VU VRIJE UNIVERSITEIT AMSTERDAM

VU Research Portal

Advanced bioactivity screening analytics for rapid identification of environmental toxicants

Jonker, L.W.

2018

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Jonker, L. W. (2018). *Advanced bioactivity screening analytics for rapid identification of environmental toxicants*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

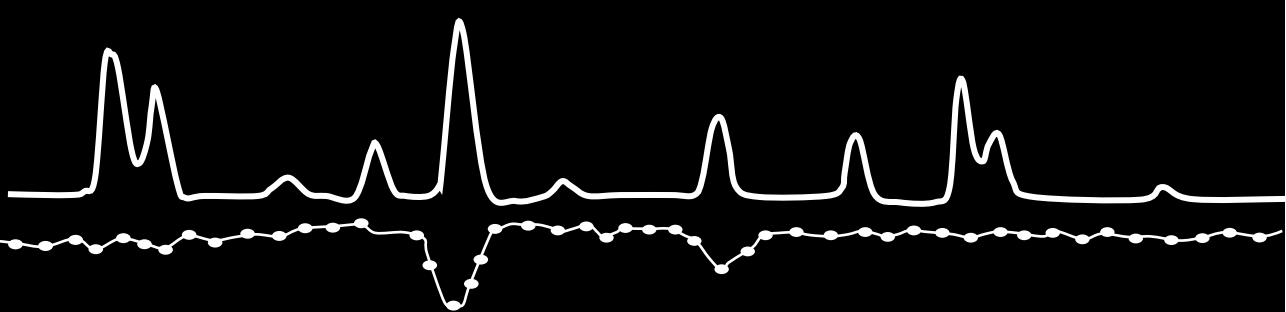
General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

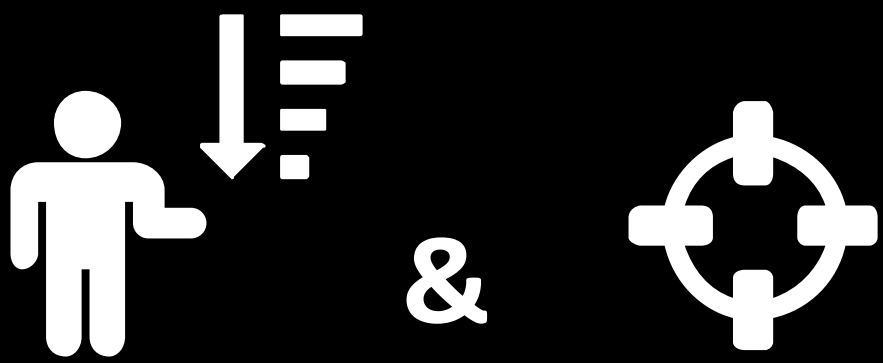
E-mail address:
vuresearchportal.ub@vu.nl

Download date: 13. Mar. 2024



Chapter 1

Introduction and scope



As a result of modern day production and use of organic compounds, food, feeds, biota, sediments, soils, surface and ground waters may be contaminated with a vast number of chemicals. Research has shown that a variety of these chemicals may induce adverse biological effects posing a hazard for environmental and human health [1–5]. Surveys have shown that many organic toxicants are present in the aquatic environment. Since billions of people rely on surface waters for the production of their drinking water, this may form a life-long route of daily exposure to contaminants. In this regard, endocrine disrupting and genotoxic compounds are considered highly relevant because of their relation to reproduction toxicity and carcinogenicity [6,7]. Therefore, governments and water laboratories intensively monitor drinking water sources to safeguard quality and potentially trace pollutions and their sources.

Water laboratories use state-of-the-art technologies to determine known toxic compounds down to very low levels. Monitoring is typically performed using either liquid chromatography (LC) in combination with triple quad (QqQ) mass spectrometry (MS) or by gas chromatography (GC) with single-MS or QqQ-MS detection. These techniques are predominantly used to measure pre-listed compounds, so-called target compounds. Hence, unknown toxicants and compounds that are not included in the monitoring program will be missed. High-resolution time-of-flight (ToF) and orbitrap mass spectrometers have been introduced in water laboratories in recent years allowing the screening and identification of unknown emerging pollutants. Due to their high selectivity and resolving power, these approaches can be used in a non-targeted fashion, however, they do not provide information on the potential biological activity (e.g. toxicity) of the compounds identified. Water sample toxicity can be monitored with a wide range of bioassays, but these reveal the sample bulk (toxic) bioactivity rather than the individual compounds causing the activity. In other words, bioassays of water do not allow correlation of the observed toxicity to specific components of the probed water sample.

Effect-directed analysis (EDA) approaches combine chemical and biological analysis for the detection and identification of individual (unknown) bioactive (often toxic) compounds in environmental extracts. In EDA, the complexity of a sample is reduced by first performing a chromatographic separation and collecting separated compounds in different fractions. Subsequently, these fractions are subjected to bioassay analysis and the bioactive fractions are further analysed chemically in order to identify the potential toxicant. Since the fraction-collection frequency used in typical EDA studies is relatively low, fractions are large (i.e. several minutes). This means that part of the chromatographic resolution is lost and that most fractions still contain many different compounds when environmental samples are analysed. Consequently,

the fractions that show bioactivity need further separation and subsequent fractionation followed by another round of bioassay testing and chemical analysis. This process is repeated until the bioactive compound is isolated and can be identified unambiguously. In practice the iterative EDA process can be very labour intensive and tedious, and often takes up to weeks to months. Moreover, during the extensive and repeated sample and fraction handling, compound losses may occur. Because traditional EDA is time consuming and the success rate with respect to bioactive identification is low, it is unsuitable for routine applications where resources are scarce and fast decisions have to be made.

The research described in this thesis aims to address limitations of current EDA approaches. The work was carried out in the framework of a collaboration between the *Division of Bioanalytical Chemistry,* the *Institute for Environmental Studies* (both situated at the Vrije Universiteit Amsterdam) and *Het Waterlaboratorium*. The joined project entitled *"High-Throughput Effect-Directed Analysis: a novel platform for rapid and sensitive identification of toxic compounds in the aquatic environment"* was supported by the Dutch Technology Foundation (STW) with the main objective to increase throughput and toxicant identification success rate for water analysis, and to reduce the labour-intensity of EDA approaches. The ultimate goal was to provide water laboratories with a tool enabling to perform EDA a much higher high-throughput. To this end, we had to realize (1) an efficient coupling of bioassays with chromatography, allowing probing of (preferably) individual components of environmental mixtures without significant loss of chromatographic resolution, and (2) parallel chemical detection (e.g. MS) permitting assignment of assayed compounds. To meet these requirements the following analytical approach was proposed (Figure 1). A water sample, extract is analysed by LC or GC. The chromatographic column effluent is continuously split towards (1) a high-resolution fraction collector which distributes the flow in small fractions over a well plate, and (2) MS for real-time selective detection. Subsequently, the collected fractions are individually subjected to bioassay testing. By plotting the assay responses of each fraction against their elution time, a bioactivity chromatogram can be constructed, which can be compared with the parallel acquired MS chromatogram. Peaks indicating bioactivity in the bioassay chromatogram can then be directly correlated with parallel acquired mass spectra revealing the molecular mass of the bioactive compound, thereby allowing identification of e.g. unknown toxic compounds.

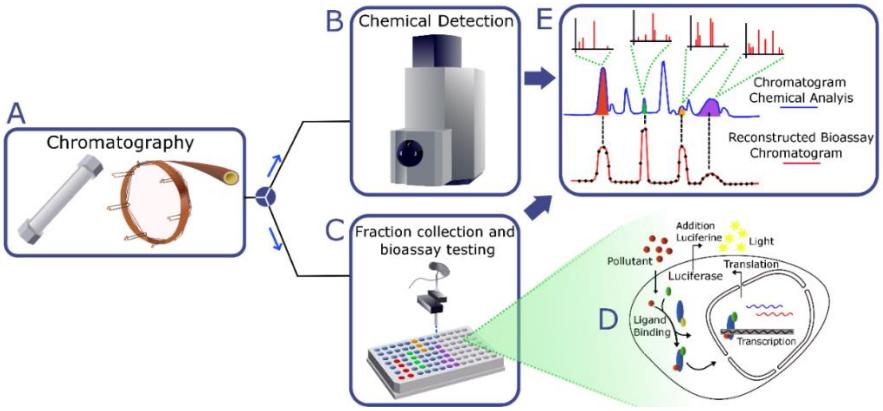


Figure 1. Proposed workflow for high-throughput effect-directed analysis. (A) Separation of the sample extract. (B) The column effluent is split towards a chemical detector (e.g. a mass spectrometer) and (C) a high-resolution fraction collector. (D) Collected fractions are subjected to bioassay testing. (E) Comparison of the constructed bioassay and MS chromatograms indicates the molecular masses of the bioactive components.

The thesis starts with an overview of current methodologies for EDA in environmental, food and drug-discovery research (Chapter 2). The chapter discusses sample preparation, fractionation approaches, bioassays, and identification methods used amongst the different research fields for the EDA-based identification of bioactive compounds in mixtures such as environmental, food and natural extracts.

A new method for the detection and identification of (unknown) estrogenic and anti-estrogenic compounds in environmental waters is described in Chapter 3. An LC-nanofractionation platform is developed which combines LC both with a sensitive and selective human cellular reporter gene assay, and with a high-resolution mass spectrometer. A modified autosampler enabled high resolution fraction collection in 96-well plates maintaining chromatographic resolution and allowing straightforward connection to the cell-based bioassay. As the chromatographic resolution is essentially maintained during fractionation, the bioassay readout can directly be correlated to the parallel acquired MS chromatogram, permitting unequivocal determination of the masses of observed bioactive substances and thus preventing the need for additional fractionation cycles. Subsequent compound identification was based on molecular formula calculation from recorded accurate masses, isotope-pattern matching with candidate molecular formulas, and compound database searching (e.g. Chemspider) based on calculated molecular formula.

In addition to the aquatic environment, the chemical safety of consumer products in the domestic environment is an issue of emerging concern. Studies have shown that plastics used in these products may be an important source of potential contaminants [9,10]. Additives, such as light stabilizers, flame retardants, antioxidants and phthalates have been reported to leach from plastics via volatilization and abrasion thereby forming a route of indirect exposure through contact with house dust or inhalation [11], while direct exposure may occur by dermal contact with the materials [12]. Some additives have been reported to induce adverse health effects and estrogenic activity has been measured in leachates [13–16]. In Chapter 4, the platform described in Chapter 3 was applied to screen plastics from consumer products such as printers, decorative items, televisions and routers for the presence and identification of estrogenic compounds that could leach into the environment. Eight products were analysed and in four samples estrogenic activity could be detected. With the MS data the estrogenic activity could be appointed to 2,4-di-tert-butylphenol, bisphenol A (BPA) and a compound showing features similar to bisphenol A. The MS data also showed the presence of flame retardants but estrogenic activity could not be directly related to these compounds. BPA might however be a degradation product originating from the flame retardant tetrabromo-BPA or analogues of this compound. Since additives have shown to successfully migrate from plastics into the environment, consumer products may act as an important source of estrogenic compounds.

A new tool for fraction collection of LC separations in high-density well-plates is described in Chapter 5. Common LC fraction collectors release column effluent droplets from a tip by gravitational force and collect a fraction in e.g. a well for a certain time interval until the tip moves to the next well. Since droplet release only occurs when adhesion with the tip is overcome, the droplet release rate may be insufficient when small fraction volumes (<10 s/fraction) have to be collected. In such a scenario, the fraction collector may switch to the next well without droplet release resulting in fractions with variable collection volumes. Fraction collectors for nano-LC often make use of deposition of the LC column effluent by moving the tip down for forced droplet release by contact with the target surface. This downwards movement takes additional time and adjustment may be tedious. In Chapter 5, a fraction collector is developed for accurate, contact-free fraction collection of LC separations. A low dead volume solenoid valve was implemented in an autosampler enabling droplet ejection at a frequency between 1 and 30 Hz. An external electronic signal converter and software script were developed to control the solenoid valve, the x-y movement and valve switching between waste and fraction collector. The performance was tested for different solvent compositions and multiple flow rates. Visual assessment of the droplet release gave an indication of suitable working ranges in terms of the relation between

flow and frequency which were further investigated quantitatively by continuous fractionation of a fluorescent solution into a buffer solution. Subsequently, the fluorescent readout of the buffer solutions after fractionation were compared. A dye mixture and fluorescent compound were fractionated to study the repeatability and resolution. To this end the readout was compared with the signal from the in series connected UV/VIS and fluorescence detector. Fraction collection for analyte enrichment and analysis by nuclear magnetic resonance spectroscopy was demonstrated by fractionation of an academic mixture. To show the potential for bioactivity screening a bioactive mixture containing hormones and hormone like compounds was fractionated and the bioactives were successfully detected using two reporter gene assays.

Although chromatographic isolation of analytes is predominantly carried out by LC fractionation, GC remains an important separation technique in many research fields, yielding superior separations for a wide range of compounds. Especially for environmental research, GC is the method of choice for many compound classes such as pesticides and flame retardants. However, fractionation of GC separations is not straightforward and current fractionation platforms are limited in the number of fractions that can be collected. Furthermore, analyte loss due to aerosol formation may occur for systems relying on cold trapping. Previously, a new GC fractionation principle was proposed by the *Division of BioAnalytical Chemistry* circumventing these limitations. The system employs post-column infusion of a trap solvent prior to fraction collection in 96 well-plates [17]. However, the initial device did not contain a detector for parallel chemical detection, and therefore fractions could not be correlated to peaks in a chromatogram. Chapter 6 describes the development of a GC fractionation platform featuring fraction collection in 384-well plates for bioassay detection with on-line parallel flame ionization detection (FID) via a post-column split. The y-split at the column outlet divides the effluent towards the FID detector and an inverted y-piece where vaporized trap solvent is infused. Different trap solvents are assessed for optimal analyte collection. Implementation of a trap-solvent pre-heater is studied, allowing the infusion of vaporized trap solvent. This enhances mixing with the GC eluate, improving the fraction collection by minimizing extra band broadening. The platform was also used in combination with an acetylcholinesterase assay for bioactivity screening. A wide range of pesticides act on acetylcholinesterase and a mixture of two pesticides was fractionated in a 384 well plate for subsequent bioassay testing. By plotting the assay response against the corresponding fraction number a bioassay chromatogram was reconstructed and compared with the in-parallel acquired FID signal. Using this setup, peaks in the reconstructed bioassay chromatogram - indicating affinity to acetylcholinesterase - could directly be appointed to peaks in the FID chromatogram.

Since FID does not provide structural information on eluting compounds, the identity of bioactive compounds detected in the post-column bioassays cannot be revealed directly. In order to obtain selective information on compounds, in Chapter 7 the GC fractionation platform was extended with MS detection parallel to fraction collection in 384 well plates. The system setup is similar to that described in chapter 6 but an MS detector is installed instead of a FID. Restriction capillaries with different lengths and diameters were connected to the y-split to adjust the split ratio. Determination of the split ratio was achieved by comparing the MS signal of an n-alkane mixture measured with and without split. The fraction collection repeatability was demonstrated by collecting multiple fraction collection cycles in the same well plate followed by GC analysis of each fraction. Comparison of the fraction-reconstructed and online acquired chromatogram showed the separation is maintained during fractionation. The platform was coupled with a reporter gene assay for offline detection of anti-androgenic compounds. The platform can be used for environmental research of pollutants showing to induce anti-androgenic activity, but may also be of interest for the pharmaceutical industry to screen natural products. The MS data acquired in parallel to the fraction collection creates the possibility for direct toxicant identification by MS-database searching utilizing electron ionization-based MS libraries.

The thesis is summarized in Chapter 8. In addition, a section discussing future perspectives is provided. The latter briefly discusses potential next steps to improve the platforms described in this thesis such as online solid phase extraction for LC fractionation and large volume injection for GC fractionation. The option to use the platforms to isolate and enrich analytes for analysis with nuclear magnetic resonance spectroscopy is also discussed as well as the added value of using different ionization sources and implementation of an online electrochemical conversion cell to mimick metabolic activation allowing to capture analytes not bioactive in their native form. Finally, bioassay miniaturization is addressed and fractionation strategies to increase the confidence level of analyte identification are discussed.