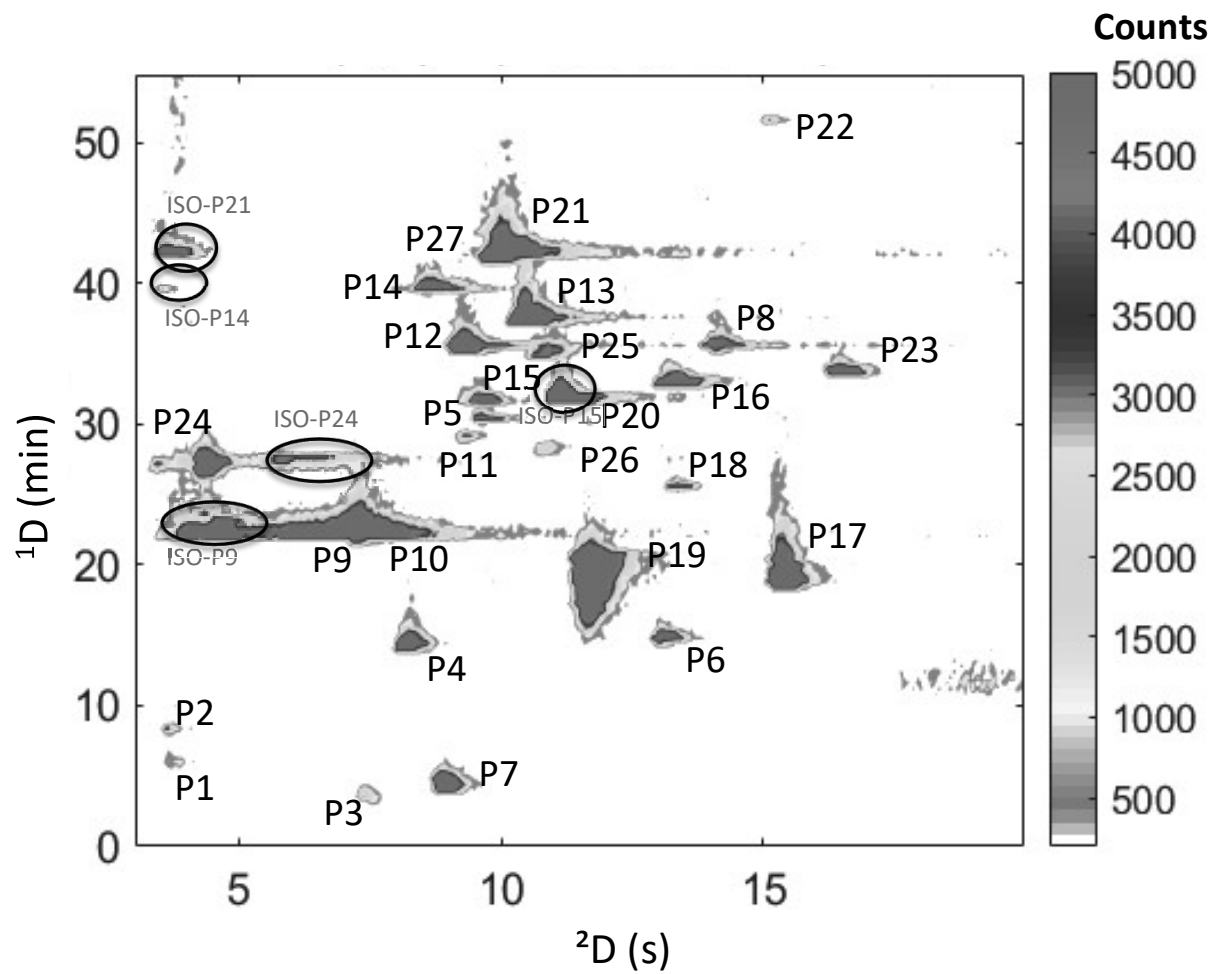


FIG3

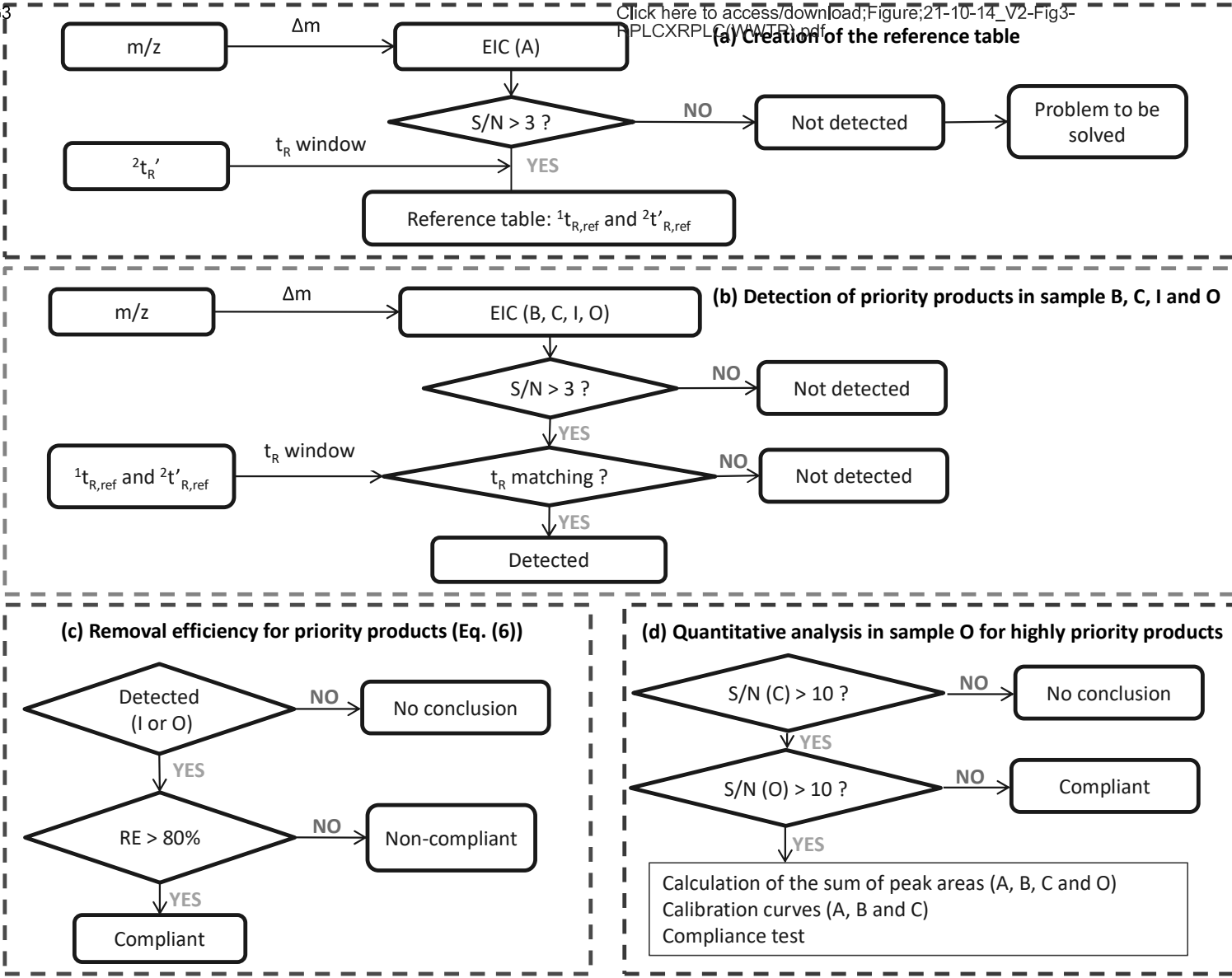


FIG4

[Click here to access/download;Figure;21-10-14_V2-Fig4-RPLCXRPLC\(WWTP\).pdf](#)

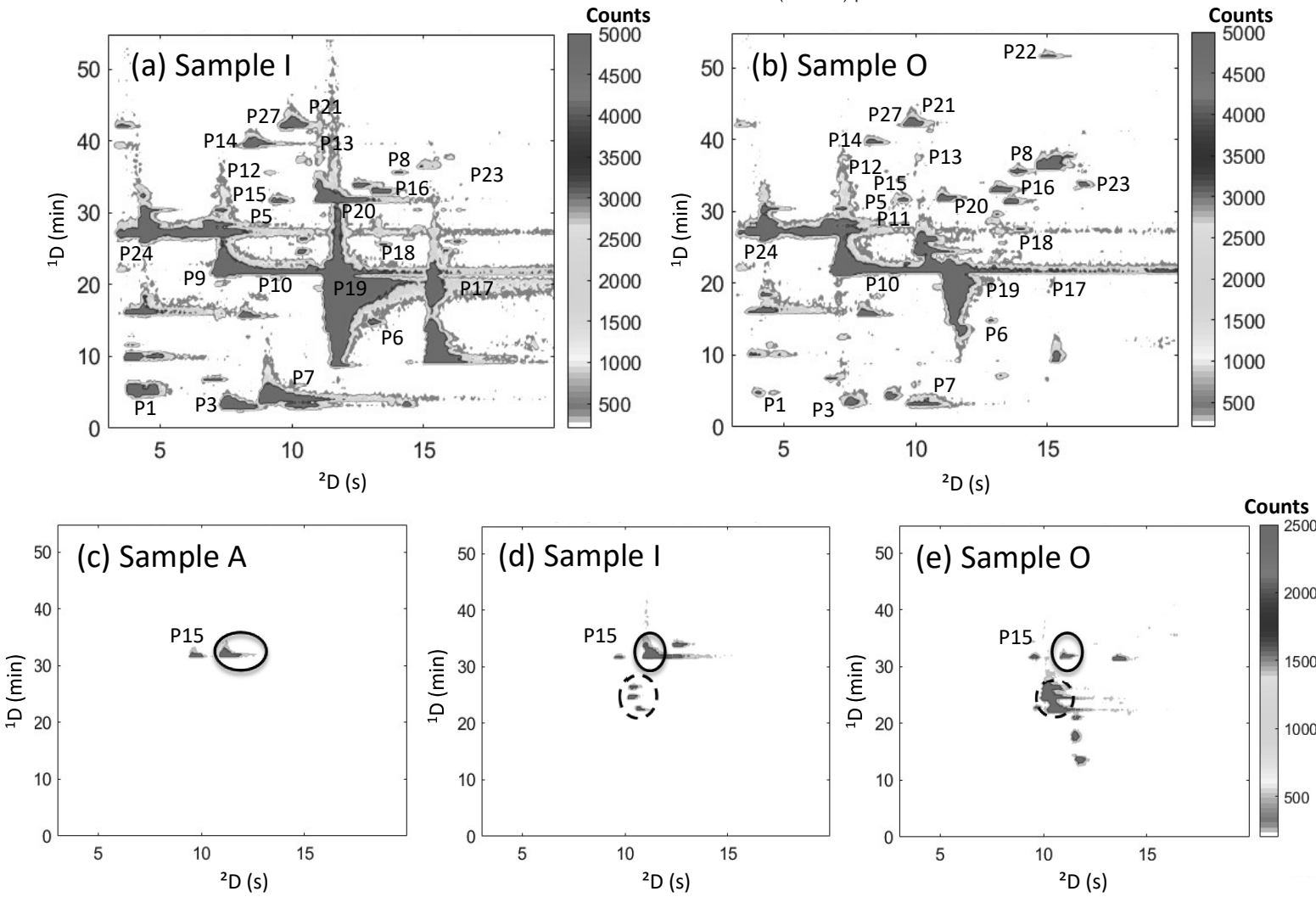
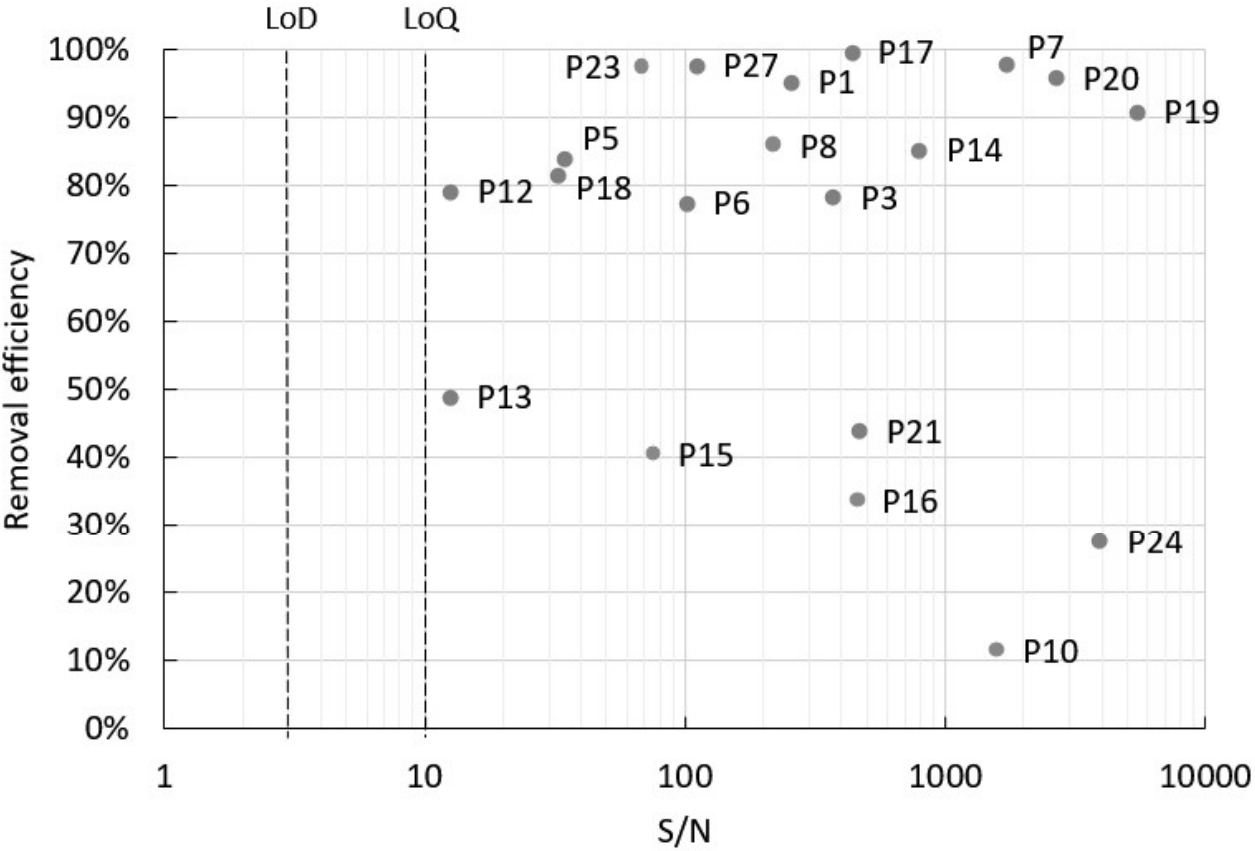


FIG5

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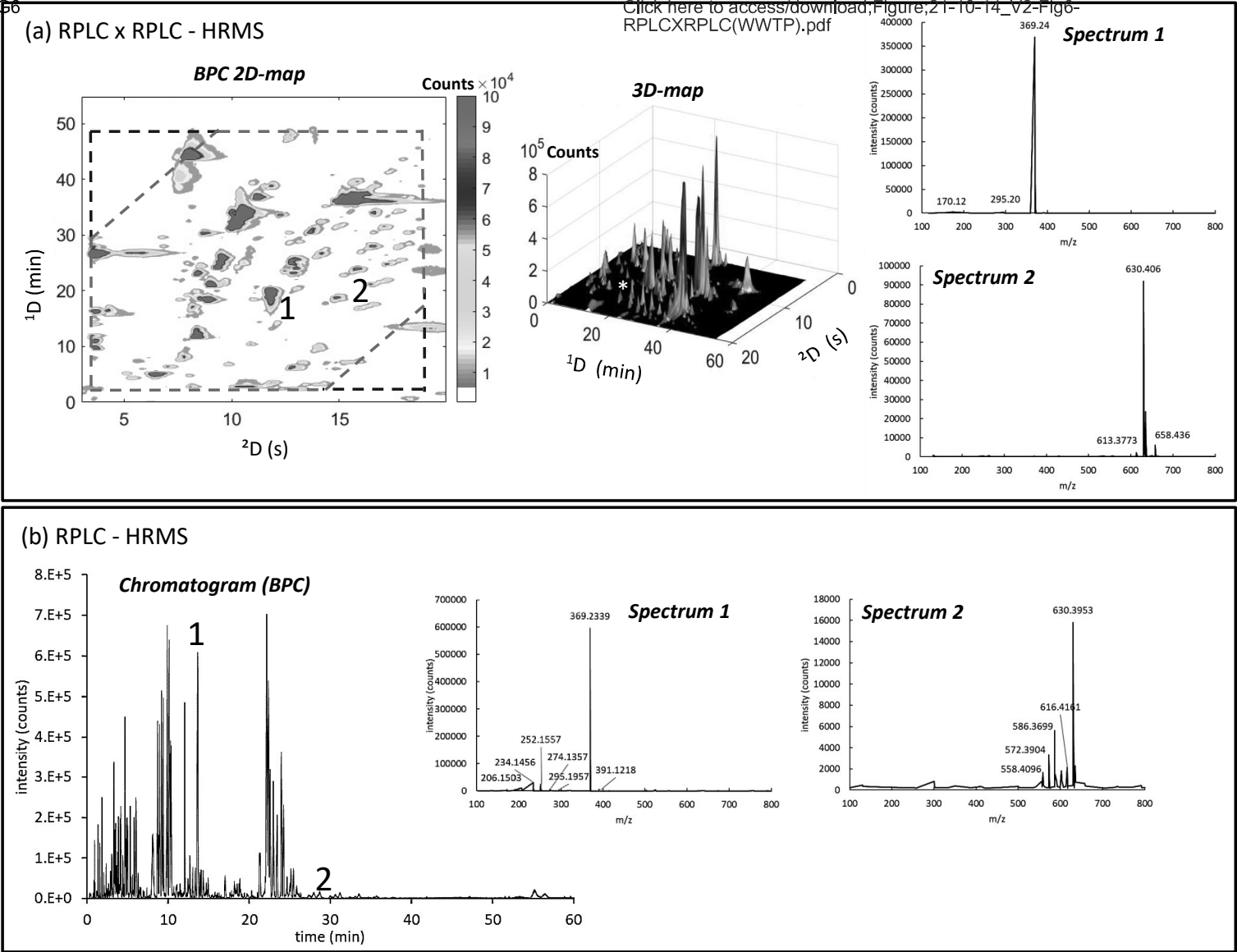
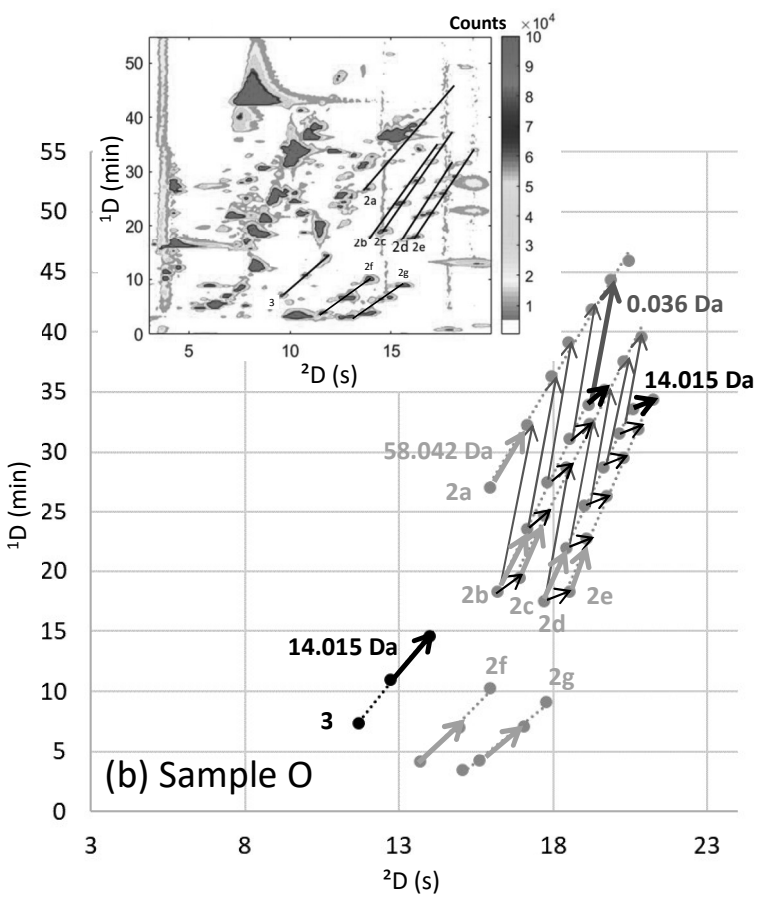
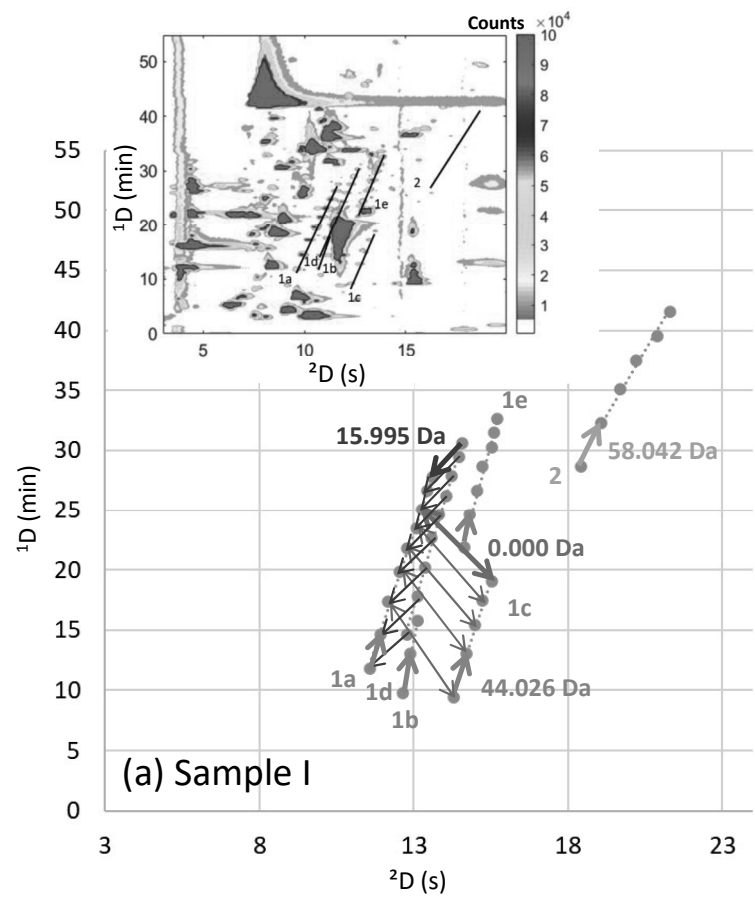


FIG7

[Click here to access/download;Figure;21-10-14_V2-Fig7-RPLCXRPLC\(WWTP\).pdf](#)



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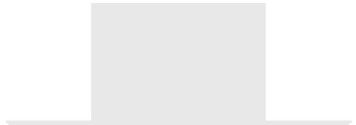
Fleur Marie Saint-Germain: Investigation; Methodology; Formal Analysis; Writing - original draft;

Karine Faure: Investigation; Writing - original draft.

Estelle Corbel : Conceptualization; Writing - review & editing

Jean-Michel Lerestif: Conceptualization; Resources ; Writing - review & editing

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