New solvent systems to separate some estrogenically active compounds by high-performance thin-layer chromatography (HPTLC)

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Abstract

Two solvent mixtures for high-performance thin-layer chromatographic (HPTLC) separation of some compounds showing estrogenic activity in the yeast estrogen screen (YES) assay are presented. The new method, planar yeast estrogen screen (pYES) combines the YES assay and a chromatographic separation on silica gel HPTLC plates with the performance of the YES assay. For separation, the analytes were applied bandwise to HPTLC plates (10 × 20 cm) with fluorescent dye (Merck, Germany). The plates were developed in a vertical developing chamber after 30 min of chamber saturation over a separation distance of 70 mm, using cyclohexane–methyl ethyl ketone (2:1, V/V) or cyclohexane–CPME (3:2, V/V) as solvents. Both solvents allow separation of estriol, daidzein, genistein, 17β-estradiol, 17α-ethinyl estradiol, estrone, 4-nonylphenol and bis(2-ethylhexyl) phthalate.

Keywords High-performance thin-layer chromatography · Thin-layer chromatography · Estrone · 17β-Estradiol · 17α-Ethinyl estradiol

Abbreviations

HPTLC High-performance thin-layer chromatography
TLC Thin-layer chromatography
E1 Estrone
E2 17β-Estradiol
EE2 17α-Ethinyl estradiol
E3 Estradiol
DEHP Bis(2-ethylhexyl) phthalate
4NP 4-Nonylphenol
BPA Bisphenol A
CPME Cyclopentyl-methyl ether
MEK Methyl-ethyl ketone

1 Introduction

With the European Decision EU 2015/495, three steroidal estrogens, estrone (E1), 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2), were included in the “watch list” of the Water Framework Directive (WFD) [1]. Consequently, these substances must be chemically monitored, as the European Commission has set the maximum acceptable detection limits for E1 and E2 at 400 pg/L and for EE2 at 35 pg/L [1]. Most routine analytical methods used by the Member States cannot meet these requirements, especially for EE2 [1–3].

High-performance thin-layer chromatography (HPTLC) is the only chromatographic method in which detection takes place in the stationary phase. This makes the method ideal for combination with biological detection systems. The planar yeast estrogen screen (pYES) combines chromatographic separation on silica gel thin-layer plates with performing the YES test on the planar surface of such a plate [4–9]. The method is well suited for monitoring E1, E2 and EE2 in the environment. A standardization of the method according to DIN is in progress and should consider only environmentally friendly solvents. A solvent is considered to be environmentally friendly if it is safe for the user (non-mutagenic, carcinogenic, and generally non-toxic), if it is safe to use (non-flammable, explosive, or peroxide-forming), and if it is safe for the public and the environment (i.e., the solvent is not ecotoxic, persistent, and has no ozone depletion potential or global warming potential). An environmentally friendly solvent should also be inexpensive and recyclable.

Separating EE2 from E2 is not a problem [10], nor is separating E2 and E1 [11, 12]. No problem is the separation of E1 and EE2 on RP-18 [13–15] or CN plate [15] or
with programmed multiple development technique [4, 16] either. Published is the separation of E1 from EE2 on silica gel using the programmed multiple development technique with CH₂Cl₂–acetone–petroleum ether of various compositions [4]. Separation in a single run is described in [6, 7] using the solvent mix chloroform–acetone–petroleum ether (55:20:25, V/V). This solvent mix was first published in [17].

The purpose of this paper is to describe an environmentally friendly solvent mixture capable of separating E1, E2, estriol (E3) and EE2 without the use of hazardous or environmentally problematic compounds such as chloroform.

2 Experimental

2.1 Preparation of standards and application on HPTLC plates

All the chemicals used were of analytical reagent grade. Estrone (E1), 17 β-estradiol hemihydrate (E2), 17α-ethyl estradiol (EE2) and estriol (E3) were from Sigma-Aldrich (Seelze, Germany). Equol and genistein were from Caymen Chemical Company (Ann Arbor, MI, USA). Daidzein was from Alfa Aesar (Karlsruhe, Germany). Bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEHP) and 4-nonylphenol (4NP) were from Fluka (Steinheim, Germany). All the chemicals used were of analytical reagent grade. Standards had a purity of ≥ 98.0% except equol, which is a technical product with a purity of ≥ 90%.

The solvents methanol, acetone, methyl-ethyl ketone (MEK) and cyclopentyl methyl ether (CPME) came from Carl Roth (Karlsruhe, Germany). Cyclohexane from Th. Geyer (Renningen, Germany), and the silica gel 60 F₂₅₄ plates (1.05554, 1.05642 or 1.05586) with a fluorescent dye used as stationary phase from Merck (Darmstadt, Germany).

Test solutions were prepared by dissolving standards in amounts of 2 to 7 mg in 1 mL of methanol using an Orion Cahn® C-33 microbalance from Environmental Instruments (Beverly, MA, USA). Standards were spotted bandwise over 7 mm in amount of 0.2–1 µL using a CAMAG Automatic TLC Sampler (ATS 4) device (Muttenz, Switzerland). Bands were spotted at a distance of 10 mm from the bottom plate edge and at a distance of 1.5 cm from the plate edges.

2.2 Separation and spectral measurements

For separation, silica gel plates (10 × 10 cm) were developed in a vertical developing chamber at vapour saturation (30 min) to a distance of 70 mm calculated from the application. A mixture of MEK–cyclohexane (1:2, V/V) or cyclohexane–CPME (3:2, V/V) was used as solvent. It might be necessary to focus the application line. For this, acetone or methanol was used over a distance of 5 to 10 mm. After this short development step, the plate was dried and then subjected to chromatographic separation.

For the spectral measurements of the plate, a TIDAS TLC S700 system from J & M (Aalen, Germany) with a reflection attachment consisting of two rows of optical fibers was used, which has a wavelength resolution of 0.8 nm and a spatial resolution on the plate of 100 µm. The measurement time for a single spectrum in the wavelength range from 190 to 1000 nm was 25 ms. Evaluation of the plate was performed using expression (1) derived from the extended Kubelka–Munk equation [18].

\[ KM = \frac{J_0}{J} - 1 \quad \frac{a}{1 - a}, \] (1)

where $a$: absorption coefficient; $J_0$: reflected light intensity measured from a neat plate part; $J$: reflected light intensity measured from a track.

3 Results

3.1 Solvent mixture CPME and cyclohexane

Separation of E1, E2, E3 and EE2 is possible with the environmentally friendly solvent mixture cyclohexane–CPME (3:2, V/V). Figure 1 shows the unfocused separation of the nine estrogenic active compounds E3, daidzein, genistein, E2, equol, EE2, E1, 4-nonylphenol and DEHP. The separation time at 24 °C is 24 min for a separation distance of 68 mm. The amounts of separated standards range from 300 to 600 ng per band. The scan was evaluated in the wavelength range from 271 to 273 nm by averaging 3 diode signals. E1 is separated from EE2, but no baseline separation could be achieved. Remarkable is the very good separation of equol and E2. A disadvantage of the solvent mixture is that E3 and daidzein move only a few mm and DEHP is nearly moving in the front. A real disadvantage of this solvent mixture is that EE2 and BPA, with their $R_F$ values of 0.36 and 0.37, cannot be completely separated. All $R_F$ values of the separation are shown in Table 1.

3.2 Solvent mixture methyl-ethyl ketone and cyclohexane

Compounds E1, E2, EE2 and E3 can also be baseline-separated using the environmentally friendly solvent mixture methyl-ethyl ketone (MEK)–cyclohexane (1:2, V/V). A densitogram of this (unfocussed) separation is shown in Fig. 2 and was measured in the wavelength range from 222 to 228 nm by averaging 7 diode signals. The $R_F$ values are also summarized in Table 1. The disadvantage of this solvent mixture is that E2 cannot be baseline-separated from...
equol because its $R_F$ values are 0.35 and 0.34, respectively. The separation between E2 and EE2 with $R_F$ values of 0.35 and 0.415 is quite good. Unfortunately, the compound BPA with an $R_F$ value of 0.38 is in-between and may spoil the separation.

### Table 1 Log $P$ and $R_F$ values of 10 estrogenic active compounds for two solvent mixtures

| Analyte     | Log $P$ | $R_F$ CPME/ Cyclohexane | $R_F$ MEK/ Cyclohexane | $R_F$ MEK/ Cyclohexane 2 × 4 °C |
|-------------|---------|-------------------------|------------------------|----------------------------------|
| DEHP        | 7.4     | 0.975                   | 0.89                   | 0.9                              |
| 4NP         | 5.9     | 0.815                   | 0.73                   | 0.73                             |
| E1          | 3.1     | 0.42                    | 0.48                   | 0.645                            |
| EE2         | 4.15    | 0.36                    | 0.415                  | 0.48                             |
| BPA         | 3.3     | 0.37                    | 0.38                   | 0.46                             |
| E2          | 4       | 0.225                   | 0.35                   | 0.435                            |
| Equol       | 3.2     | 0.32                    | 0.34                   | 0.43                             |
| Genistein   | 3.04    | 0.165                   | 0.275                  | 0.37                             |
| Daidzein    | 2.51    | 0.04                    | 0.13                   | 0.28                             |
| E3          | 2.5     | 0.01                    | 0.055                  | 0.09                             |

$Y = ax + b$

| $A$ | 0.184 | 0.138 | 0.108 |
| $B$ | −0.352 | −0.116 | 0.087 |
| $r^2$ | 0.8705 | 0.8816 | 0.7748 |

### 3.3 Solvent mixture methyl-ethyl ketone and cyclohexane at 4 °C

The separation time at 24 °C is 22 min for 70 mm separation distance. This solvent mixture works in a wide temperature range between 24 and 4 °C, the temperature of a refrigerator. At higher temperatures, the peaks become wider and wider, a result of diffusion. Separation at lower temperatures, on the other hand, suppresses diffusion. Figure 3 shows the contour plot of a double separation at 4 °C. For this, the trough with the solvent mixture was placed in the refrigerator for 30 min. The plate was then placed in the trough for separation, dried, and separated again over the entire distance. In a second development step, all spots were re-concentrated, as the solvent front first contacts the lower position of the analyte zone, pushing the analyte molecules together. This concentration process always leads to sharper peaks [19].

As a result, the compounds E2, EE2 and E1 show smaller peaks compared to Fig. 2. The disadvantage is that a single separation at 4 °C requires 24 min instead of 22 min at 24 °C and that equol and E2 cannot be separated.
Fig. 2 Separation of estriol (E3), daidzein, genistein, 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estrone (E1), 4-nonylphenol and bis(2-ethylhexyl) phthalate (DEHP), measured at 225 nm. Application point is located at 3 mm separation distance, the front signal at a distance of 73 mm.

Fig. 3 Contour plot of a double separation at 4 °C of estriol (E3), daidzein, genistein, 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estrone (E1), 4-nonylphenol and bis(2-ethylhexyl) phthalate (DEHP) (separation from left to right). The densitogram was taken at 276 nm (above) as an average of 7 diodes, the UV spectrum of daidzein is plotted at left.
4 Discussion

4.1 The solute retention factor \( k \)

In both solvents presented above, E3 (with three OH groups in the molecule structure) shows a lower \( R_F \) value than E1 (with only one OH group in the molecule). All compounds can be brought to higher \( R_F \) value by increasing the MEK or CPME concentration in the solvents. Lower \( R_F \) values are achieved by increasing the amount of cyclohexane in the solvents. Thus, both solvents have the same properties: “normal phase” and probably “distribution separation”. “Normal-phase separation” means that the stationary phase is more polar than the mobile phase. Polar compounds remain “dissolved” in the stationary phase longer than less polar compounds and therefore showing lower \( R_F \) value than less polar compounds. “Distribution separation” means that analytes adsorb little or not at the stationary phase. The separation is mainly determined by solving effects, and therefore, the analyte distribution between stationary and mobile phase can be described by the capacity factor.

The solute retention factor \( k \) (capacity factor) in chromatography is defined as the quotient of the molar substance amount in the stationary \( (n_s) \) and the mobile phase \( (n_m) \) \[18\]:

\[
 k = \frac{n_s}{n_m} \tag{2}
\]

Any isocratic chromatographic system has its optimal resolution at \( k = 2 \). The optimal retention time \( (t_{Ro}) \) for the best peak resolution in isocratic flow-through chromatography [column chromatography such as high-performance liquid chromatography (HPLC)] is three times the columns void time \( (t_0) \), i.e., \( t_{Ro} = 3t_0 \) (derived from Eq. 7 in [19]).

According to the Martin–Syng equation \( R_F \) value for \( k = 2 \) is \( R_F = 1/3 \) \[18\].

\[
 R_F = \frac{1}{1+k} \tag{3}
\]

To achieve optimal separation of analytes, the most critical analyte pair to be separated in the system should be brought to an \( R_F \) value of 0.333, which can be easily done by changing the solvent composition.

In Fig. 4, the Martin–Syng equation is shown as a blue line with the \( R_F \) value on the Y-axis and logarithmised \( k \) values (log \( k \) values) on the X axis. If we assume HPTLC separation in the \( R_F \) range of 0.1 to 0.9, we can see from Eq. (3) that this covers an \( k \) range of no more than two magnitudes of power. In other words: in this \( R_F \) range, compounds with \( k \) values that differ by a factor of more than 100 cannot be separated. It can also be seen that in the \( R_F \) range from 0.2 to 0.8, there is a good linear relationship between \( R_F \) and \( k \) values. Extending to the \( R_F \) range from 0.1 to 0.9, a poorer but acceptable linear relationship is obtained (as shown in Fig. 4).

4.2 \( R_F \) value compared with log \( P \) values

Log \( k \) is a logarithmic partition coefficient that describes the distribution of an analyte between the stationary and the mobile phase, as shown in Eq. 2. An important logarithm partition coefficient is also the log \( P \) value, which describes the distribution of a compound in the two-phase system of \( n \)-octanol and water \[20\]. To measure the log \( P \) value, the ratio of the concentrations of a compound in the mixture of these two immiscible solvents at equilibrium is measured analytically according to Eq. (4).
The log $P$ value is a good indicator for the polarity of a compound. Its values are published for all substances of interest [21–23]. According to Fig. 4, we can expect a negative linear relationship between the $R_F$ values of an HPTLC separation and the corresponding log $P$ values of the separated compounds (listed in Table 1). This describes expression (5) with the linear regression parameters $a$ and $b$.

$$R_F = a \times \log P + b$$

In Fig. 5, the $R_F$ values of the MEK/cyclohexane separation at 24 °C are plotted against the log $P$ values of the separated compounds. It can be seen that there is a linear relationship between the two sets of measurements. (E3 is not included because its $R_F$ value is less than 0.1 and thus lies not in the linear range.) The linearity between the two sets of measurements indicate that the separation is primarily determined by distribution rather than by adsorption chromatography. That is plausible because the high concentration of the ketone MEK or the ether CPME in the solvent mixture makes it likely that all absorption centers of the silica gel layer are blocked by these molecules. Thus, a partition of analytes occurs between a more polar stationary phase, which immobilizes more MEK (or CPME) on silica gel than cyclohexane, and a mobile phase consisting mainly of cyclohexane. As described above, all analytes can be brought to higher $R_F$ values simply by reducing the amount of cyclohexane in the solvent mixture.

Figure 5 describes two further aspects of the separation. HPTLC opens a polarity window in which the analytes can move. If we define this window in terms of $R_F$ values from 0.1 to 0.9, the separation covers approximately log $P$ values from 2.5 (daidzein) to 7.5 (DEHP), corresponding to 5 orders of magnitude. All compounds with log $P$ values in this range should move on plate. Furthermore, from a given log $P$ value, the probable $R_F$ value of a chromatographic system can be calculated if the linear regression parameters ($a$, $b$) of a system are known (listed in Table 1). Thus, from the measured $R_F$ value of an unknown compound, the probable log $P$ value can be calculated, providing initial information about the molecule possibly present in the sample.

5 Conclusion

HPTLC is a cost-effective and easy-to-use separation technique suitable for monitoring estrogenically active compounds in water. In combination with the YES test performed on the planar surface of an HPTLC plate, this method is very sensitive. The two HPTLC solvents presented are well suited for the separation of E1, E2, E3 and EE2 on silica gel, using environmentally friendly solvents. From a given log $P$ value of an analyte, the probable $R_F$ value of that compound in both chromatographic systems can be calculated. Conversely, from the measured $R_F$ value of an unknown compound, its probable log $P$ value can be estimated.

**Fig. 5** Plotted are the $R_F$ of the MEK/cyclohexane separation at 24 °C versus the log $P$ values of the separated compounds daidzein, genistein, equol, 17β-estradiol, 17α-ethinyl estradiol, bisphenol A, estrone, 4-nonylphenol and bis(2-ethylhexyl) phthalate
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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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