

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.

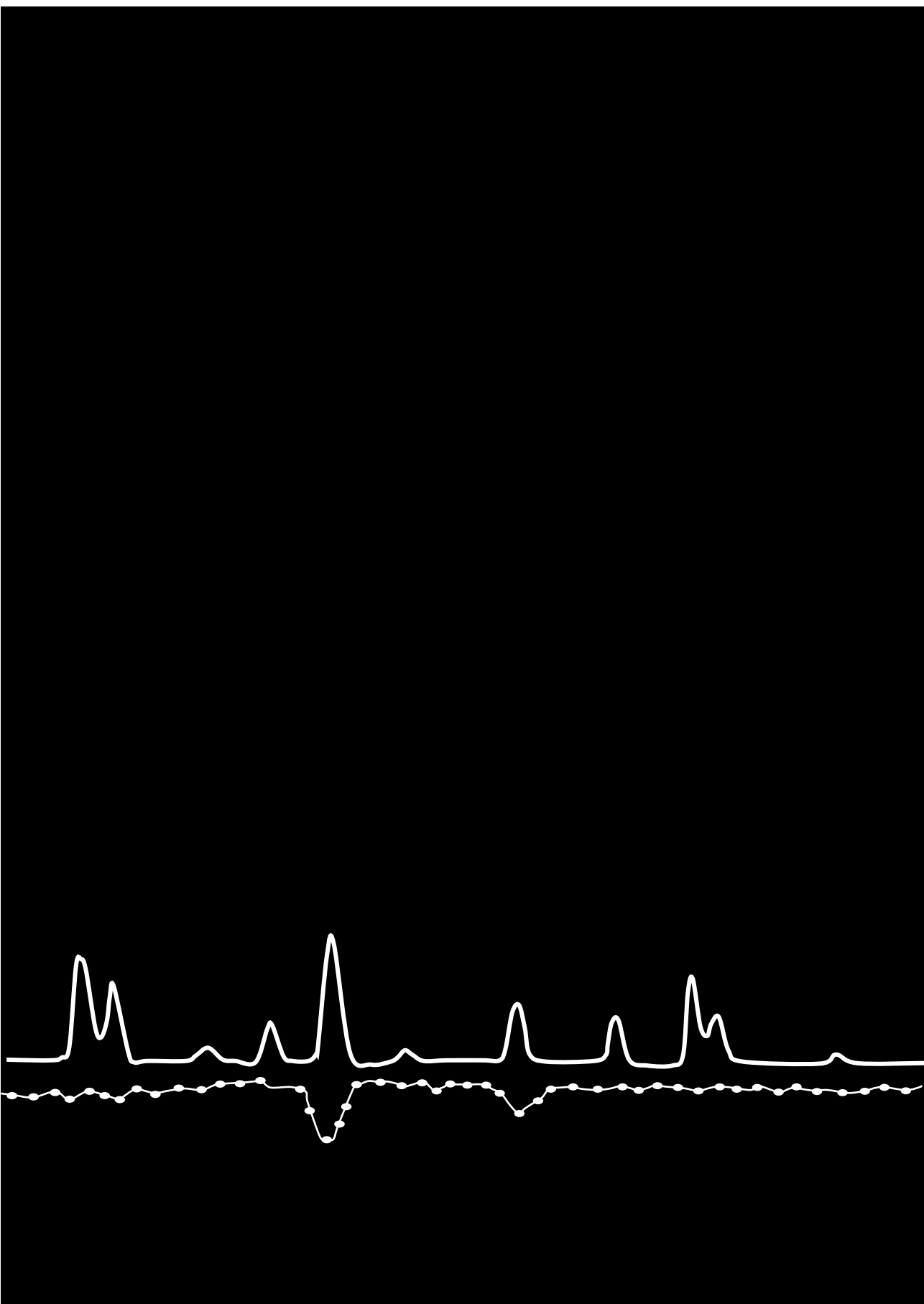
- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in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

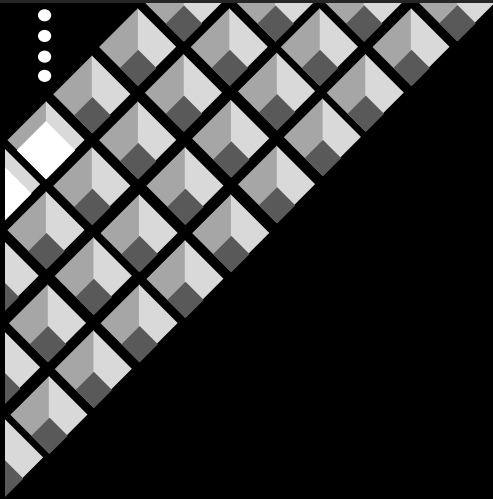
Take down policy

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:

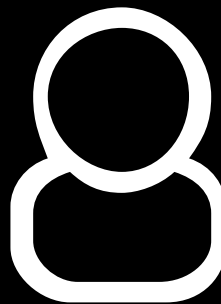
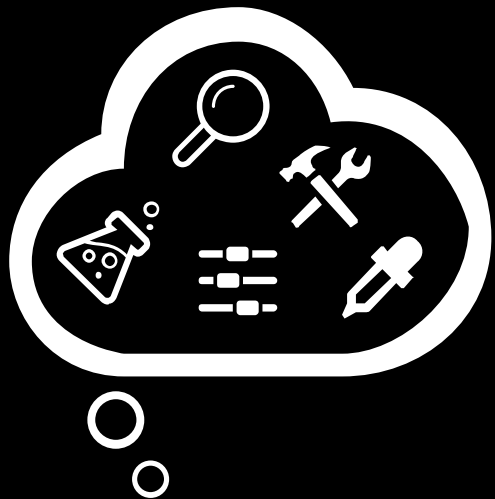
vuresearchportal.ub@vu.nl





Chapter 8

Summary and future perspectives



8.1 Summary

This thesis describes the development of new analytical methods for the detection and identification of bioactive substances in environmental extracts. These analytical methods are based on integrating chromatography, mass spectrometry and bioassays. The rationale for developing and applying new analytical methods in environmental screening settings lies in current bottlenecks in traditional effect-directed analysis (EDA) or bioassay guided fractionation (BGF) approaches, such as their success rate and labor intensity. Although many EDA approaches have resulted in the detection of environmental toxicants, they are typically very low throughput and require multiple fractionation cycles for analyte isolation. During this process sample losses may occur and eventually full structural identification of the bioactives is often not achieved. An overview of the current technology and approaches used for the profiling of bioactive mixtures for environmental and food research but also pharma settings are discussed in chapter 2.

Chapter 3 describes the development of a liquid chromatography (LC) based high resolution fraction collection platform. The platform is based on the chromatographic separation of a bioactive mixture after which the column eluate is split towards a fraction collector for subsequent bioassay analysis and MS for bioactive identification. This analytical method was designed for the efficient identification of endocrine disrupting toxicants in environmental mixtures. Because fraction collection is performed at a high frequency, the chromatographic separation is essentially maintained thereby potentially preventing the need for repeated fractionation cycles and increasing EDA throughput. Selection of the reporter gene assay for estrogen receptor activity enabled sensitive and selective detection of estrogenic and anti-estrogenic compounds commonly encountered in environmental extracts at low levels. As such, it provided information on the functional activity of the bioactives. The strength of the platform lies in its ability to reconstruct bioassay chromatograms by plotting the assay response of each fraction against its corresponding fraction time. Subsequent comparison with the parallel acquired MS chromatogram allowed for rapid pinpointing of bioactives in complex chromatograms. Since only a small percentage of the fractions is used for bioassay testing multiple biological endpoints could be tested using a battery of assays. Compared to traditional EDA studies this approach significantly increased throughput which was also shown in chapter 4 where successful detection of estrogenic compounds in plastic casings from electronic products (television, router, decorative items, printer) was demonstrated using the LC fractionation platform described in chapter 3. A total of eight products were analysed and estrogenic activity could be detected in four samples. Because additives have shown to

successfully migrate from plastic casings into the environment this may be an important source of estrogenic compounds.

In chapter 5, the development of analytics involving a fraction collector based on solenoid valve technology is described for accurate and contact-free high-resolution fraction collection of LC separations. Fraction collection in conventional LC is mostly based on the collection of droplets released by gravitational force from a fraction collection tip. In this scenario, repeatable fractionation may not be possible when small fractions need to be collected and the droplet release rate has to be increased. This can result in alternating fractions containing different volumes of LC effluent thereby affecting the repeatability of bioassay analysis. The implementation of a solenoid valve enabled non-gravitational ejection of very small droplets at a high frequency, thereby improving the fraction collection accuracy. To this end mechanical and electronic modifications to existing robotics were made. A mounting unit for the solenoid valve and a low dead volume unit to connect the solenoid valve to the LC tubing was designed. Also, an electronic signal converter was developed for accurate control of the pulse width and solenoid-valve supply voltage. Droplet ejection of the platform was tested with multiple LC solvents, and mixtures thereof, while covering a wide range of flow rates. Results showed there is little variation in the collected volumes and therefore the fraction collection was very repeatable, also at low flow rates. Peak broadening induced by the system was negligible. The potential of the system for bioactivity screening was successfully demonstrated by fractionating mixtures containing estrogenic and androgenic compounds, and a spiked environmental extract. All bioactives present in the analysed mixtures were successfully identified. Repeated fractionation showed that analytes were consistently collected in the same fractions indicating the system can be used for analyte purification and enrichment. The latter was also performed to demonstrate the feasibility of analyte enrichment for NMR analysis.

While bioactive isolation is predominantly performed with LC fractionation, GC remains the method of choice for many compound classes and is the preferred technique to study the occurrence and fate of many classes of environmental pollutants. However, compared to LC fractionation, collecting fractions from a GC separation is much more challenging. In contrast to available devices capable of collecting only a few fractions after GC, in chapters 6 and 7 a fractionation technique is described for the collection of complete GC separations. The platform is based on the post-column infusion of a vaporized trap solvent that condenses once directed outside the GC oven via an exit capillary allowing the collection of trap solvent droplets containing separated compounds. Parallel flame ionization detection (FID) (chapter 6) and MS (chapter 7) detection created the possibility to directly correlate fractions to chromatographic peaks. The

specific advantage of the MS detector is the availability of electron-ionization (EI) spectral databases useful for analyte identification. The technique's feasibility for toxicity screening purposes was demonstrated for environmental profiling of acetylcholinesterase inhibiting and anti-androgenic compounds thereby demonstrating its potential impact on EDA research. The technique proved to perform repeatable fractionation cycles (30x) without significant band broadening and could be used for analyte enrichment purposes.

8.2 Future Perspectives

8.2.1 Sample preparation

The implementation of online SPE for EDA could be a further advancement of the in this thesis presented LC-fractionation platform for HT-EDA in terms of sensitivity. The SPE procedure could be fully automated, would circumvent liquid handling, evaporation and reconstitution steps, and would require less sample material since the whole extract is used in the analysis rather than a fraction. Furthermore, it would be beneficial if also breakdown products and metabolites of the original pollutants could be detected. As indicated in Chapter 4, some compounds require bioactivation [3] and are not bioactive themselves. Using the current setup these compounds would be missed. Metabolic incubations with microsomes on environmental extracts could be performed prior to fractionation but would add another step to the EDA process and increases the sample complexity since metabolites formed will also become part of the mixture. Although electrochemical conversion (EC) is not exactly mimicking human metabolism, EC might function as an efficient alternative in the generation of metabolite-like products for EDA profiling [4]. Additionally, oxidation and/or degradation products of environmental pollutants that might be formed in the body or in the environment could artificially be produced by heat, acid or base induced degradation and/or by hydrogen peroxide oxidation [5]. In case of on-line generated EC metabolites, when placed in between the analytical column and fraction collector, eluting compounds can be electrochemically converted before fraction collection to test the bioactivity of EC products. Performing a fraction collection cycle with and without EC oxidation will clarify whether bioactivity can be attributed to the parent compound or an EC oxidation product which might mimic breakdown or oxidation products that can also be formed in the environment, or *in vivo*.

Stir bar sorptive extraction could be an interesting sample preparation technique for GC fractionation. In addition to simplifying sample preparation also the amount of material loaded on the column is increased since the whole extract is injected. This would reduce the number of fraction collection cycles to be performed to obtain sufficient material for offline analysis.

8.2.2 Fraction collection and miniaturization

Although high resolution fraction collection ensures that chromatographic separation is maintained for bioassay analysis, when the obtained peak capacity is insufficient, coelution will result leaving it unclear which analyte is responsible for the bioactivity. Recently, a strategy was presented where a second fractionation cycle was performed with the same crude mixture using an orthogonal separation [1]. Following this approach previously coeluting compounds were resolved in the orthogonal separation but were then coeluting with other compounds. Since both bioactivity and MS data was obtained in parallel for both separations, matching bioactives with parallel measured masses for both separations clarified the masses of the bioactive analytes of interest. An alternative approach could be to increase the peak capacity by applying a multidimensional separation as recently demonstrated by Ouyang et al. [2]. The success of the multidimensional approach, however, depends entirely on the selection of the correct stationary phase combination. Therefore, it would be beneficial to have some information about the sample composition before fractionation is started.

Fraction collection on flat surfaces would be interesting for MS analysis using MALDI. Even though MALDI spotter devices are available, they are almost exclusively developed and used for nano-LC separations. The fraction collection device presented in chapter 5 showed potential for droplet collection on flat surfaces and different strategies may be investigated to achieve MALDI-MS analysis such as spraying MALDI matrix solution on the target plate prior to and after fraction collection creating a sandwich like matrix-analyte-matrix MALDI spot. Yet another approach could be to post column infuse MALDI matrix prior to the spotting unit. This requires an additional investigation regarding optimal mixing and matrix concentration conditions. Finally, a recently published technology called e-MALDI might be interesting to combine with the solenoid valve based fraction collector. e-MALDI uses electrostatic forces for enhanced droplet drying and results show the sensitivity can increase with a factor of 2 to 30 times compared to traditional MALDI [6].

Bioassay miniaturization to microarray dimensions would be a future prospective/advancement of the current LC-nanofractionation spotter system and would be beneficial when only small sample amounts are available besides reducing reagent costs. Working on such a small scale would require highly accurate fraction collection and may be achieved by hyphenating (nano)LC with nanoliter dispensers. Successful nanoliter dispensing generally demands optimization of the dispensing parameters in relation to the dispensing solvents. When coupled to nanoLC, such a device needs to be capable to adjust the dispensing parameters along the nanoLC gradient for successful dispensing.

Furthermore, it is important to take measures preventing evaporation of subsequent microarray format bioassays.

8.2.3. *Analyte identification*

Toxicant identification is mostly depending on chromatographic and MS data. Although accurate mass data reduces the number of possible candidates, subsequent database searching can still return multiple compounds. Furthermore, many emerging pollutants are not present in current databases. A myriad of useful programs and software packages are available, but they often require an experienced user or a high level of programming skills for successful operation. Since in recent years MS manufacturers start to implement these tools in user friendly software, it is expected that identification success rates based on databases will further increase. Next to this, developments and advancements in ion-mobility MS could aid in bioactive identification and might especially be of interest to distinguish positional isomers. Furthermore, while ESI remains the method of choice for MS ionization after LC separations, additional analysis with APCI would benefit the ionization and detection of not readily ionizable bioactives. Especially certain classes of endocrine disruptors are notoriously difficult to ionize with ESI and complementary ionization techniques such as APCI and APPI are well known methods to increase sensitivity. Additionally, ionization in negative mode instead of the commonly applied positive ionization mode will, for example, result in much higher sensitivity for many compounds in this compound class. Another excellent technique for structural identification is NMR however, its use for toxicant identification is limited due to its low sensitivity. In chapter 5 the feasibility of analyte enrichment by repeated fraction collection cycles was demonstrated and could be useful for analyte identification with NMR when the toxicant identity cannot be revealed by MS.

The GC fractionation platforms presented in chapter 6 and 7 would benefit from the implementation of a large volume injector (LVI) thereby reducing the number of fraction collection cycles required to obtain a sufficient amount of material collected for off-line analysis (e.g. bioassay or NMR). The application of the GC-fractionation platforms in other fields than environmental research is also of interest. In GC-olfactometry the human nose functions as biological detector but it may occur that an analyte of interest is not recognized by the olfactometrist due to blocking of receptors in the nose by a previously eluting compound. Performing GC-fractionation cycles using water as a trap solvent would allow to taste fractions offline and could function as a complementary tool to traditional olfactometry approaches.

The analytical nanofractionation based HT-EDA platforms presented in this thesis, enable EDA with enhanced throughput compared to traditional EDA approaches, and therefore may encourage implementation of the technology as an additional monitoring tool in, for example, water laboratories. Conventional monitoring is mostly based on target analysis of selected compounds, but may leave toxic compounds overlooked. To overcome this shortcoming, suspect and non-target screening methods are increasingly implemented in water laboratories, and there is a growing interest in effect-based analysis by means of bioassay testing. In its current status, the LC fractionation platform could function as a tool to investigate bioactivity that cannot be explained by current target, suspect and non-target screening. However, the implementation of such a platform with the necessary facilities may still be a hurdle for smaller laboratories. Moreover, operation of the platform requires an operator trained in both the analytical and the biological aspects of EDA. To create a general acceptance and stimulate use of the HT EDA approach as presented in this thesis, the development of an easy to use commercially available device would be needed. Such a device should include an advanced chromatographic system, a high density well plate holder preferably allowing automated nano-fractionation of multiple samples for bioassay analysis (or e.g. NMR pre-purification), and a well plate transfer unit to a liquid handler enabling a battery of assays to be run in an automated fashion. In parallel, via a post-column split, a high-resolution MS capable to perform MS/MS will be essential for successful structural compound identification. Finally, automated data processing including molecular formula calculation based on accurate mass and isotope pattern, database searching, retention time matching, and structure activity prediction of the potential bioactives should result in a HT EDA device returning lists of only a few candidates per bioactivity found. Once such a device is available, EDA might become a commonly used tool for environmental non-targeted toxicity profiling. This tool may eventually also be applied routinely in, for example, pharmacological, food and doping research.

References

- [1] R.A. Otvos, M. Mladic, G. Arias-Alpizar, W.M.A. Niessen, G.W. Somsen, A.B. Smit, J. Kool, At-Line Cellular Screening Methodology for Bioactives in Mixtures Targeting the 7-Nicotinic Acetylcholine Receptor, *J. Biomol. Screen.* 21 (2016) 459–467. doi:10.1177/1087057115625307.
- [2] X. Ouyang, P.E.G. Leonards, Z. Tousova, J. Slobodnik, J. De Boer, M.H. Lamoree, Rapid Screening of Acetylcholinesterase Inhibitors by Effect-Directed Analysis Using LC x LC Fractionation, a High Throughput in Vitro Assay, and Parallel Identification by Time of Flight Mass Spectrometry, *Anal. Chem.* 88 (2016) 2353–2360. doi:10.1021/acs.analchem.5b04311.
- [3] M.M.H. Van Lipzig, J.N. Commandeur, F.J.J. De Kanter, M.C. Damsten, N.P.E. Vermeulen, E. Maat, E.J. Groot, A. Brouwer, M.H.A. Kester, T.J. Visser, J.H.N. Meerman, Bioactivation of dibrominated biphenyls by cytochrome P450 activity to metabolites with estrogenic activity and estrogen sulfotransferase inhibition capacity, *Chem. Res. Toxicol.* 18 (2005) 1691–1700. doi:10.1021/tx0501233.
- [4] D. Falck, A.J. Oosthoek-De Vries, A. Kolkman, H. Lingeman, M. Honing, S.S. Wijmenga, A.P.M. Kentgens, W.M.A. Niessen, EC-SPE-stripline-NMR analysis of reactive products: A feasibility study, *Anal. Bioanal. Chem.* 405 (2013) 6711–6720. doi:10.1007/s00216-013-7158-9.
- [5] L.A. Marques, J. Kool, F. de Kanter, H. Lingeman, W. Niessen, H. Irth, Production and on-line acetylcholinesterase bioactivity profiling of chemical and biological degradation products of tacrine., *J. Pharm. Biomed. Anal.* 53 (2010) 609–16. doi:10.1016/j.jpba.2010.04.015.
- [6] O. Kudina, B. Eral, F. Mugele, E-MALDI: An Electrowetting-Enhanced Drop Drying Method for MALDI Mass Spectrometry, *Anal. Chem.* 88 (2016) 4669–4675. doi:10.1021/acs.analchem.5b04283.