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Separation of eight selected flavan-3-ols on cellulose thin-layer chromatographic plates

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Abstract

The potential of microcristaline cellulose as sorbent in the separation of eight compounds: (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg), procyanidin B1 and procyanidin B2 was studied. Cellulose HPTLC plates prewashed in water (not necessary, when water was used as developing solvent) and dried with a hair dryer, bandwise application and development in horizontal developing chamber (sandwich configuration) gave the best results. Detection was performed using vanillin–H_3PO_4 reagent. Four new developing solvent systems were proposed: water, 1-propanol–water (20:80, v/v), 1-propanol–water–acetic acid (4:2:1, v/v) and 1-propanol–water–acetic acid (20:80:1, v/v), and at least two of them were needed for the differentiation between all eight compounds. Surprisingly, water enabled the separation of epimers C from EC and GC from EGC, as well as the dimers procianidin B1 and B2. Additionally, C, EGC, B1 and B2 were separated from all the other compounds. The best choice for developing solvent is given for each of the studied compounds. The best separation of the five main catechins (EC, GC, EGC, ECg, EGCg) present in green tea extract was achieved using 1-propanol–water–acetic acid (20:80:1, v/v). The chromatograms of oak bark extract developed in solvents with higher water content (1-propanol–water (1:4, v/v) and 1-propanol–water–acetic acid (20:80:1, v/v)) showed less bands than chromatograms developed in solvents with higher organic modifier content (e.g. 1-propanol–water–acetic acid (4:2:1, v/v)). It was proved that such behavior was due to the presence of procyanidins beside the main component catechin.

Keywords: Flavan-3-ols; Catechins; Procyanidins; Thin-layer chromatography; Cellulose HPTLC plates; Green tea; Oak

1. Introduction

Flavan-3-ols (catechins) are polyphenolic compounds that have been shown to exhibit a wide variety of beneficial effects on human health [1–3] when using in appropriate concentrations. These compounds are present in relatively high concentrations in tea leaves [4,5], oak [6], cocoa beans [7,8], grapes [9,10] and many other plants. They are also the building blocks of oligomeric procyanidins (condensed tannins).

The analysis of flavan-3-ols has usually been performed by HPLC using UV, electrochemical or MS detection [10–16] and also by thin-layer chromatography (TLC) [7,10,16–21]. The resolution of critical pairs in some TLC studies on silica gel [16,19] is relatively poor and does not allow reliable separation, although it is sometimes sufficient for qualitative screening purposes. Recently, some new TLC systems for the separation of (+/−)-catechin and (−)-epicatechin based on different modified silica sorbents [17] and cellulose [18,20,21] as sorbent were published. Additionally, TLC cellulose plates and pure water as developing solvent enabled us to distinguish between the (+)-catechin and (+/−)-catechin [21].

The aim of this study was to investigate the separation of selected flavan-3-ols and dimeric procyanidins (Fig. 1) using cellulose TLC or HPTLC plates. The applicability of the developed method was checked for screening of the extracts of oak bark and green tea.

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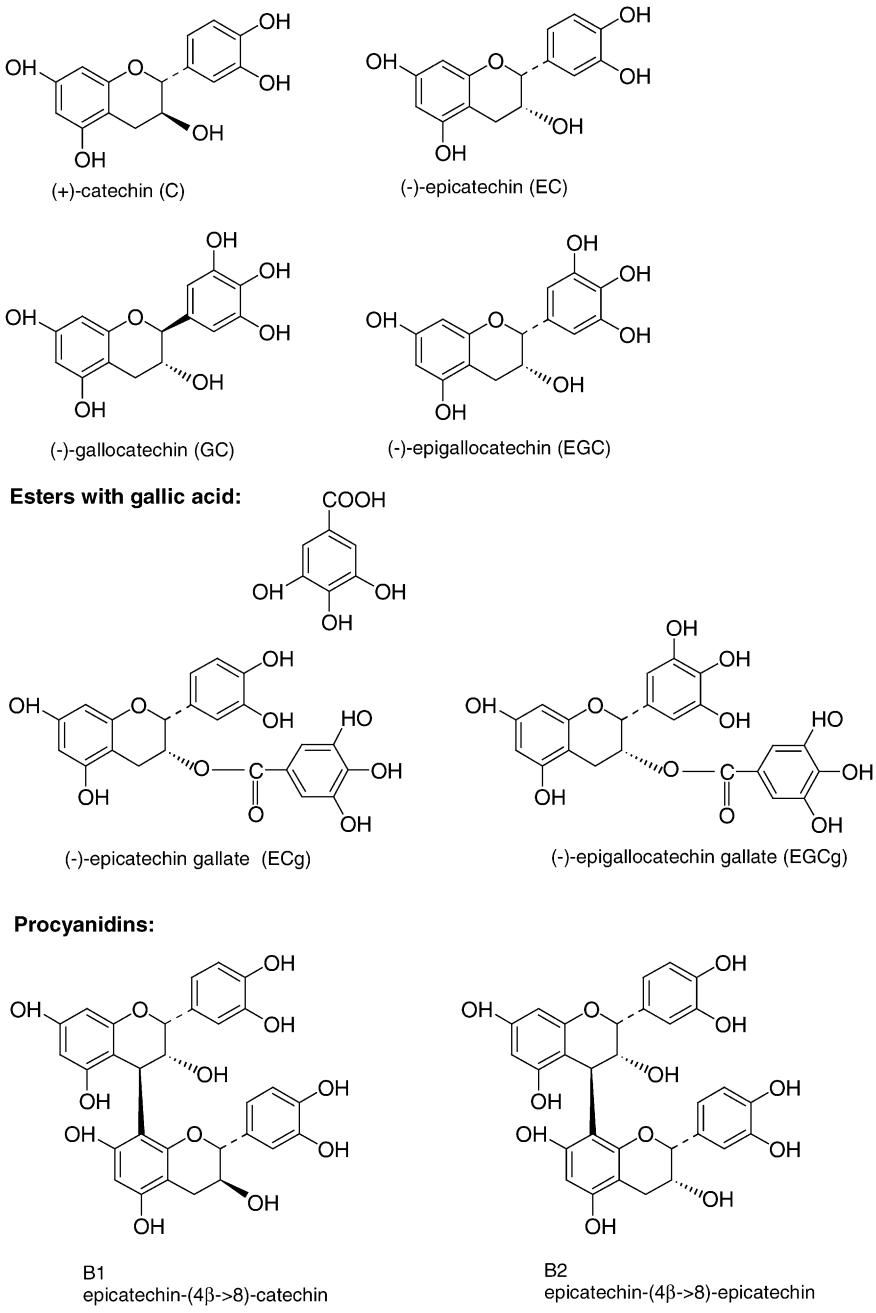

Fig. 1. The structures of the studied flavan-3-ols.

2. Experimental

2.1. Chemicals

Methanol, ethanol, 1-propanol, 1-butanol, acetone, ethyl acetate, acetonitrile, tetrahydrofuran, phosphoric acid and vanillin were of analytical grade and were purchased from Merck (Darmstadt, Germany). Acetic acid was from Scharlau Chemie (Barcelona, Spain). Standards of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-gallocatechin were obtained from Sigma (St. Louis, MO, USA). Procyanidin B1 and procyanidin B2 were from Extrasynthese (Genay Cedex, France).

2.2. Preparation of standard solutions and detection reagent

Separate stock solutions (1 mg/ml in methanol) were prepared. Application solutions were prepared by diluting the separate stock solutions with methanol to a concentration of 0.1 mg/ml. In addition, a standard solution M6 containing all the standards of six flavan-3-ols (0.1 mg/ml of each in methanol), and a standard solution M8 containing all the

standards of six flavan-3-ols and procyanidin B1 and procyanidin B2 (0.1 mg/ml of each in methanol) were prepared. All the solutions were kept in a refrigerator. Vanillin–H_3PO_4 reagent [22] was prepared by adding 10 ml of *ortho*-phosphoric acid to a solution of 1 g of vanillin in 70 ml of ethanol.

2.3. Preparation of plant extracts [23]

Green tea (Green leaf tea Taylors of Harrogate, UK) and oak bark (*Quercus robur* L. bark, purchased from University Pharmacy, *Quercus cortex* lot No. 65/0–2000; Helsinki, Finland) extracts were prepared by extracting 10 g of the plant material by 150 ml of boiling water. After filtration, 100 ml of the water extract was further treated in the separation funnel with 40 ml of chloroform to remove chlorophyll. Water phase was extracted 2 times with 50 ml ethyl acetate. The ethyl acetate phase was collected, filtered through anhydrous sodium sulfate and evaporated to dryness.

2.4. Thin-layer chromatography

TLC was performed on precoated cellulose 20 cm × 20 cm TLC plates (Merck No. 5716) and 10 cm × 20 cm HPTLC cellulose plates (Merck No. 1.05786). The plates were cut into 20 cm × 10 cm, 10 cm × 10 cm or 4 cm × 10 cm. Solutions of standards and extracts of oak bark (2 mg/ml) and green tea (7.5 mg/ml) were applied as 10 mm or 8 mm bands, 10 mm from the bottom of the plates and 15 mm from the left edge of the plate, 5 mm or 7 mm apart with the application speed 6 s/μl using a Linomat IV application device (Camag, Muttenz, Switzerland). Application volume for the extracts and the standards was 2 μl, except for ECg and EGCg for which it was 3 μl.

The developing solvents consisted of aqueous solutions containing acetone, acetic acid, tetrahydrofuran, acetonitrile, ethyl acetate, methanol, ethanol, 1-propanol and 1-butanol as organic modifiers. TLC plates were developed to a distance of 5 cm in a twin-trough chamber (unsaturated or saturated with 30 min preconditioning) for 10 cm × 20 cm plates (Camag) and in horizontal developing chamber (Camag; 6 ml of developing solvent) using tank (without preconditioning) and sandwich configuration. In case of development in the twin-trough chamber, 10 ml of developing solvent was poured into each trough; for development in saturated chamber two sides of the chamber were lined with filter paper. The developed plates were dried in a stream of warm air for 2 min and then immersed for 1 s into vanillin–H_3PO_4 dipping reagent by means of Camag immersion device III. Drying in a stream of warm air for 2 min furnished colored bands for separated compounds.

A Camag Video Documentation System coupled to a Reprostar 3 transilluminator and a frame grabber system equipped with a 3 × 1/2 in. (1 in. = 2.5 cm) CCD video camera (model HV-C20, Hitachi Denshi, Japan), operated with VideoStore 2 V2.30 and VideoScan V1.01 software (Camag), was used for recording and analyzing the images of the developed TLC plates.

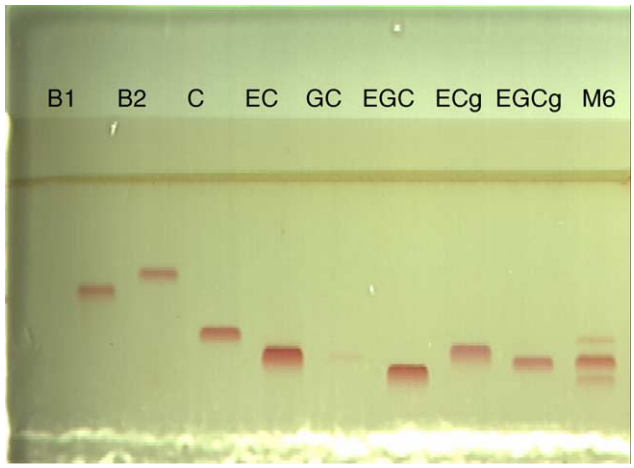

Fig. 2. Separation of flavan-3-ols on cellulose HPTLC plate developed using water in horizontal developing chamber (sandwich configuration).

3. Results and discussion

We have already reported, that epimeric flavan-3-ols, (+)-catechin and (−)-epicatechin can be separated by analytical ultra-micro rotation planar chromatography (U-RPC) as well as by conventional TLC on cellulose plates with pure water as developing solvent. This separation enabled us to distinguish even between the (+)-catechin and (+/−)-catechin [21]. Therefore, we decided to test the potential of cellulose as sorbent and water as developing solvent in the separation of additional related compounds. The results showed, that it is also possible to separate epimers GC and EGC, as well as the dimers procyanidin B1 and B2 (Fig. 2). Additionally, C, EGC, B1 and B2 were also separated from all the other compounds EC, GC, ECg and EGCg from the mixture M8 (Table 1, solvent I), which were not separated from each other (Fig. 2).

Table 1
Separation of eight compounds from the mixture M8 on cellulose HPTLC plates developed using five different developing solvents: water (I), 1-butanol–water–acetic acid (4:2:1, v/v; II), 1-propanol–water–acetic acid (4:2:1, v/v; III), 1-propanol–water (20:80, v/v; IV), 1-propanol–water–acetic acid (20:80:1, v/v; V)

Compound	Solvent I	Solvent II	Solvent III	Solvent IV	Solvent V
C	+	–	+	+	–
EC	–	–	+	+	+
GC	–	–	–	+	+
EGC	+	+	+	+	+
ECg	–	+	+	+	+
EGCg	–	–	+	–	–
B1	+	+	+	–	–
B2	+	–	–	–	–

(+) Separated from all the other compounds; (−) separated only from some compounds.

To further improve the separation, we started with the method of Friedrich and Wiedemeyer [11], who performed the separation of C, EC, GC and EGC on self-made cellulose TLC plates using 1-butanol–water–acetic acid (4:2:1, v/v) as developing solvent. Application of additional compounds ECg, EGCg, B1 and B2 on the commercially available cellulose TLC plates resulted only in separation of EGC, ECg and B1 (Fig. 3A). However, C, EC, GC, EGCg and B2 from the mixture M8 (Table 1, solvent II) were not separated from each other. This experiment showed that the selectivity of water as developing solvent was better compared to the selectivity of the published three component developing solvent. Another disadvantage of using 1-butanol–water–acetic acid (4:2:1, v/v) as developing solvent is that it is necessary to perform the separation on the plates prewashed in water (Fig. 3A), as shown for C and EC in our earlier studies [20]. The bands on the not prewashed plate were quite diffuse, especially for C, ECg and EGCg, while the band of ECg was diffused also on the prewashed TLC plate.

The replacement of 1-butanol with 1-propanol in the developing solvent (1-propanol–water–acetic acid, 4:2:1, v/v) enabled the separation of most of the studied compounds from each other, except GC and B2 (Fig. 3B, Table 1, solvent III). The resolution was not good enough for the densitometric evaluation of standards, but it was sufficient for the visual examination. Nevertheless, the new developing solvent 1-propanol–water–acetic acid (4:2:1, v/v) was also more informative for the both plant extracts.

In order to achieve the separation of all studied compounds, we tested the influence of the addition of organic modifiers to water as developing solvents: 20% (v/v) tetrahydrofurane, 20% (v/v) acetic acid, 9.5% (v/v, at 20 °C) ethyl acetate, 20% (v/v) aceton, 20% (v/v) acetonitrile, saturated (9.8% (v/v) at 20 °C) 1-butanol, 20% (v/v) 1-propanol, 20% (v/v) ethanol, 20% (v/v) methanol. Compared to water the addition of any of the studied organic modifiers to water resulted in better separation of compounds from the mixture M6 (Fig. 4).

Since 20% (v/v) 1-propanol in water produced the best separation out of the tested developing solvents, various concentrations of 1-propanol in water were used to study the effect of the concentration of 1-propanol on the retardation of the six flavan-3-ols—an experiment which could not be performed with butanol because of its limited miscibility with water (Fig. 5). There is an interesting and unexpected dependence of R_F values of the studied compounds from the content of 1-propanol in water: at concentrations of 1-propanol between 40 and 60%, the compounds achieve maximum R_F values and thereafter the R_F values drop despite of the higher content of 1-propanol in the developing solvent mixture (Fig. 5). These results showed that the separation mechanism is complex. The best separation of flavan-3-ols

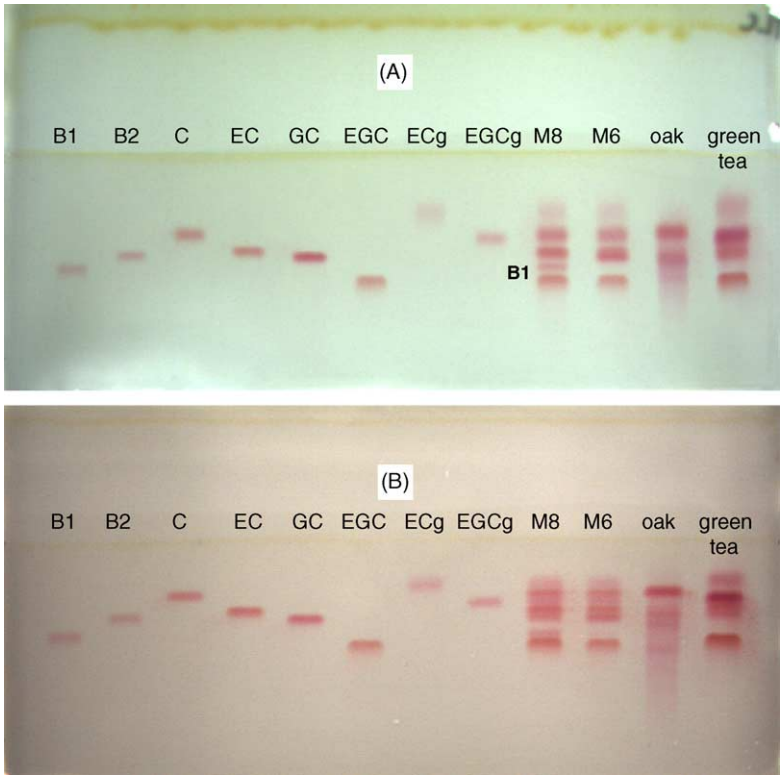

Fig. 3. The separation of flavan-3-ols test mixtures (M6 and M8), oak and tea extracts on prewashed cellulose HPTLC plate developed in horizontal developing chamber (sandwich configuration) using different developing solvents. Solvent II (A): 1-butanol–water–acetic acid (4:2:1, v/v); solvent III (B): 1-propanol–water–acetic acid (4:2:1, v/v).

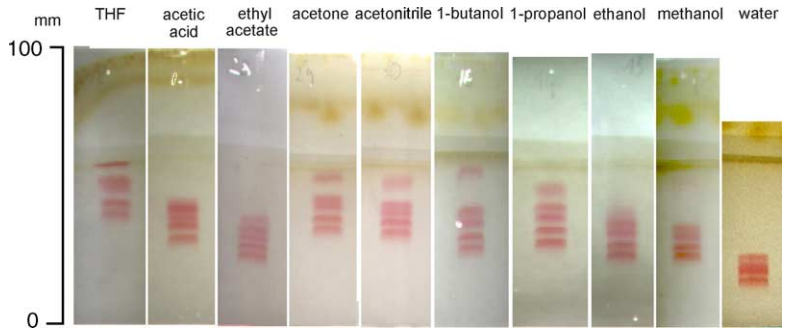

Fig. 4. The separation of flavan-3-ols test mixtures (M6) on cellulose TLC plates using water and aqueous developing solvents containing 20% (v/v) of organic modifiers, except: 9.5% (v/v) ethyl acetate and 9.8% (v/v) 1-butanol. Plates were developed in unsaturated normal chamber.

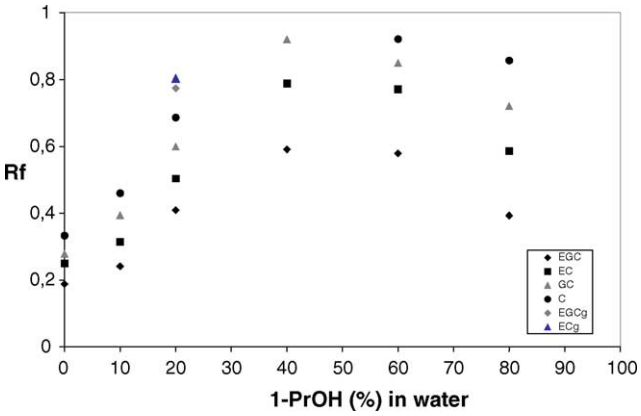

Fig. 5. The influence of 1-propanol content in water on the separation of flavan-3-ols test mixtures (M6) on cellulose HPTLC plates in unsaturated normal chamber.

from the mixture M6 was achieved with 20% (v/v) 1-propanol in water (Table 1, solvent IV), whereas developing solvent with higher or lower concentrations of 1-propanol partially merged the bands together. The separate application of all the standards showed that ECg and EGCg (the upper two bands) were not good resolved (Fig. 6). The effect of addition of 1% of acetic acid to developing solvent (resulting in 1-propanol–water–acetic acid (20:80:1, v/v)) was evident, especially in better resolution between the two gallates, ECg and EGCg (Figs. 6 and 7). Surprisingly higher concentrations of acetic acid (5 and 10%) in the developing solvent caused band broadening, which was especially remarkable for the EC and EGC. The use of 1-propanol–water–acetic acid (20:80:1, v/v) as developing solvent resulted in the separation of EC, GC, EGC and ECg from all the other compounds from the mixture M8 (Table 1, solvent V), while the other four compounds were not separated from each other (Fig. 7).

Developing solvent is usually considered as the most important parameter affecting the selectivity in TLC [24]. However, optical activity of the cellulose sorbent was crucial for the separation of catechins. For this reason a wide choice of developing solvents with appropriate elution strength was possible for the separation of six related compounds. However, some differences in selectivity between different solvents have been observed and could be explored for the characterization of plant extracts as shown for the green tea and oak extracts.

As expected the bands were a bit more diffuse on TLC plates compared to those obtained on HPTLC plates. Testing of three different application distances (5, 10 and 15 mm) showed the best resolution for the application at 5 mm [24]. The saturation (30 min) of the twin-trough chamber slightly improved the separation and gave more compact bands, however development in the horizontal developing

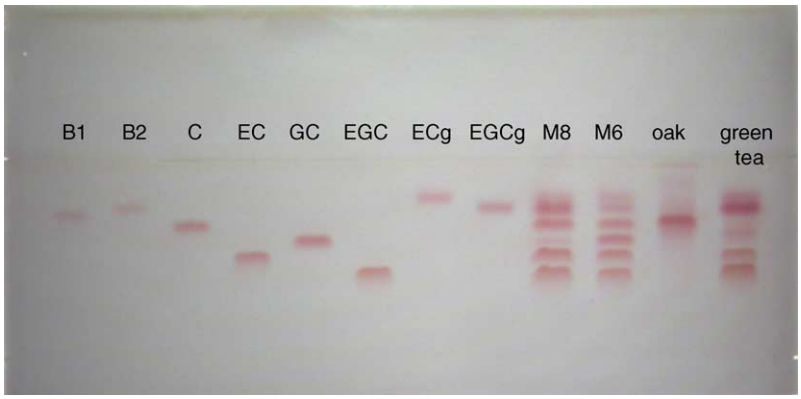

Fig. 6. The separation of flavan-3-ols test mixtures (M6 and M8), oak and tea extracts on HPTLC plates developed using 1-propanol–water (20:80, v/v) in horizontal developing chamber (sandwich configuration).

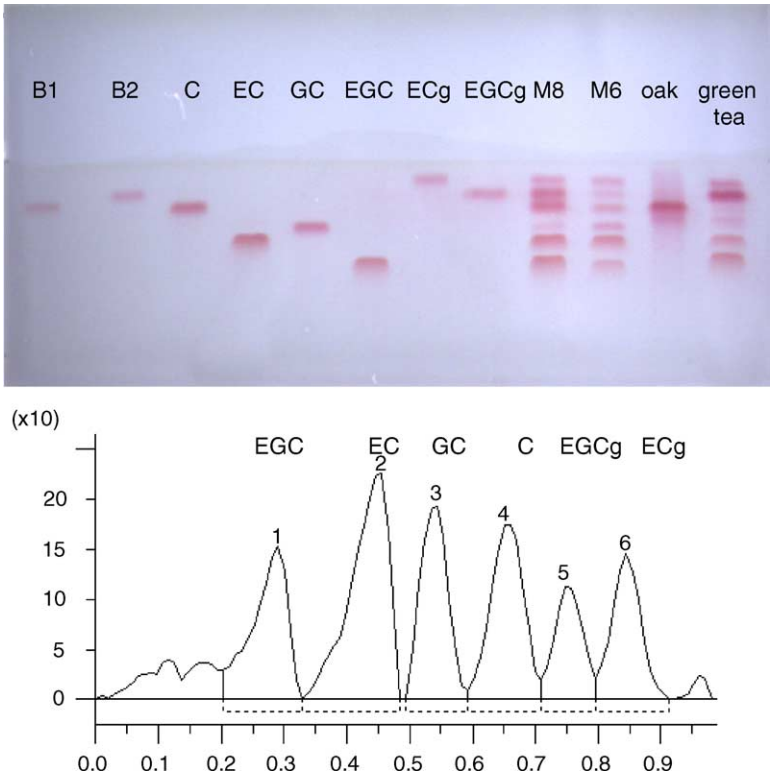

Fig. 7. The separation of flavan-3-ols test mixtures (M6 and M8), oak and tea extracts on prewashed HPTLC plates developed using 1-propanol–water–acetic acid (20:80:1, v/v) in horizontal developing chamber (sandwich configuration). Lane M6 is presented also as densitogram.

chamber, especially sandwich configuration showed further improvements.

Finally, we can recommend the following chromatographic conditions for the separation of the eight studied compounds: cellulose HPTLC plates prewashed in water (not necessary, when water is used as developing solvent) and dried with a hair dryer, horizontal developing chamber (sandwich configuration), bandwise application 5 mm from the bottom of the plate, developing distance 5 cm. Suitable developing solvents for each of the studied compounds are presented in Table 2. It is evident that two solvent systems are needed for differentiation between all eight compounds. Considering that catechin represents the main flavan-3-ol in many plant extracts and that procyanidins B1 and B2 are minor but important components with pronounced biological activity [25], water (solvent I) is recommended as the first choice. The fastest way to separate epigallocatechin gallate, which is the main flavan-3-ol in green tea, is by using 1-propanol–water–acetic acid (4:2:1, v/v).

Table 2
Recomended developing solvents for the separation of each of the eight studied compounds from all the others on cellulose HPTLC plates

Compound	Developing solvents
C	I, III, IV
EC	III, IV, V
GC	IV, V
EGC	I, II, III, IV, V
ECg	II, III, IV, V
EGCg	III, (I + II, I + V)[a]
B1	I, II, III
B2	I

Water (I), 1-butanol–water–acetic acid (4:2:1, v/v; II), 1-propanol–water–acetic acid (4:2:1, v/v; III), 1-propanol–water (20:80, v/v; IV), 1-propanol–water–acetic acid (20:80:1, v/v; V).

[a] Development of two plates is needed.

The extracts of oak bark and green tea were chosen for testing the applicability of the developed methods for screening of the extracts due to the fact, that they differ in the main components being catechin in oak bark [21,26] and epigallocatechin gallate in green tea [23]. Our results showed that all newly proposed developing solvents enabled better separation of those two compounds than the solvent in the published method [18]. The best separation (Fig. 7) of the five main catechins (EC, GC, EGC, ECg, EGCg) present in green tea extract [23] was achieved using 1-propanol–water–acetic acid (20:80:1, v/v). The situation was more complicated in the case of oak bark extract, probably due to the presence of catechin oligomers (procyanidins). The chromatograms developed in solvents with higher water content (solvents IV and V, Figs. 6 and 7) showed less bands than chromatograms developed in solvents with higher organic modifier content (solvents II and III, Fig. 3A and B). At the same time, we observed that the relative position of the procyanidin dimers B1 and B2 compared to the position of catechin moved down

with increasing content of organic modifier in the developing solvents. At developing in pure water B1 and B2 were above catechin, while at developing in solvents II–V they gradually merged with other studied flavan-3-ols. Chromatogram of oak bark extract developed in water resulted in one band above the band of catechin [21]. This band did not belong to B1 neither to B2, because it was too closed to catechin. However, according to its position on the chromatogram, high content of catechin [21,26], and available literature data about dimers present in oak (*Q. robur* L.) bark [26] we assume that that band could be procyanidin B3 (catechin-(4α→8)-catechin).

A comparison of the selectivity of solvents I–V in the analysis of oak bark extract showed that water was crucial for the detection of the dimer. The presence of a dimer in oak bark extract was additionally confirmed on silica gel with a published developing solvent [28]. It is known that on silica gel the procyanidin oligomers with the highest polymerization degree are closed to the start and the monomers on the top of the chromatogram [27,28]. However, compared to silica gel the advantage of cellulose is that it enables the separation of optical isomers. In order to show the applicability of the developed methods in screening of plant extracts, further investigations of additional plant extracts are needed.

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