# Chapter 6

General discussion and outlook

# 1. General Discussion

The often diffuse chemical pollution of the environment requires novel analytical and bioanalytical tools to unravel the chemical composition of complex environmental samples. This thesis focused on comprehensive two-dimensional liquid chromatography coupled with time of flight mass spectrometry (LC × LC-ToF MS) as a powerful tool to assist non-target analysis and effect-directed analysis (EDA) in environmental research. Different environmental matrices including wastewater treatment plant (WWTP) effluent, indoor dust and laundry dryer lint have been analyzed by LC × LC-ToF MS on a non-target basis (Chapters 2, 3). This approach was combined with LC × LC fractionation followed by bioassay screening of the fractions in an EDA approach. A $T_4$-TTR binding assay using a fluorescence probe was miniaturized to 96 well plate format to facilitate high throughput bioactivity screening in EDA (Chapter 4). Finally, EDA was enhanced by LC × LC fractionation, parallel MS detection and a high throughput acetylcholinesterase (AChE) bioassay testing. Environmental contaminants which caused the effect in the bioassays were rapidly identified and confirmed.

**Application of LC × LC-ToF MS for non-target analysis**

The main challenges for the application of LC × LC-ToF MS for non-target analysis in environmental research were the selection of the stationary phase combinations, the coupling of the LC × LC system to the MS and software compatibility between different software packages used for controlling the LC × LC system, MS data acquisition and the visualization of the 2D chromatogram. Until now, these difficulties hampered the applicability of LC × LC coupled with MS in environmental analysis. However, in this thesis several examples were presented that show that LC × LC has great potential and can be applied on a more routine basis in environmental analysis.

Theoretically, the combination of reversed-phase liquid chromatography (RPLC) and normal phase liquid chromatography (NPLC), size exclusion chromatography (SEC) or ion exchange chromatography (IEX) can achieve good orthogonality and, consequently, effective two-dimensional separation. However, in practice, SEC and IEX are seldom used as most of the environmental organic contaminants are small and uncharged molecules. The combination of RPLC and NPLC is an interesting option but it requires

extra instrumentation such as a solvent evaporation interface to control incompatible mobile phases.[1–3]

In hydrophilic interaction chromatography (HILIC) generally compatible mobile phases are applied while employing complimentary separation mechanisms to RPLC. However, the two-dimensional separation of RPLC and HILIC was unsuccessful to capture the entire LC × LC separation space as was demonstrated for WWTP effluents, because most of the compounds retained on HILIC do not have retention on RPLC stationary phases (Chapter 2). The assessment of the orthogonality of two-dimensional separation is important for the selection of the most optimal stationary phases. Yet, most of the reported methods were not straightforward and required sophisticated software or programming skills.[4] Therefore, a simple approach to rapidly estimate orthogonality of two-dimensional separations was developed. Using this approach, the orthogonality of three different stationary phase combinations (C18 and phenyl-hexyl, C18 and pentafluorophenyl, C18 and HILIC) were assessed and the combination of C18 and pentafluorophenyl showed the best two-dimensional separation (Chapter 2).

As a result of the ultra-fast separation in the second dimension of LC × LC, the MS should be able to run at high scan frequency (≥5 Hz), which makes ToF-MS a suitable choice. For most of the commercialized ionization sources, the flowrate in the second dimension of LC × LC is too high (≥2 ml/min) and, therefore, a post column flow splitter is necessary. Also, a straightforward software solution for LC × LC-ToF MS in environmental research was not available at the start of this study. We therefore combined the LC software (Agilent ChemStation), MS software (Bruker Compass) and the two-dimensional chromatography software (GC Image) to create a clear workflow for non-target analysis in environmental research (Fig. 1).

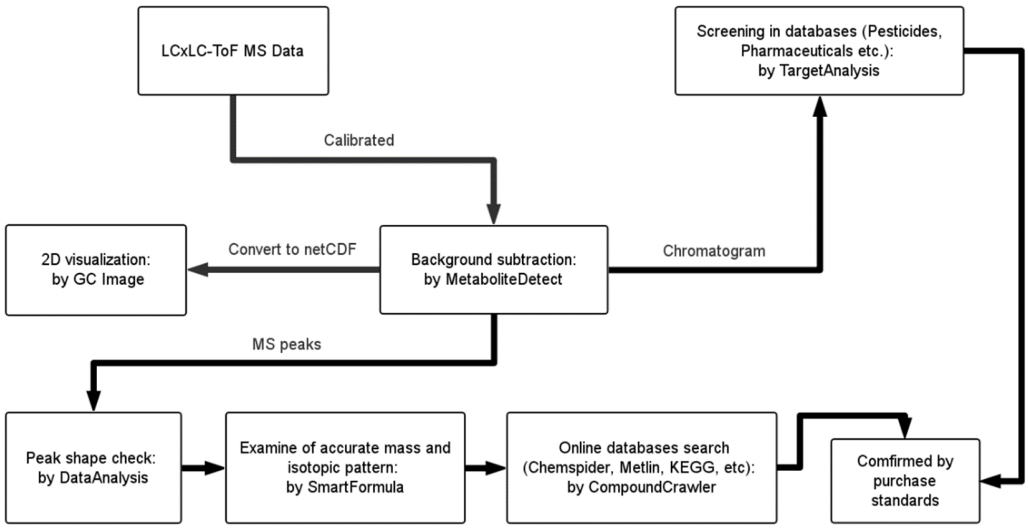


**Figure 1.** Workflow of LC × LC-ToF MS non-target analysis and suspected screening in environmental research, as developed in this study.

**High throughput bioassays and LC × LC-ToF MS facilitated EDA**

EDA is a powerful approach to identify which environmental contaminants cause toxicity in complex samples. However, the conventional one-dimensional LC (1DLC) fractionation that is generally used in EDA shows to be insufficiently capable to unravel the composition of complex samples, because the fractions still contain too many compounds to make a direct correlation between the compound's identity and bioactivity. The relatively low identification success rate of many EDA studies limits the wider application of this approach to find key toxicants in various matrices. Besides, another aspect hampering EDA application is the low throughput of the bioassay. As proof of principle for LC × LC facilitated EDA, a high throughput AChE assay was applied in this study as it is a simple and straightforward assay which not requiring any cell experiment. In addition, a fluorescence T4-TTR binding assay had been adapted to 96 well plate format in this study, to support the future demand of high throughput screening of thyroid hormone (TH) disruptors.

The LC × LC-ToF MS facilitated EDA together with high throughput bioassays developed in this study significantly simplified the approach (Figure 2). With a post column flow splitter, the flowrate of LC × LC became compatible with MS, which

enabled simultaneous chemical analysis in parallel with micro-fractionation in four 96 well plates for high throughput bioassay screening (Chapter 5). On the other hand, direct and straightforward identification would not be possible without the greater separation power provided by LC × LC due to matrix effect and too many candidate peaks. In the end, LC × LC assisted by ToF MS and high throughput bioassays reduces the total analysis time of EDA from months to a couple of weeks.

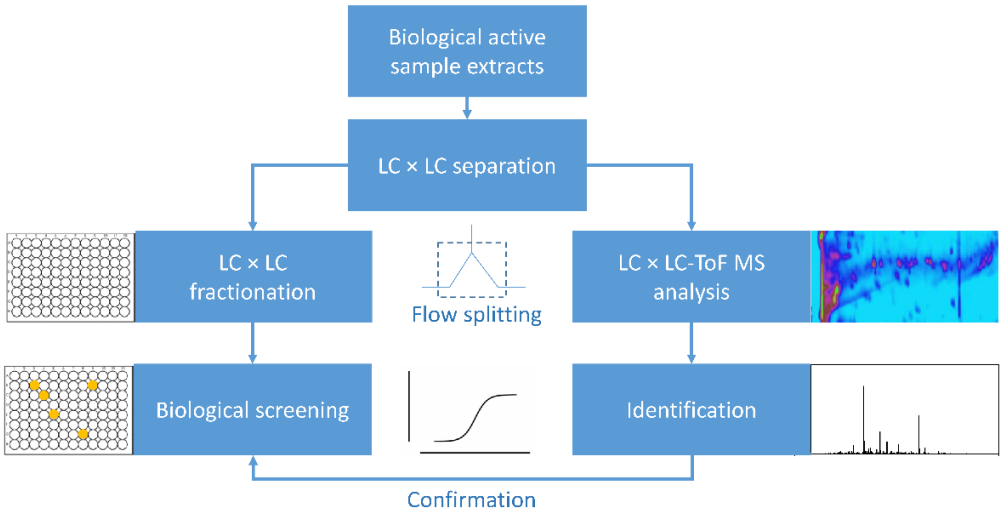


**Figure 2.** The EDA workflow developed in this study using LC × LC for fractionation into 96 well plates.

## 2. Outlook

Although in this study many advantages of LC × LC have been achieved, it should be noted that further steps need to be made before LC x LC can routinely serve as a fully convincing alternative to the existing 1DLC. First of all, the performance of LC × LC beats 1DLC only when the samples are sufficiently complex. Besides economic considerations such as instrument investment and higher costs related to the higher consumption of mobile phase solvents and buffers, the limited sensitivity of LC × LC needs to be addressed before LC × LC becomes mainstream in environmental analysis. Briefly, in LC × LC sensitivity is compromised to obtain an increase in speed. The nature of LC × LC requires very fast separation in the second dimension, in order to maintain the separation achieved in the first dimension as much as possible. Because of that,

columns with larger internal diameters are usually applied in the second dimension, with the consequence of lower sensitivity. A solution for this problem is to run the two-dimensional liquid chromatography system in heart-cutting mode (LC-LC), but then the comprehensive character will be lost and the technique will not be well suitable anymore for non-target environmental analysis, as in fact only part of the sample is analyzed and important environmental contaminants may be missing. Nevertheless, recent developments in LC × LC show other options such as active modulation and multiple heart-cutting approaches.[4,5]

Therefore, there is certainly potential for LC × LC in environmental analysis. One of the advantages of the technique is the flexibility in possible stationary phase combinations. In this study only three of them (C18 and HILIC, PFP and phenyl hexyl, respectively) were tested for environmental samples. There are, however, still dozens of other possibilities worth exploring. The emerging field of mixed-mode HPLC stationary phases is for example highly interesting to be used in LC × LC. These can provide more separation mechanisms and extra flexibility of combining the two dimensions.[6] Besides, other MS ionization sources may be coupled with LC × LC, such as APCI (atmospheric pressure chemical ionization) and APPI (atmospheric pressure photoionization), in order to also include analytes with a lower polarity which may be more difficult to ionize using the commonly used ESI.

The miniaturized fluorescence T4-TTR binding assay developed in this study showed great potential for application in not only EDA of environmental samples, but also for other areas such as food and pharmaceutical products which require high throughput screening of TH disruptors. Hundreds of samples can be screened and analyzed within an hour, using a low-cost fluorescence probe. In the future, the sensitivity of assay may be improved by further purification of the fluorescence probe.

Furthermore, the EDA approach using LC × LC may also be applied in other areas of research to screen for biologically active compounds in a complex sample matrix. In the future, the approach may be used to explore effective ingredients from natural products such as herbal medicines and for body fluid e.g. breast milk, blood and urine) diagnosis of specific groups of disease markers.

## References

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