Journal of Chromatography A, xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Full length article

# Selective two-dimensional effect-directed analysis with thin-layer chromatography

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ARTICLE INFO

*Article history:*
Received 11 May 2017
Received in revised form 17 August 2017
Accepted 4 October 2017
Available online xxx

*Keywords:*
Two-dimensional separation
Acetylcholinesterase inhibition assay
Elution head-based extraction
Peak capacity
Orthogonality

ABSTRACT

There are thousands of organic trace substances in the environment that are not fully characterized, and evaluation of their relevance to the ecosystem is difficult. Effect-directed analysis (EDA) is a suitable tool to assess the effects of a substance via in-vitro bioassays, which can provide information about the relevance of the substance. High-performance thin-layer chromatography (HPTLC) has been shown to be a good method for fractionation. Environmental samples, however, often have high complexity, which is why the peak capacity of HPTLC is not sufficient. Therefore, this study focused on the development of selective two-dimensional (2D) HPTLC-EDA to increase the peak capacity and facilitate the identification of effective compounds. Thus, only effective zones were selected in the first dimension in terms of heart-cutting and were transferred to the second dimension through elution head-based extraction. Three 2D approaches were developed and validated. The best results in terms of peak capacity and orthogonality were achieved when the retardation factors of the first dimension were used to adjust the mobile phase (MP) for the second dimension. Applying the acetylcholinesterase (AChE) inhibition assay as an example EDA, analysis of spiked surface water by 2D HPTLC-EDA allowed zones with neurotoxic effects to responsible substances to be assigned. The 2D separation reduced the complexity of effective zones and thus facilitated the subsequent identification of effective compounds. Knowledge about a substanceís effects enabled assessment of its relevance to the environment.

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## 1. Introduction

Because of human influences, the environment gets increasingly polluted with anthropogenic trace substances in addition to naturally occurring ones [1]. Many of these substances have not been characterized, and their impact on humans and the ecosystem has not been sufficiently studied [2]. The challenge is to evaluate the relevance of these various contaminants and to select the important ones. Therefore, effect-directed analysis (EDA) – a combination of physicochemical fractionation, bio-testing and chemical analysis – is a suitable technique [3]. Through fractionation of pollutants in complex samples it is possible to assign bioactive substances to the detected effects in a certain end point [4].

For volatile compounds, gas chromatography coupled with mass spectrometry (GC–MS) is used for EDA because of the good peak capacity and the wide availability of mass spectral databases [5]. However, the limitation of GC with thermo-labile and non-volatile compounds and the challenge to trap the compounds from gaseous phase after separation enables the use of high-performance liquid chromatography (HPLC), which gathers broader substance diversity. Today, HPLC is a frequently used separation technique for EDA [6]. High-performance thin-layer chromatography (HPTLC) has proven to be a particularly suitable separation technique for EDA [7,8]. In contrast to column chromatography, HPTLC is an open separation system, and since the layer is solvent-free after separation, an in-vitro bioassay can be applied directly [9]. It is also possible to vary the applied sample volume over a wide range to increase the sensitivity of the method. Identification of effective compounds is realizable through coupling to a mass spectrometer (MS) or a nuclear magnetic resonance spectrometer (NMR) [10,11].

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https://doi.org/10.1016/j.chroma.2017.10.009
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However, despite all these advantages, the main disadvantage of HPTLC is the lower peak capacity compared to HPLC [6].

For improvement of HPTLC peak capacity, one-dimensional (1D) gradient development was already shown to be a good strategy [12,13]. In that method, the plate is developed in a stepwise manner with increasing migration distances and different solvent composition within each step. For environmental samples, the peak capacity of such gradient development is often insufficient, which is why a two-dimensional (2D) separation strategy was developed in this study. Many studies have described comprehensive 2D HPTLC where the complete sample was separated on the same plate but in two directions [14–16], but a selective 2D HPTLC-EDA is presented here for the first time. Also a study about 2D HPLC-AChE was identified, where a 2D on-line HPLC separation was applied to sewage water. But the main difference of the study to our one is, that the bioassay was not applied in two dimensions but only after the second separation [17]. We used the elution head-based extraction technique to transfer effective zones from the first to the second dimension [18]. Thereby the elution head is pressed to the layer to seal the zone which should be extracted. The elution solvent flows over the sealed zone and washes out the compounds, where a frit in the elution head restrains solid components from the layer and matrix [19]. After the separation in each dimension, the acetylcholinesterase (AChE) inhibition assay, an enzyme test for the detection of neurotoxic effects, was used [20]. The coupling of HPTLC and AChE assay is applied frequently for investigation of acetylcholinesterase inhibiting compounds in plant extracts [21]. But the assay procedure is quite different depending on the user. A significant variation is the application of AChE onto the HPTLC plate. This is done either by spraying [22,23] or by immersing the plate in the enzyme solution [24]. Various substrates are also used, e.g. 1-naphtyl acetate or 5,5′-dithiobis-(2-nitrobenzoic acid) [21]. For the present study, the plate was immersed in the AChE solution and 3-indoxyl-3-acetate was used as substrate. With our developed workflow, only effective zones were transferred to the second dimension so that substances without any effect were already excluded after the first dimension. Through the second separation, a further fragmentation of the effective zones could be achieved with the aim of prioritizing effective compounds of interest. The next important step was the coupling of this method to further analytical techniques such as MS or NMR with the aim of identifying the prioritized effective compounds.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ultrapure water was received with an ultrapure apparatus (Purelab Ultra, Elga LabWater, Lane End, UK). Acetonitrile (Rotisolv ≥ 99.95 %, LC–MS-Grade), 2-propanol (Rotisolv ≥ 99.95 %, LC–MS-Grade) and acetone (Rotisolv ≥ 99.9 %, HPLC) were purchased from Carl Roth (Karlsruhe, Germany). Dichloromethane and *n*-hexane were purchased from J.T. Baker (Center Valley, USA). Methanol (AMD Chromasolv ≥ 99.9%) was supplied by Sigma-Aldrich (Steinheim am Albuch, Germany). Chloroform (HiPerSolv Chromanorm) and ammonia (25%, AnalaR Normapur) were purchased from VWR International (Bruchsal, Germany), and formic acid (98–100%), ethyl acetate (Uvasol) and toluene (Uvasol) were supplied by Merck (Darmstadt, Germany).

For the AChE inhibition assay, acetylcholinesterase Type VI-S (2000 U/vial) from electric eel and ascorbic acid and 3-indoxyl-3-acetate from Sigma-Aldrich were used. Dimethyl sulfoxide, bovine serum albumin, hydrochloric acid (32%) and 2-amino-2-(hydroxymethyl)-1,3-propanediol were supplied by Merck (Darmstadt, Germany).

### 2.2. Standards

Pure standard substances were acquired from various suppliers (Carl Roth, Karlsruhe, Germany; LGC Standards, Teddington, UK; Merck, Darmstadt, Germany; Sigma-Aldrich, Steinheim am Albuch, Germany; Thermo Fischer, Karlsruhe, Germany; VWR International, Bruchsal, Germany). Standard stock solutions were prepared by dissolving 10 mg of each pure substance in 100 mL methanol and storing at −18 °C. To prepare respective standard solutions, the stock standards were diluted in methanol to the required concentration. Standard solutions were used for four weeks.

For mobile phase (MP) development, five standard solutions (A–E) with 5–8 compounds (10 ng/μL) were used. Their exact composition is given in Fig. 2. To gather a possibly broad retention area the substances were chosen according to their polarity. The $\log K_{ow}$ values were determined using the KOWWIN software (v1.68) from US EPA. For the 35 model compounds the middle value of $\log K_{ow}$ was 3.0 with a range from 0.1 (cefixime) up to 6.1 (bromophos-ethyl). The stability of chromatography was examined with a mix consisting of nine substances (1,3,6-naphthalenesulfonic acid, 1,5-naphthalenesulfonic acid, 1-naphthalenesulfonic acid, theobromine, caffeine, thiourea, *N*-phenylacetamide, benzamide, and *N*,N-dimethyl-4-[(*E*)-phenyldiazenyl]aniline) with 10 ng/μL [8]. A multi-component standard (83 μg/L) with 448 environmentally relevant anthropogenic trace substances, such as pharmaceuticals, herbicides, X-ray contrast agents, and others, was used for the determination of a frequency distribution across the retardation factors ($hR_F$). The spiking of surface water was performed with a mix consisting of 50 neurotoxic substances (Table S1).

### 2.3. Sampling and sample preparation

A grab sample of surface water was taken from the river Danube (Leipheim, Germany). The sample (1 L) was spiked with the neurotoxic mix to a final concentration of 4 μg/L. Enrichment was performed by solid phase extraction (SPE) with an enrichment factor of 1000.

Enrichment was performed by solid phase extraction (SPE) with Bond Elut Plexa cartridges (Agilent Technologies, Santa Clara, USA) containing a non-polar divinylbenzene-based neutral polymeric sorbent. The cartridges were conditioned with each 5 mL *n*-hexane, dichloromethane, acetone, methanol, and ultrapure water. The sample was filled in a storage vessel and ran over the cartridge, powered by a peristaltic pump with a flow of 2.5 mL/min. After loading, the cartridges were dried for 45 min in an airstream. Three solvents were used for elution. At first, 3 mL methanol (0.4% v/v ammonia) was used. The second eluent consisted of a 50:50 v/v mixture of methanol (0.4% v/v ammonia)-ethyl acetate (4 mL), and the third eluent was dichloromethane (3 mL). Evaporation was performed in a gentle stream of nitrogen, and 50 μL of ultrapure water was added as keeper before evaporation of dichloromethane. The residue was diluted with methanol to a volume of 1 mLs conditioned and eluted the same way the sample cartridge was treated but without any contact with a sample.

Recovery rates of SPE were determined for the 35 model compounds used for method development. The substances were spiked to deionized water before and after solid phase extraction to get the same matrix in both samples. Concentrations were chosen that 100% recovery would lead to 10 μg/L after the enrichment. Measurement was done via HPLC-HRMS (see Chapter 2.4). The median of recovery achieved 87.5% with a 10% quantile of 46.1%. One substance could not be detected in the positive ESI mode, why it was not considered (cefixime). With that a medium enrichment factor of 875 could be calculated.

Please cite this article in press as: L. Stütz, et al., Selective two-dimensional effect-directed analysis with thin-layer chromatography, J. Chromatogr. A (2017), https://doi.org/10.1016/j.chroma.2017.10.009

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