Chapter 1

General introduction

1. Background

In 1736, the British pharmacist Joshua Ward set up a factory in Twickenham for the manufacture of sulfuric acid, which was later known as the “Great Vitriol Works”. It is probably the first practical production of chemicals at an industrial scale. The development of chemical industry is integral to the industrial revolution. Since then, the mass production of synthetic chemicals, such as pesticides, fertilizers, pharmaceuticals and plastics, has brought great prosperity to human society and revolutionized the way we live. However, every silver lining has a cloud. While we are enjoying the convenience of modern industrialization, millions of chemicals have been diffused to lands, seas, freshwaters and the atmosphere of the planet. Many of these chemicals are environmental contaminants which may greatly impact the ecosystem and threaten human health. They are believed to be able to induce carcinogenicity, mutagenicity, neurotoxicity and endocrine disruption.[1–4]

Therefore, it is crucial for human beings to be fully aware of the occurrence, fate and effects of (emerging) contaminants in the environment. The modern analytical techniques including, but not limited, to gas chromatography (GC),[5] high performance liquid chromatography (HPLC),[6–9] inductively coupled plasma mass spectrometry (ICP-MS),[10,11] gas chromatography coupled with mass spectrometry (GC-MS) [12,13] as well as liquid chromatography coupled with mass spectrometry (LC-MS) [14,15] and spectroscopic methods [16–18] are effective tools to chemically characterize environmental samples. The main strength of the chemical analytical approach is the capability of the rapid determination of the level of chemicals of concern in the environment. With the recent development of high resolution mass spectrometry (HR-MS) such as time of flight (ToF), Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR) analyzers, direct chemical identification of unknown contaminants present in environmental samples based on accurate mass and isotopic pattern has become possible, and broadened the scope of environmental analysis from target screening to non-target analysis.[19,20] Furthermore, tandem HR-MS such as quadrupole ToF (Q-ToF) and linear trap quadrupole (LTQ) Orbitrap provide extra confidence of identification by specific fragmentation patterns generated in the first stage MS.[21–23]

Solely based on chemical analysis it is not possible to evaluate the actual toxicity of

environmental samples, or to figure out novel chemicals responsible for the toxicities. Bioanalytical methods including different *in vivo* and *in vitro* bioassays such as zebra fish embryo toxicity test (zFET),[24] estrogen receptor mediated chemically activated luciferase gene expression (ER-CALUX) assay,[25] Ames assay,[26] transthyretin (TTR) binding assay [27] and acetylcholinesterase (AChE) inhibition assay [28] have been applied for the assessment of e.g. developmental toxicity, estrogenic activity, mutagenic potential, thyroid hormone disruption and acetylcholinesterase inhibition in environmental samples. Complementary to the chemical tools, they are also important approaches for environmental monitoring and risk assessment.

2. Effect-directed analysis

One of the major challenges environmental scientists face nowadays is the analysis of a large number of environmental contaminants in a variety of complex environmental sample matrices. The environmental chemicals of concern not only include the well-known high production volume synthetic chemicals such as pesticides,[29] polychlorinated biphenyls (PCBs) [30] and polybrominated diphenyl ethers (PBDEs),[31] but also novel emerging contaminants like pharmaceuticals and person care products (PPCPs),[32] metabolites and transformation products (TPs).[33] To perform target analysis for all these contaminating compounds is a mission impossible. On the other hand, it would be unnecessary to characterize the complete chemical composition of an environmental sample if the focus is to find the chemicals that cause the toxicity. In light of that, the concept of effect-directed analysis (EDA) was introduced. The EDA approach is a combination of biological analysis, fractionation techniques and chemical analytical methods.[34] So far, it has been applied to unravel various complex environmental matrices, such as wastewater treatment plant (WWTP) effluent,[35] sediment,[36] indoor dust [37] and biota samples.[38] In EDA, a complex sample is first extracted, purified and tested with a bioassay with a specific toxicological end point. Then the extract is fractionated and several fractions, typically in the order of three to several dozens, are collected, usually via a HPLC based approach. Subsequently, the fractions are tested with the bioassay to screen for the bioactive, “hot” fractions. Finally, the hot fractions are analyzed, usually by chromatographic techniques combined with high resolution

mass spectrometry, to identify the chemicals responsible for the observed toxicity (Figure 1).

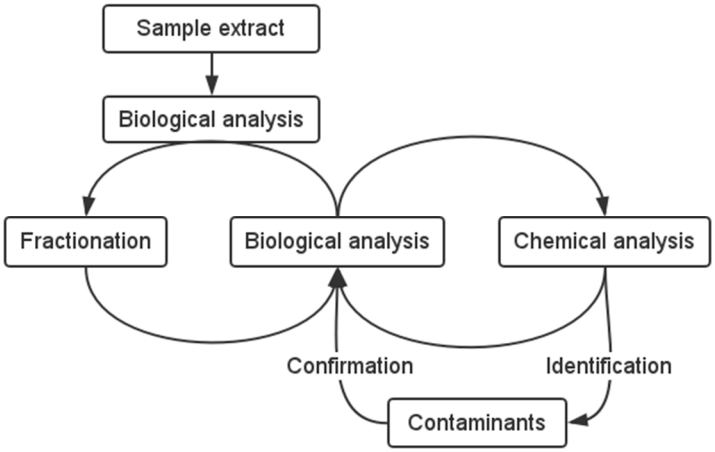

Figure 1. Scheme of a complete EDA workflow.

However, the efficiency of the current EDA approach is limited by the separation power of the fractionation procedures as well as the throughput of the bioassays. Low peak capacity in separation usually leads to difficult MS identification and possibly extra steps of fractionation.[39–41] Recently, ultra-high performance LC based fractionation in 96 well microplates was developed to improve the throughput in EDA of photosynthesis inhibitors of pelagic marine algae, by enhancing the separation power and reducing the total analysis time.[42] However, challenges remain when very complex samples need to be analyzed, as the contaminants present in the environment are not only large in number, but also possess various physicochemical properties. For instance, polycyclic aromatic hydrocarbons (PAHs), mainly generated by industrial combustion and motor vehicle exhaust are generally non-polar compounds, while a large portion of PPCPs and their metabolites are strongly polar compounds. Ideally, a more efficient and effective EDA approach includes high resolution and comprehensive separation, straightforward fractionation into ≥ 96 well plate format and high throughput bioassays.

3. Comprehensive two-dimensional liquid chromatography

The separation capability of a chromatography system is represented by the peak capacity (n), which is defined as the number of peaks that can be separated with

unit resolution in a given time interval (t_1-t_n). In unidimensional chromatography it can be expressed as the following equation, where N is the theoretical plate number:[43]

$$n = 1 + \int_{t_1}^{t_n} \frac{\sqrt{N}}{4} \frac{dt}{t}$$

Obviously, one way to enhance the total peak capacity of a HPLC system in a certain time interval is to improve the theoretical plate number, which can be achieved by applying smaller sized particles in HPLC columns and operating the separation in extreme conditions i.e. higher pressure and higher temperature, according to the simplified Van Deemter equation:

$$HETP = A + \frac{B}{u} + Cu$$

In the equation, HETP is the height equivalent to a theoretical plate, A is the eddy diffusion coefficient, B is the longitudinal diffusion coefficient, C is the resistance to mass transfer coefficient and u is the linear velocity of the mobile phase. With this concept UPLC™ and high temperature HPLC was developed.[44,45] Recently, further development of HPLC separation at 1500 bar using columns packed with 1.5 μm core–shell particles was reported to further improve the peak capacity per unit time.[46]

Another strategy for delivering greater peak capacity in chromatography is by introducing another separation dimension. Comprehensive two-dimensional liquid chromatography (LC × LC) is an emerging technique and its application has been widely reported in a variety of field, analyzing e.g. pharmaceutical,[47] polymer,[48–51] food and beverage,[52–54] proteomics [55] and traditional Chinese medicine samples.[56,57] Theoretically, the total peak capacity of a comprehensive two-dimensional chromatography system is the product of the two separation dimensions, provided that they are orthogonal.[58]

$$n_c^{2D} = n_c^1 \cdot n_c^2$$

However, the application of LC × LC in the field of environmental analysis is so far scarce, although the technique may greatly reduce the sample complexity by providing enhanced peak capacity. Besides, by applying different stationary phases in the two separation dimensions, multiple or combined selectivity can be achieved. Furthermore, when coupling with HR-MS, more effective separation using LC × LC

results in less ion suppression in the interface, which enables accurate identification of chemicals in complex samples. By using a post column flow splitter, high resolution fractionation and optimal flowrate for HR-MS interface can be delivered simultaneously after LC × LC. Therefore, LC × LC combined with HR-MS and parallel post column fractionation for high throughput bioassays may provide an ideal platform to perform EDA.

4. Endocrine disruption

Endocrine disruption has manifested itself as early as the 1940s, with the now famous case of the thinning of egg shells of peregrine falcons, leading to reduced breeding success. According to the International Programme on Chemical Safety (IPCS), World Health Organization (WHO) and United Nations Environment Program (UNEP), an endocrine disruptor is “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population”. Research into the phenomenon of endocrine disruption has been in the spotlight since the late 1990s, with e.g. the observation of the feminization of male fish after exposure to estrogenic compounds in wastewater treatment plant effluents.[59,60] In recent years, a number of valuable scientific reviews on endocrine disruption have been published and the term is no longer limited to the obvious endocrine pathways such as estrogenicity and androgenicity, but also to other endpoints such as progestagenic, glucocorticoid and thyroidogenic activities.[61] To evaluate and access the endocrine disrupting activities, applications of *in vitro* bioassays with different toxicological endpoints in environmental research have been widely reported in recent years.[59,62–64]

The thyroid system is a rather complex system that comprises different glands, i.e. the hypothalamus, the pituitary and the thyroid gland, that secrete the natural hormones T_3 an T_4 into the blood circulation, where they are transported to their target organs and tissues by carrier proteins. There are several assays available to measure thyroid hormone disruption potential of chemicals and environmental samples. The most classical one uses ^{125}I labelled T_4 as the radioactive ligand in a competitive binding assay.[27] The method was successfully applied in many studies

and until now showed the best sensitivity to many of the thyroid hormone disrupting compounds.[38,65,66] However, the throughput of this approach is yet still rather limited, due to the costly radioactive labeled ligand, relatively complicate assay steps and safety issues related to the handling of radioactive tracer. Besides, other assays such as the TR-CALUX (thyroid hormone responsive chemical activated luciferase gene expression) assay [67] and 8-anilino-1-naphthalenesulfonic acid ammonium (ANSA)-TTR competitive fluorescence displacement assay [68] have been developed to assess the TTR binding capacity of thyroid hormone disruptors in the environment. The possibility to perform high throughput EDA focusing on thyroidogenicity in environmental matrices turned out to be somewhat hampered by the lack of a fast and reliable bioassay. Recently, similar to the ANSA-TTR assay, another method was introduced to study the binding of OH-PBDEs to TTR and thyroxine-binding globulin (TBG) using the fluorescence probe fluorescein isothiocyanate (FITC) associated to T4 [69] based on a previous study.[70] The assay was performed in cuvette and the reported sensitivity of detecting OH-PBDEs was comparable to the radio-ligand assay. The basic principle of the displacement assay is the fluorescence intensity enhancement of the probe after binding to TTR, or the fluorescence polarization enhancement of the probe after binding to TBG. Although the assay was only performed in cuvette and was not yet tested for real environmental samples, after proper miniaturization it may have a promising potential of being applied in high throughput EDA due to its simple concept and low cost fluorescence probe.

5. EDA-EMERGE and MiSSE projects

The major part of the research described in this thesis focusing on developing and optimizing a comprehensive two-dimensional liquid chromatography system to support the identification of emerging toxicants in effect-directed analysis (EDA) was conducted within the EDA-EMERGE Marie Curie initial training network (MC-ITN), funded by the European Commission within the 7th framework program. This project aims to train a new generation of young scientists in the interdisciplinary techniques required to meet the major challenges in monitoring, assessment and management of toxic pollution in European river basins. Research within EDA-EMERGE is

directed towards developing powerful new analytical, bioanalytical and hyphenated tools to unravel the enormous complexity of contamination, effects, and cause-effect relationships in the environment.[71]

In addition, part of the research was carried out within a project entitled “Mixture assessment of Endocrine Disrupting Compounds (EDC) with emphasis on thyroidogenicity – using cats as model for human indoor exposure”, in short MiSSE (Mixture aSSessments of EDCs), funded by the Swedish Research Council (FORMAS). The project is aiming to assess indoor human and child exposures to anthropogenic thyroid hormone disrupting compounds (THDCs) in matrices such as household dust and consumer products, so that management of THDCs can be further improved.

6. Scope and outline of the thesis

The aim of this thesis was:

i. Develop LC × LC-ToF MS-based methods for non-target analysis of different environmental samples,
ii. Develop and miniaturize bioassays for high throughput screening,
iii. Establish LC × LC-ToF MS-facilitated high throughput EDA of complex environmental samples.

Before this study started, two-dimensional liquid chromatography was rarely applied in environmental research. Therefore, the first stage aim was to set up a two-dimensional liquid chromatography system. There are different configurations of the two-dimensional liquid chromatography interface, including the loop interface,[48] packed loop interface,[72] stop flow interface,[73] interface with parallel second dimension [74] and vacuum evaporation interface (VEI).[75] The most widely applied loop interface was selected for this work, for its relatively simple setup and applicability in delivering comprehensive two-dimensional liquid chromatography (LC × LC). To perform non-target environmental analysis, coupling of LC × LC to HR-MS is necessary. Because of the fast scan rate, ToF-MS was selected. Besides, a post-column splitter was implemented to provide an optimal flow for the MS interface. The flow-split setting, on the other hand, also provides the possibility of simultaneous collecting of fractions in parallel to the MS detection in the later EDA

study. In addition to the hyphenation of the instrumentation, an LC × LC-MS data analysis strategy including transferring of data files between different software packages was developed. Finally, the system was optimized for the analysis of environmental samples from different matrices (Chapters 2,3).

Consequently, as much finer fractionation was achieved in LC × LC facilitated EDA, a high throughput bioassay was required. In Chapter 4, a miniaturized high throughput T4-TTR binding assay based on a fluorescence probe was adapted from earlier work by Ren and Guo (year). The adapted assay was performed in 96 well microplate format and therefore suitable for high throughput EDA applications, providing a cheaper, easier to handle and faster alternative to the current radio-ligand binding assay. The performance of the assay was assessed by the determination of the dose response curves and the IC50's of several known thyroid hormone disrupting compounds from different groups of chemicals and comparing them to results obtained in the radioligand T4-TTR binding assay. The applicability of the assay for the evaluation of the thyroid hormone disrupting potential of environmental samples was demonstrated by testing a selection of herring gull (*Larus argentatus*) egg extracts sampled from two different locations in Norway.

Finally, a complete high throughput EDA study of a concentrated wastewater treatment plant effluent sample based on LC × LC fractionation, a high throughput acetylcholinesterase (AChE) inhibition assay and parallel LC × LC-ToF MS analysis was demonstrated in Chapter 5.

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