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Modern direct bioautography of endocrine active compounds

M. Sc. Ines Klingelhöfer and Prof. Dr. Gertrud Morlock, Institute of Nutritional Science and Interdisciplinary Research Center of the Justus Liebig University Giessen, are active members of the Expert Group for the development of the yeast estrogen screening in combination with planar chromatography (HPTLC-pYES). In July 2013, they showed a direct bioautography method (DB) which led for the first time to sharp substance zones. By such a meaningful, effect-directed screening of complex samples and the subsequent identification of these bioactive substances, an extensive food and natural product screening is now feasible.

Introduction

Endocrine active compounds are ubiquitous in our food, in nutritional supplements and in cosmetics. They affect the health of humans and animals by controlling and regulating essential functions of metabolism, growth and development. These substances include both natural estrogens, such as ß-estradiol and estrone, which pass through excreta of humans and animals in the environment and therefore in the food circulation, as well as plasticizers, pesticides and biocides, which contaminate the food through industrial production. Another group of substances are phytoestrogens – phytochemicals in nature. A specific detection method for these substances that act like estrogens is HPTLC-pYES.

The bioautography has been used for almost 70 years [1], however, the strong diffusion of the substance zones (during several hours of incubation with the aqueous culture medium) has been a substantial disadvantage. Any attempt to improve the DB was unsuccessful, and highly diffuse zones were not really convincing to the analyst.

The DB shown here, using the example of HPTLC-pYES, was substantially improved and for the first time carried out on RP-18 W plates [2, 3], which were considered to be not applicable for the DB (as these phases did not show the bioassay response). Since water has no elution strength on the reversed phase, and long incubation times in an aqueous medium result in less diffusion of the zones, we pursued to use water-wettable reversed phases. These as well as medium-polar layers appear clearly more suitable for DB than the previously used silica gel phase. The improved HPTLC-pYES method detects estrogen-effective substances as sharply-bounded

zones in complex sample matrices. The excellence of these new DB methods is illustrated by the fact that the biological detection can also be used for quantification.

Sample preparation

Propolis tinctures were used directly or diluted 1:10 with ethanol. The content of propolis capsules was treated with 1 mL ethanol, mixed for 3 min (vortex), centrifuged at 15,000 g for 5 min and the supernatant analyzed. Propolis lozenges were pestled and 100 mg of this material was extracted analogously to the capsules.

Standard solutions

Estrone (E1), 17-estradiol (E2), 17-ethinylestradiol (EE2), estriol (E3), bisphenol A (BPA), 4-*n*-nonylphenol (NP) and caffeic acid phenethyl ester (CAPE) were dissolved in ethanol or methanol (0.1, 1 and 50 pg/µL and 2.5 and 50 ng/µL).

Chromatogram layer

HPTLC plate silica gel 60 RP-18 W (Merck), 20 x 10 cm, cut when necessary with the smartCut plate cutter

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), band length 6.5 mm, track distance 7.5 mm, distance from lower edge 8 mm and from the lateral edge 12 mm, application volume 0.1 µL/band (standards) and 0.1 – 5 µL/band (samples)

Chromatography

In the Twin Trough Chamber 10 x 10 cm or 20 x 10 cm with 5 mL or 10 mL *n*-hexane – toluene – ethyl acetate 8:3:2, migration distance 7 cm

Bioassay

The chromatogram was immersed into the yeast cell suspension (recombinant *Saccharomyces cerevisiae* BJ3505 cells, which express the human estrogen receptor (hERα) and carry the reporter gene lac-Z) and incubated for 3 h [2]. Estrogen-effective substances result in the release of β-galactosidase, which cleaves the substrate pyranoside (placed by a second immersion step) to 4-methylumbelliferyl-β-D galactopyranoside to the blue fluorescent dye 4-methylumbelliferone (MU).

Biodensitometry

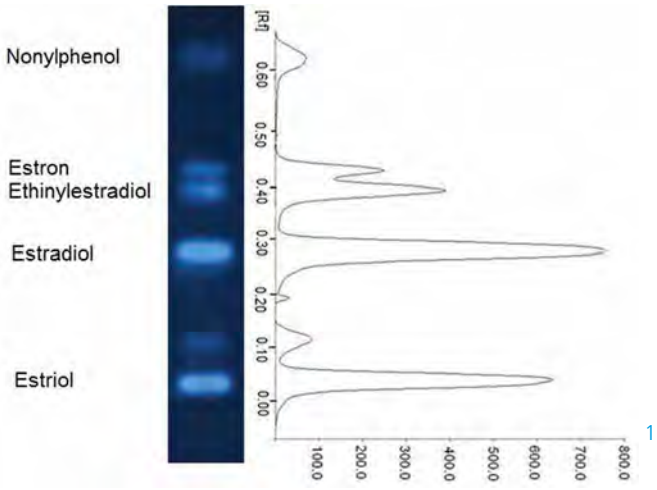
TLC Scanner 3 with winCATS, fluorescence measurement at 365/>400 nm, mercury lamp, slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, evaluation via polynomial regression

Mass spectrometry

Elution of the bioactive zones with methanol-ammonium formate buffer (10 mM, pH 4, 49:1, 0.2 mL/min) by TLC-MS Interface (elution head 4 x 2 mm) into the ESI-MS (CMS, Advion)

Results and discussion

For the detection of estrogen-effective substances in matrix-rich samples, up to 24 samples were separated on the HPTLC plate in parallel. The optimization of the HPTLC-pYES workflow and adaption of the yeast cell cultivation for the RP-18 W HPTLC plate was carried out with the standard substances E1, E2, EE2, E3 and NP. This resulted in sharp, blue fluorescent MU-zones.



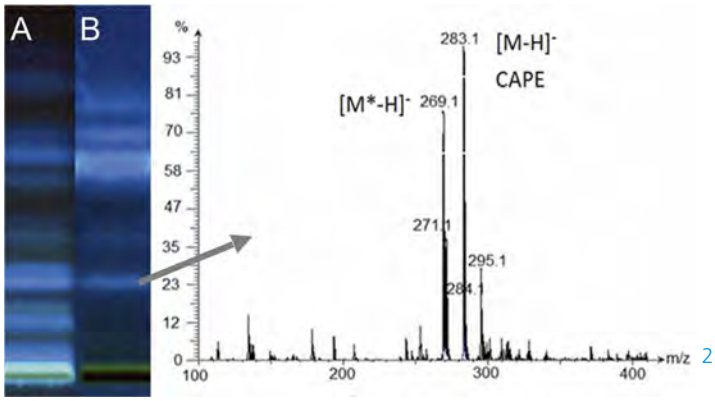
Sharply-bounded, blue fluorescent MU zones after 4 h of incubation in an aqueous medium; fluorescence measurement at UV 365/>400 nm

The response (blue MU-fluorescence) was generated by the activity of the yeast cells and subsequently measured conventionally (biodensitometry). LOD, LOQ and the maximal working range of the estrogen-effective substances were determined ($n = 3$, each with a newly prepared yeast cell culture) [2]. The detection of E2 was the most sensitive: depending on the method of calculating, the average LOD was 0.2 pg/band and the mean LOQ at 0.5 pg/band (calibration method in accordance

with DIN 32645) or at 0.5 and 1 pg/band, if it was calculated over the signal-to-noise ratio (S/N).

Substance (pg/band)						
Mean (n=3)	E1	E2	EE2	E3	BPA	NP
LOD (S/N 3)	25	0.5	2	n.d.	62.5×10^2	25×10^3
LOQ (S/N 10)	50	1	5	500	12.5×10^3	50×10^3
Working range	$25 - 25 \times 10^3$	0.5 – 50	$2 - 1 \times 10^3$	$5 \times 10^2 - 50 \times 10^3$	$62.5 \times 10^2 - 1 \times 10^6$	$25 \times 10^3 - 1 \times 10^6$

Using seven commercially available propolis samples, it was demonstrated that with no or only minimal sample preparation estrogen effective substances could be detected with excellent sensitivity, identified and quantified [3]. Many food extracts, such as the propolis samples shown here, showed natively blue fluorescent zones [2, 3]. For the exclusion of false positive MU-zones, the entire bioassay procedure was carried out without yeast cells. The corresponding negative controls showed no natively fluorescent zones. The implementation of a negative control was considered essential to guard against false positive findings.



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Chromatograms of a propolis sample: natively fluorescent zones (A) and blue fluorescent MU-zones for HPTLC-pYES (B); exemplary identification of a zone (CAPE) by ESI-MS (TLC-MS Interface)

CAPE was found in all propolis samples. For quantification, the recovery in the range of 10 to 150 ng/band was determined. The average recovery of all the concentration levels was 95 % (*%RSD* = 15 %; $n = 7$). Via biodensitometry (5-point calibration), the CAPE contents of propolis samples corrected over the average recovery rate were between 710 and 2387 µg/g and agreed very well with literature values (using SPE and HPLC-MS/MS) [3]. Through this optimized DB method, it was possible for the first time – due to the sharply defined zones – to detect individual bioactive ingredients in complex samples with sufficient sensitivity to make a unique assignment and to identify and to quantify response generated by the yeast cells. Discovered active ingredients are easier to quantify by conventional detection modes (physically/chemically), if compared to biodensitometry. The latter served here as proof of the achieved performance quality, accuracy and reliability of this effect-directed analytics.

It is good scientific practice, to carry out the LOD and concentration information by biodetection via multiple analyses, comparing the values of each newly prepared yeast cultures. LODs in very good working yeasts were likewise reproducible at 250 fg/band E2 (S/N 3), but were not always reachable by newly prepared yeast cultures.

For important estrogen-effective substances the LODs are in the fg- and pg-range and thus allow the direct detection at trace levels without enrichment.

Further information is available on request from the authors.

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[2] I. Klingelhöfer, G.E. Morlock, J Chromatogr A 1360 (2014) 288

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