Identification and quantification of endocrine active substances such as (xeno)estrogens and phytoestrogens with a bioautographic HPTLC method

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Introduction

Bioactive compounds, such as endocrine disrupting chemicals (EDCs), are an important issue due to the globally increasing environmental pollution. EDCs are of natural or anthropogenic origin. Natural EDCs include, for example estrone (E1), 17β-estradiol (E2) and estriol (E3). Another group of natural EDCs are phytoestrogens such as genistein, apigenin and caffeic acid phenethyl ester (CAPE). Anthropogenic substances which are released into the environment are pesticides, fungicides, preservatives, plasticizers (4-nonylphenol, NP) or come from drugs (17α-ethinylestradiol, EE2). They can bind to human estrogen receptor α (ERα) and thereby activate or inhibit the endocrine system of humans or wildlife and are known as xenoestrogens. According to the relevance of the substances to mammalians, research focuses on the component analysis of the environmental samples as well as on their bioactivity.

Results and discussion

The possibility to transfer the liquid YES (L-YES) to the TLC/HPTLC field has already been demonstrated [1-4]. However, the clear substance assignment and quantification of substance mixtures always failed due to arising diffuse zones. The key was to prevent the extreme diffusion during several hours of plate incubation with aqueous cell suspensions. As water has a very high elution power on silica gel plates, zone diffusion effects have been rigorously after several hours of aqueous plate incubation. However, aqueous bioassays were *conditio sine qua non* for the surviving of microorganisms. Instead, lipophilic plate surfaces such as RP-8 or RP-18 were not water-wettable. A wettening of the chromatogram was not possible by immersion into the aqueous bioassay suspension or by spraying. Nevertheless, these lipophilic surfaces would substantially reduce the zone diffusion effect, as water has no elution power on such layers and thus the compounds would remain as a narrow and sharp band. Combining all this knowledge resulted in the usage of wettable lipophilic layers such as RP-18 W. For the first time, sharply bounded bands were obtained after 4 hours of aqueous incubation of the chromatogram (Fig. 1).

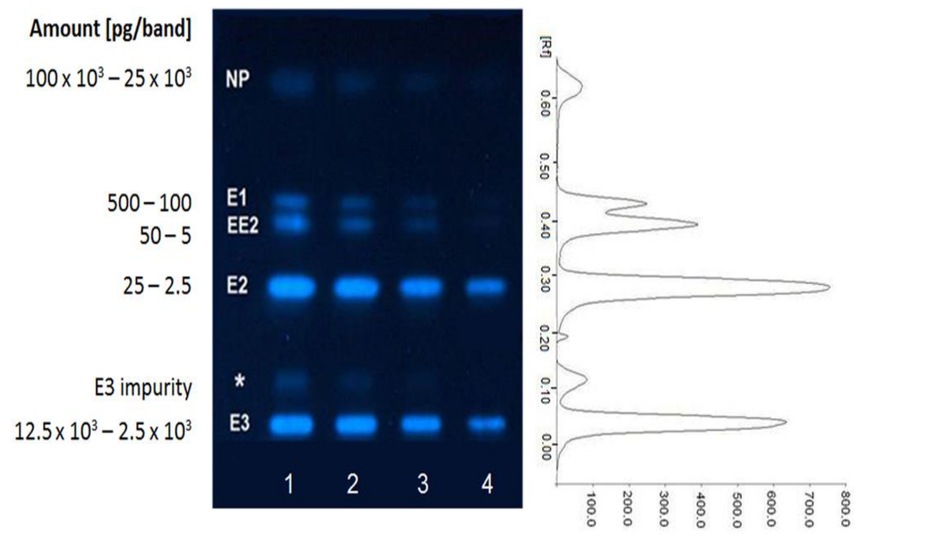

Fig.1 Sharp-bounded, clearly separated zones after 4 h aqueous bioassay application; natural estrogens E1, E2, E3, synthetic estrogen EE2 and xenoestrogen NP are detected as blue fluorescent zones, for some down to the femtogram-per-zone range

Substantial improvement of the bioautographic workflow

Within 5 hours, 20 samples can be analyzed in parallel with regard to any bioactive compounds, exemplarily shown for estrogens in complex food samples (Fig. 2). Step 1 is simultaneous development of up to 20 samples in parallel by HPTLC, step 2 is the pYES bioassay application for effect-directed detection and step 3 is the bioactive compounds characterization by MS). Pharmacologically active compounds were discovered in food due to their reaction with the human estrogen receptor down to the ng/L range.

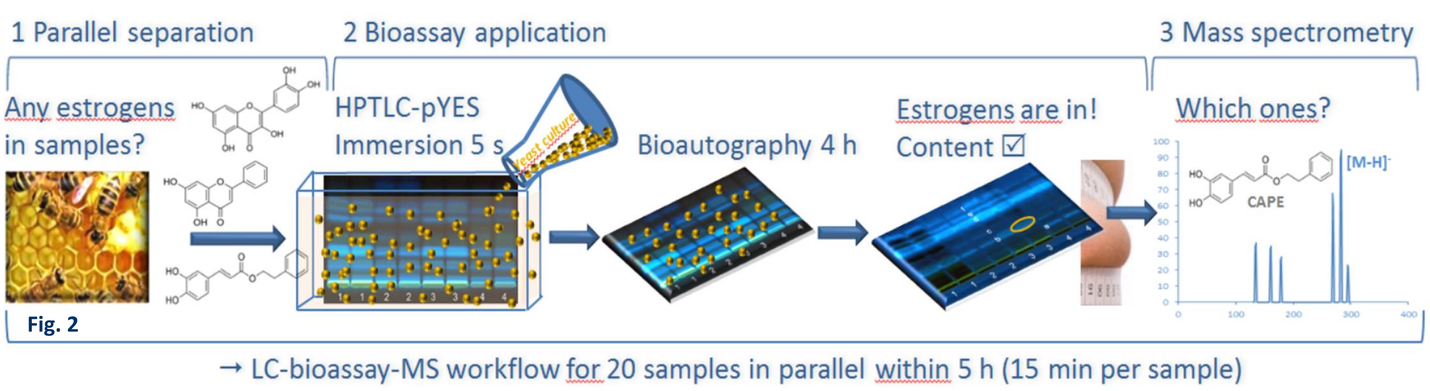

Fig. 2 Stepwise bioautographic workflow for many samples in parallel

Estrogenic properties of propolis samples

Commercially available propolis fluids were investigated without further sample preparation, except for dilution. The propolis samples as capsule and tablet formulations were extracted with ethanol. Standard and sample solutions (0.1-20 µL) were applied as 6.5 mm bands on the HPTLC plate RP-18 W. After development and bioautography (Fig. 3), the unknown bioactive zone in the propolis sample, preliminarily assigned as CAPE based on chromatographic and spectral characteristics, was verified through HPTLC-ESI-MS via the elution-head based TLC-MS Interface (Fig. 4).

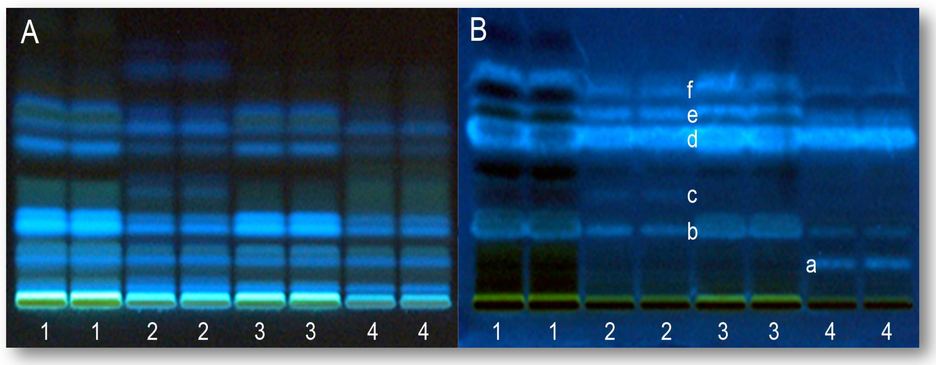

Fig. 3 Chromatograms of commercially available propolis samples at UV 366 nm for four propolis samples (0.3 µL, applied twofold) (A) after chromatography (native fluorescence) and (B) after direct bioautography (HPTLC-pYES) showing bioactive zones a-f; sample 1 is overloaded and fluorescence quenching is visible in the upper hR_F range

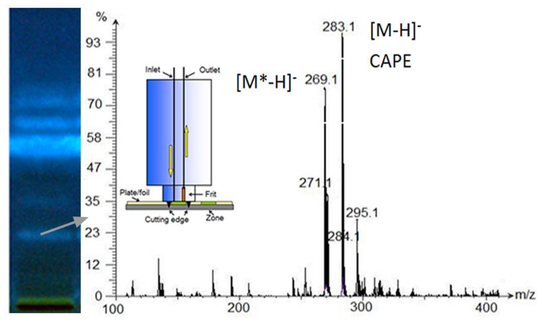

Fig. 4 HPTLC-ESI⁻-MS of an unknown ECD confirmed the unknown's identity to be CAPE: mass spectra of a CAPE standard zone eluted via the elution head-based TLC-MS Interface into the ESI-MS

Conclusions

For the first time, such an accurate and precise quantitation via direct bioautography was shown. The direct link to single active compounds in complex mixtures is most influential in the biological and analytical fields and across fields such as food science, biotechnology, biochemistry and medicine. The fundamental improvement shown led to clear compound assignments and reliable quantitation for the first time, despite long aqueous incubation times (investigated for up to 24 h). This achievement is valid for aqueous bioassays in general and thus applicable to all other bioassays.

References [1] M.B. Mueller *et al.* Chromatographia 60 (2004) 207–211. [2] S. Buchinger *et al.* Anal Chem 85 (2013) 7248–7256. [3] D. Spira *et al.* J Planar Chromatogr 26 (2013) 395–401. [4] A. Schönborn, A. Grimmer J Planar Chromatogr 26 (2013) 402–408.

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Interested in practical performance? Join the international pYES expert group!

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