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Sharp-bounded zones link to the effect in planar chromatography-bioassay-mass spectrometry

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ABSTRACT

The traditional direct bioautography workflow was substantially altered to yield narrow, sharp-bounded effective zones. For the first time, microorganisms quantitatively detected the single effective compounds in complex samples, separated in parallel on a planar chromatogram. This novel effect-directed workflow was demonstrated and optimized for the discovery of endocrine disrupting compounds (EDCs) reacting with the human estrogen receptor down to the femtogram-per-zone range, like 250 fg/zone for 17β-estradiol (E2). For application volumes of up to 0.5 mL, estrogen-effective compounds could directly be detected in complex samples at the ultratrace level (ng/kg-range). Sharp-bounded, estrogen-effective zones discovered were further characterized by direct elution into the mass spectrometer. HPTLC-ESI-MS mass spectra of (xeno)estrogens were shown for the first time. Owed to the substantially improved zone resolution, compound assignment was reliable and a comparison of the receptor affinities was conducted for six (xeno)estrogens. Also, long-term cell cultivation of the genetically modified yeast was demonstrated on the HPTLC plate. The optimized HPTLC-pYES workflow was proven for real food samples, exemplarily shown for beer. The general applicability of generating sharp-bounded zones was successfully proven by transfer of the fundamentally improved workflow to the *Bacillus subtilis* bioassay used for discovery of antibiotics in plant extracts. This new era of quantitative direct bioautography in combination with mass spectrometry will accelerate the scientific understanding in a wide application field via the streamlined access to fast and reliable information on effective components in complex samples.
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1. Introduction

Over 80 million low molecular weight substances are registered in Chemical Abstracts Service. Daily increasing in number, the threat of potential (xeno-)contaminants and residues, inclusive of their degradation products and metabolites, to the global food/feed chain increases. Even excellent multi-residue methods will not cope with this challenge or can only contribute to a certain extent in identifying noxious matter [1–4]. Additionally, food is complex in matrix and less is known about foodborne noxious matter or effects on health and wellness after intake. Consequently, a kind of effect-directed analysis (EDA) that directly combines bioassays with chromatography (the domain of bioautography) would increasingly attract attention in the field of food science with regard to functional food, food safety, foodborne diseases and health care. It could fill the present lack in comprehensive information [5] and contribute answers to current contradictory analytical results [6]. However, for almost 70 years [7], traditional bioautography lacked to convince analysts due to diffuse, broad spots after long incubation times on the plate. Using bioassays performed under aqueous conditions in combination with planar chromatography, a poor zone resolution was obtained. Being not suited for identification and quantitation, it was mainly used for qualitative profiling [8–11]. Hence, in this study, the traditional direct bioautography workflow was substantially altered to generate sharply bounded zones.

The improved workflow was applied to the analysis of endocrine disrupting compounds (EDCs). EDCs are important bioactive target compounds being not only naturally present in food, but also a source of contamination due to an increasing xeno-pollution. Natural EDCs in vertebrates include, for example, estrone (E1), E2 and estriol (E3). Foodborne EDCs are mostly either phytoestrogens or contaminants, i.e. plasticizers like nonylphenol (NP) and bisphenol A (BPA) [12] or drugs like 17α-ethinylestradiol (EE2) [13]. These compounds can effect the endocrine system of mammalians due to their binding to the human estrogen receptor (hERα or β). The

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current EDA of EDCs is complex. In many experimental procedures, environmental samples were purified and fractionated, including high-performance liquid chromatography (HPLC) [14], solid phase extraction [15] and thin-layer chromatography (TLC) [16]. Quantitative detection was performed by HPLC-tandem mass spectrometry (MS/MS) [15,17,18], gas chromatography-MS/MS [19] or HPLC-quadrupole time-of-flight mass spectrometry (QTOF MS) [20]. The most common *in vivo* assays are the mouse uterotrophic assay [21] as well as the rainbow trout, zebrafish [22] or mollusks experiment (*Potamopyrgus antipodarum*) [23]. The major advantage of these *in vivo* assays is the systemic analysis of EDC influences, by covering all areas of the endocrine system, such as metabolism, absorption, distribution and excretion. The disadvantage is that the environmental samples have to be purified to avoid inflammatory reactions *in vivo* (cannot directly be used). More time- and cost-effective and independent from animal experiments are *in vitro* assays, such as human breast cancer cells MCF-7 [24], a proliferation assay termed E-screen, various yeast estrogen screens (YES) [25], hepatocytes assay [26], or receptor-binding assays [27].

For example, the liquid (L)-YES used recombinant *Saccharomyces cerevisiae* carrying the DNA sequence of the hER together with the reporter gene lac-Z which is fused to an estrogen responsive element (ERE) [28]. Compounds with estrogen activity induced the expression of the lac-Z gene, which encoded the enzyme β-galactosidase, reacting with a substrate to generate a colored or fluorescent product. For the latter, 4-methylumbelliferyl-β-D-galactopyranoside (MUG) was used reacting to the blue fluorescent methylumbelliferone (MU) [29]. L-YES was initially applied on a planar plate (without chromatography), but broad spots were obtained after several hours of plate incubation with the aqueous yeast cell suspension [29]. Although the transfer of L-YES to the TLC/HPTLC field has been demonstrated [30–32], the clear substance assignment and reliable quantification of substance mixtures always failed due to arising zone diffusion. In the present study, a substantial improvement was demonstrated resulting in sharp-bounded zones even after several hours of incubation with aqueous bioassay media. This achievement is valid for aqueous bioassays in general, which was demonstrated by transfer to the *Bacillus subtilis* bioassay.

2. Materials and methods

2.1. Chemicals and materials

Bidestilled water was generated using a Heraeus Destamat Bi-18E (Thermo Fisher Scientific, Schwerte, Germany). Dimethyl sulfoxide, glycerol, ethanol, methanol, toluene, ethyl acetate, *n*-hexane, sodium hydroxide pellets, glycine and 4-methylumbelliferyl-β-D-galactopyranoside (MUG) (all HPLC grade, Roth, Karlsruhe, Germany) as well as agar, trypan blue, copper sulfate, ammonium formate, D-glucose and yeast nitrogen base (all Fluka Sigma–Aldrich, Steinheim, Germany) were purchased. *Saccharomyces cerevisiae* BJ3505 (protease-deficient, MATα, PEP4: HIS3, Prb1Δ1.6R, HIS3Δ200, lys2-208, trp1Δ101, ura3-52) were generated by McDonnell et al. [28,33] and obtained from W. Schwack, University of Hohenheim, Stuttgart, Germany, who received it from S. Buchinger, German Federal Institute of Hydrology, Koblenz, Germany. HPTLC plates RP-18 W and plates of Table S-1, citric acid and disodium hydrogen phosphate were delivered from Merck Millipore, Darmstadt, Germany. 17β-estradiol (E2, 98.5%) and 17α-ethinylestradiol (EE2, 98%) were from Dr. Ehrenstorfer, Augsburg, Germany, whereas estrone (E1, 95%) and estriol (E3, 95%) were from Cayman Chemical Company, Ann Arbor, MI, USA. 4-*n*-Nonylphenol (NP, 98%) and bisphenol A (BPA, 97%) were bought from Alfa Aesar, Karlsruhe, Germany.

2.2. Stock and standard solutions

Stock solutions of 2 mg/mL (S0) were prepared with ethanol (for E2, E3, EE2 and BPA) or methanol (for E1 and NP) and stored at −20 °C until use. For standard solutions, stock solutions were diluted to 50 ng/μL (S1), 2.5 ng/μL (S2), 50 pg/μL (S3), 1 pg/μL (S4) and 0.1 pg/μL (S5).

2.3. pH measurement of TLC/HPTLC sorbents

The plate was cut into sections using the TLC Plate Cutter (CAMAG, Muttenz, Switzerland). One plate strip was dipped into lac-Z buffer (pH 7) and the other in citrate buffer (pH 12), then dried. A third strip was used without any treatment. About 3 cm^2 of each buffered and not-buffered silica gel phases were scraped off the glass carrier into a beaker and suspended in 10 mL water. The pH value of this suspension was measured using a pH meter.

2.4. Cell cultivation

Experimental steps involving the recombinant estrogen-sensitive yeast cells *Saccharomyces cerevisiae* BJ3505 [28,33] were performed in a S1 certified biochemical laboratory. All experimental studies were based on stock (−80 °C) of the recombinant yeast cells stored in 30% glycerol supplemented growth medium consisting of D-glucose (1 g/L), yeast nitrogen base (6.8 g/L) and 14 amino acids [34]. From the stock, cells were streaked on an agar plate [35] with growth medium (30 °C, 2 days) to supply colonies for further suspension cultivation. The suspension cultures were inoculated in 20 mL growth medium and cultured at 30 °C by shaking at 100 rpm for 18 h. Prior to the assay, cell concentration and viability determination was performed using a hemocytometer and trypan blue staining (0.4%). For the pYES, the cell number was adjusted to 2.5×10^8 cells in 50 mL with 150 μM copper sulfate supplemented growth medium. Cells were split daily by 1:10 dilution to remain constant cell quality within the exponential growth phase for further studies.

2.5. Long-term (24 h) cell cultivation on the HPTLC plate

One recombinant yeast cell colony on the agar plate was suspended in the culture medium (20 mL) and pre-cultivated for 24 h in the incubator at 30 °C. This culture suspension was diluted 1:10 with the medium, in which the developed plate was immersed as described. The plate was incubated in a horizontal position in a plastic box under almost 100% humidified air at 30 °C for 24 h.

2.6. HPTLC-bioassay

Standard solutions were applied in the range of 0.1–25 μL on TLC/HPTLC plates as 6.5 mm bands by the Automatic TLC Sampler 4 (ATS4, CAMAG). Respective standard mixtures were obtained by overspraying of the individual standard solutions. After drying the start zones with cold air of a hair dryer for 1 min, development was performed in a Twin Trough Chamber (20 × 10 cm, CAMAG) using n-hexane–toluene–ethyl acetate 4:1.5:1 (*V*/*V*/*V*) up to a migration distance of 70 mm. After analogous drying of the developed zones for 2 min, the chromatogram was documented at UV 366 nm illumination (TLC Visualizer, CAMAG). The chromatogram was automatically immersed into the prepared cell suspension (Chromatogram Immersion Device, CAMAG) at a speed of 3.5 cm/s and an immersion time of 5 s. The wet plate was incubated in a horizontal position in a plastic box under almost 100% humidified air at 30 °C for 3 h and dried (analogously for 2 min). For determination of the resulting β-galactosidase, the plate was analogously immersed into the substrate solution. Therefor 20 mg MUG

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