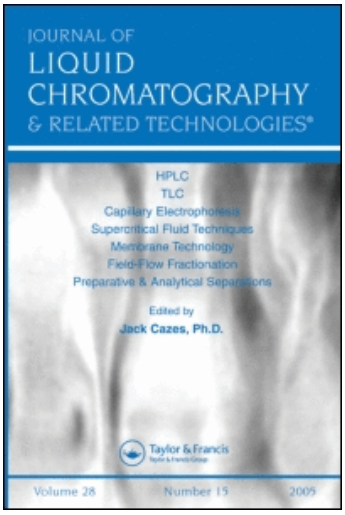

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Analysis of Some Natural Antioxidants by Thin-Layer Chromatography and High Performance Thin-Layer Chromatography

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Analysis of Some Natural Antioxidants by Thin-Layer Chromatography and High Performance Thin-Layer Chromatography

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Abstract: (HP)TLC is a technique with large applicability in the fields of plant material analysis and stability tests of extracts and finished products. The implementation of a modern standardized methodology led to the increasing of acceptance and recognition of (HP)TLC as competitive analytical method. (HP)TLC has many advantages, such as lower costs, short time analysis, the possibility of multiple detection, and specific derivatization on the same plate, etc.

The natural antioxidants (polyphenols) are present in plant extracts; they play a key role in antioxidative defence mechanisms in biological systems and they act as free radicals scavenging agents. The separation of polyphenols from each other and from other components of the plant extracts can be done by a great number of (HP)TLC developed techniques. Mostly, complex crude plant extracts are screened for antioxidant activity or to distinguish the components of plant extracts with antioxidant character or radical-scavenging properties. (HP)TLC has been used for the determination of individual antioxidant capacity of target compounds and it might be of interest to the routine chemical or biological screening and they can solve real analytical problems.

Keywords: Natural antioxidants, Polyphenols, (HP)TLC, Antioxidant capacity

INTRODUCTION

Recent years have seen tremendous growth in the use of plants in the pharmaceutical and food industries. The demand for analytical methods to

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ensure quality and safety of products is rapidly increasing because new ones enter the market almost daily.

The interest in research concerning the compounds from plants and their biological activity has significantly increased in the last few years as a result of the constantly increasing popularity of phytotherapy. As a consequence, most of the pharmacopoeias throughout the world are revising their monographs on medicinal plants, including monographs for plant extracts.

Diets rich in fruits and vegetables are associated with a reduced risk of diseases associated with oxidative stress, such as coronary heart disease, some cancers, and neurodegenerative disease.[1] Recent researches have focused on trying to identify those natural components that contribute to good health. Protection provided by vegetables, fruits, and beverages might be associated with phenolic compounds (polyphenols) with antioxidant activity.[2] Polyphenols are the active substances found in many plants, being secondary metabolites and have antioxidant properties and several other specific biological actions that are yet poorly understood; they are involved in defense against UV radiation or aggression by pathogens. As a consequence, the effects of phenolic compounds on health and the identification of polyphenols provide the greatest protection of health. Moreover, it is essential to determine the nature and distribution of such compounds in plants because several thousand compounds have polyphenolic structures. The polyphenol content of plants may be affected by many factors, such as: variety of compounds, ripeness at the time of harvest, environmental factors, and processing and storage.[3] The pedoclimatic and agronomic factors and exposure to light have major effects on polyphenolic content of plants.

The natural antioxidants (phenolic compounds) play a key role in antioxidative defense mechanisms in biological systems. They act as free radical scavenging agents. Free radicals can be generated by metabolic pathways within body tissue, can be introduced by external sources, and can be caused by environmental pollution. Attention has turned to natural antioxidants because the use of synthetic antioxidants has been falling off due to their suspected action as cancer promoters. There is a considerable interest, in recent years, in finding new, natural antioxidants for application in food, pharmaceuticals, and cosmetics. Moreover, searching for novel biologically active compounds sometimes leads to the discovery of new properties of known plant constituents.

This paper presents an overview of polyphenolic compounds analysis and screening of their antioxidant activity, without being exhaustive.

NATURAL ANTIOXIDANTS

The natural antioxidants are a potential source of compounds possessing biological activities, employed in foods, pharmaceuticals, and cosmetics. The

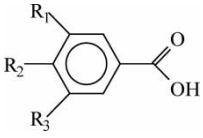
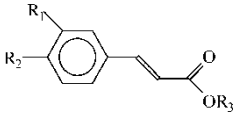
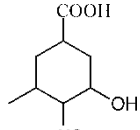
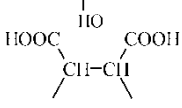
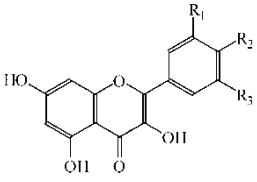
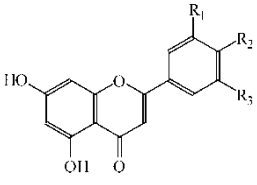
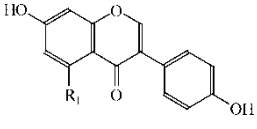
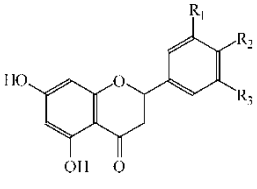
natural compounds having antioxidant properties are phenolic compounds (polyphenols), which are widely distributed in plants. Polyphenols have an important role as antioxidants in the defense mechanism of the body. Polyphenols scavenge superoxide radicals and hydroxyl radicals, reduce lipid peroxyl radicals, and inhibit lipid peroxidation by interrupting the free radical pathway.[4]

Polyphenols can be classified, according to their structures, into different groups. The groups and the structures of the most common phenolic compounds are presented in Table 1. Without compounds from Table 1, a large diversity of phenolic compounds occur in nature, due to the association of polyphenols with various carbohydrates and organic acids and with each other. Phenolic compounds may be divided into three groups: phenolic acids, flavonoids, and tannins.

Two classes are distinguished within the group of phenolic acids: derivatives of hydroxybenzoic acid and derivatives of hydroxycinnamic acid. The hydroxybenzoic acid content of plants is very low, but these compounds are components of hydrolyzable tannins, such as gallotannins, ellagitannins, etc. The hydroxycinnamic acids are more common and they are found in plants as glycosylated derivatives or esters, rather than in free form. The main representative and abundant compound of this class is caffeic acid.

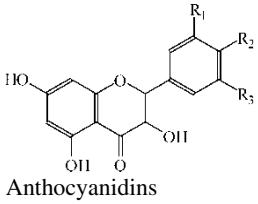
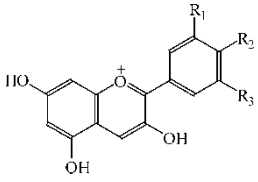
Flavonoids occur in a variety of structural forms. Flavonoids are divided into six classes: flavonols, flavones, isoflavones, flavanones, flavanols, and anthocyanidins. Different flavonoid classes may be linked through a common acyl moiety. Flavonols are one of the most omnipresent flavonoids in plants, and they are generally presented in glycosylated forms by association with a sugar moiety (glucose, rhamnose, etc.). The main representative compounds are quercitin and kaempferol. Flavones are less common than flavonols in plants. Usually, the flavones are present as glycosides or polymethoxylated derivatives. The principal compounds of this class are luteolin and apigenin. Isoflavones are structures similar to estrogens and they are found in leguminous plants. The main compounds are genistein and daidzein, present as aglycones, glucosides, acetylglucosides, and malonylglucosides. It is important to mention that aglycones are highly resistant to heat. Flavanones are found in aromatic plants, being usually glycosylated by a disaccharide such as neohesperidose and rutinose. The main aglycones are naringenin, hesperitin, and eriodictyol. Flavanols are found in fruits and leguminous plants, either in the monomer form (catechins), or in the polymer form (proanthocyanidins). The richest sources of flavanols are chocolate and green tea, but they are also present in red wine. The main flavanols are catechin and gallocatechin. Proanthocyanidins (tannins) are dimmers, oligomers, and polymers of catechins, with polymerization ranges between 4 and 11. Tannins are responsible for astringent and bitterness character of plants. Anthocyanidins are pigments dissolved in plant tissues and they exist in both colored and uncolored forms, depending on pH, copigmentation, and metal chelation. In plants, anthocyanidins are stable to

Table 1. The structures of most common compounds of polyphenolic groups

Group	Compound	R_1	R_2	R_3
Hydroxybenzoic acids 	Protocatechuic acid	OH	OH	H
	Gallic acid	OH	OH	OH
Hydroxycinnamic acids 	Coumaric acid	OH	H	
	Caffeic acid	OH	OH	
	Ferulic acid	OCH_3	OH	
	Chlorogenic acid			
	Chicoric acid (dimer of caffeic acid)	OH	OH	
Flavonols 	Kaempferol	H	OH	H
	Quercitin	OH	OH	H
	Myricetin	OH	OH	OH
	Morin	OH	H	OH
	Datiscetin	OH	H	H
	Galangin	H	H	H
	Kaempferide	H	OCH_3	H
	Isorhamnetin	OCH_3	H	H
Flavones 	Apigenin	H	OH	H
	Luteolin	OH	OH	H
	Chrisyn	H	H	H
	Acacetin	H	OCH_3	H
Isoflavones 	Daidzein	H		
	Genistein	OH		
Flavanones 	Naringenin	H	OH	H
	Eriodictyol	OH	OH	H
	Hesperetin	OH	OCH_3	H
	Pinocembrin	H	H	H
	Isosakuranetin	H	OCH_3	H

(*continued*)

Table 1. Continued

Group	Compound	R_1	R_2	R_3
Flavanols	Catechin	OH	OH	H
	Gallocatechin	OH	OH	OH
	Pinobanksin	H	H	H
	Procyanidin	H	OH	OH
Anthocyanidins	Pelargonidin	H	OH	H
	Cyanidin	OH	OH	H
	Delphinidin	OH	OH	OH
	Petunidin	OCH_3	OH	OH
	Malvidin	OCH_3	OH	OCH_3
	Peonidin	OCH_3	OH	H

conditions that could degrade them, such as light, pH, and oxidation. Moreover, anthocyanidins are stabilized by glycosylation, esterification, or complexation with other flavonoids. The main compound is cyanidin. The number of identified anthocyanins (anthocyanidine glycosides) has increased dramatically in recent years to a total of about 600.[5]

Many attempts have been made in order to explain the structure-activity relationships of polyphenols.[6–8] It can be concluded, from these researches, that the monophenols are less efficient than polyphenols, but the methoxy substitution of monophenols increases their antioxidant capacity. The degree of antioxidant capacity could also be influenced by the accessibility of the radical center to each polyphenol.

(HP)TLC IN PLANT ANALYSIS

TLC is generally regarded as a common analytical technique, being widely used for the analysis of plants. Although this technique is used in almost every laboratory of the world, there is considerable resistance against any modernization of methodology. There is a trend to consider TLC as an old-fashioned technique which should be replaced by other chromatographic techniques, like high performance liquid chromatography (HPLC). But, (HP)TLC, which takes into account the latest technical and methodological developments, is a reliable and powerful chromatographic technique. (HP)TLC is able to provide adequate results with less effort.[9]

One of the oldest fields of TLC application is for the identification of plants; the TLC fingerprints of medicinal plants and extracts were

implemented in the most pharmacopoeias. Among the chromatographic methods recommended in the pharmacopoeias for the analysis of medicinal plants, extracts, tinctures, essential oils, and other plant products, the proportion of TLC is: 100% in Romanian Pharmacopoeia, 84% in Italian Pharmacopoeia, 82% in European Pharmacopoeia, and 79% in Hungarian Pharmacopoeia.[10] (HP)TLC offers some advantages, such as:

- lower costs;
- the equipment is easy to use;
- short time of analysis;
- many samples can be evaluated and compared on the same plate;
- sample preparation needs little or no cleanup;
- the possibility of multiple detection and specific derivatization on the same plate;
- the samples can be analyzed in different chromatographic systems for different classes of compounds, thus obtaining a fingerprint;
- the results can be communicated as images;
- the flexibility, as a consequence of any restriction absence, of the choice of mobile and stationary phases.

The identification of separated compounds, the main application of (HP)TLC, can be done on the basis of retention parameter (R_f) and colors of spots. It can be stated that the compound is identical with a standard substance if the difference between R_f values is less than 0.03.[11] An unambiguous identification can be obtained by using three different stationary phases eluted with the same mobile phase, or three different mobile phases with same stationary phase (usually silica gel), or three different detection types. On this basis, the fingerprints of samples can be obtained and the components can be detected and described without the need to know the chemical nature of each chromatographic spot. Moreover, the (HP)TLC fingerprints can be generated and saved as electronic images using video or digital cameras or a flat bed scanner.[12]

A new application of the (HP)TLC technique is for the stability tests of extracts and finished products. Based on fingerprints, any changes or degradations during process development can be detected, can be documented if the composition of individual batches is kept constant and if the raw material is preserved or converted in the steps of production process. Moreover, the degradation products induced during the stress test could be detected. Stability tests using (HP)TLC are based on the visual comparison of fingerprints, which sre not used for very small changes in the sample to be detected, but significant changes are easily seen. A great advantage of (HP)TLC is the possibility to visualize the entire sample on the plate, which is not possible in on-column separations (gas chromatography and liquid chromatography). An example is the stability tests of *Vitex agnus castus* extract.[13] The extracts are stressed under drastic conditions and the

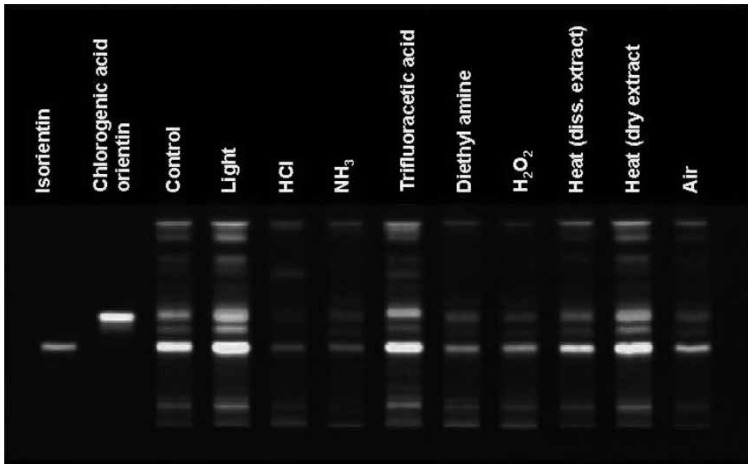


Figure 1. Results of stress tests on flavonoides (UV 366 nm).[13]

changes in the flavonoid content are investigated (Figure 1). Flavonoids are separated on (HP)TLC silicagel $60F_{254}$ plates with tetrahydrofuran-toluene-formic acid-water 16 : 8 : 2 : 1, v/v and detected by dipping of the warm plate into natural products reagent, followed by polyethylene glycol 400 solution. The results show that the flavonoides are rather stable under stress conditions. The concentrated HCl and H_2O_2 change all constituents.

ANALYSIS OF ANTIOXIDANTS

A great number of (HP)TLC techniques have been developed and successfully applied for the separation of polyphenols from each other and from other components of the plant extracts.[5,14] Some of the chromatographic conditions for the analysis of polyphenols by (HP)TLC are presented in Table 2.

It can be seen, from the many researches on plant screening, that a wide variety of (HP)TLC plates are commercially available; many of these have found applications in the natural antioxidants analyses. From an analytical point of view, (HP)TLC on silica gel will give superior results in most cases, because these plates give better separation, reproducibility, less band broadening, and they are more sensitive than TLC plates. But, a TLC method developed previously can be easily converted to (HP)TLC plates. Reversed phase (HP)TLC on chemically bonded phases based on silica gel offer some advantages, due to their different selectivity, the most promising one being diol-modified silica gel, but only a few water-miscible organic solvents can be used.

Also, it can be concluded that the quality of separation depends on positioning of the sample, size, homogeneity, and shape of the application zones.

Table 2. Chromatographic conditions for the analysis of polyphenols from different samples

Analysed compounds	Stationary phase	Mobile phase	Detection	Sample	Ref.
Gallic acid, protocatechuic acid, chlorogenic acid, ferulic acid, synapic acid, p-hydroxybenzoic acid, elagic acid, p- and o-coumaric acid	2D TLC DC-silica gel $60F_{254}$	I–benzene-acetic acid-water 6 : 7 : 3, v/v II–sodium formate-formic acid-water 10 : 1 : 200, v/v	Diazotited sulfanilic acid in 10% sodium carbonate daylight	Rudbeckia hirta	[15]
Apigenin, luteolin, caffeic acid, chlorogenic acid	TLC silica gel $60F_{254}$	Ethyl acetate-formic acid-acetic acid-water 100 : 11 : 11 : 27, v/v	1% NP[a] and %% PEG[b] UV light (366 nm)	*Rosmarini folium*	[16]
Eriocitrin, hesperidin, luteolin-7-O-rutoside, diosmin, rosmarinic acid	(HP)TLC NH_2	Acetone-glacial acetic acid 87 : 15, v/v	a–UV light (365 nm) b–2% methanolic $AlCl_3$, UV light (366 nm) c–bis-diazotized sulfanilamide, VIS light	Mentha piperita	[17]
Caffeic acid, gallic acid, p-coumaric acid, kaempferol, apigenin, naringenin	TLC silica gel $60F_{254}$	Benzene-ethyl acetate-formic acid 30 : 15 : 5, v/v	a–UV light (254 nm) b–UV light (366 nm) c–1% ethanolic $AlCl_3$ UV light (366 nm)	Croatian red wines	[18]
Naringenin, morin, chrysin, quercetin, galangin, apigenin, kaempferol, coumaric acid, caffeic acid, ferulic acid	TLC silica gel 60A	Petroluem ether-acetone-formic acid 35 : 10 : 5, v/v	a–UV light (254 nm) b–1% NP + 5%PEG UV light (366 nm)	Standard mixture	[19]

Luteolin, luteolin-4- and luteolin-7-O-glucoside, apigenin, apigenin-7-O-glucoside, amentoflavone, bartramiaflavone, philonotisflavone	TLC Sil $C_{18}-50$	Methanol-water-acetic acid 78 : 25 : 3, v/v	1% NP and 5%PEG	Phillyrea latifolia	[20]
Isoquercitin, avicularin, rutin, apigenin-7-glucoside, naringin, hesperidin	(HP)TLC silica gel $60F_{254}$	Ethyl acetate-methanol-formic acid 9 : 1 : 0.1, v/v	a–UV light (254 nm) b–1% NP + 5%PEG	Standard mixture	[21]
Caffeic acid, ferulic acid, galangin, quercitin, pinocembrin, naringenin, apigenin, chrysin, kaempferol, acacetin	2D TLC silica gel $60F_{254}$	I–chloroform-methanol-formic acid 44 : 3.5 : 2.5, v/v II–n-hexane-ethyl acetate-acetic acid 31 : 14 : 5, v/v	a–UV light (254 nm) b–UV light (366 nm) c–1% ethanolic $AlCl_3$ UV light (366 nm)	Propolis	[22, 23]
Puerarin,3′-methoxypuerarin, daidzin, daidzein	(HP)TLC silica gel F_{254}	Chloroform-methanol-ethyl acetate-water 16.2 : 18.8 : 52 : 3, v/v	UV light (254 nm)	Pueraria radix	[24]
Echinacoside, cynarine, chlorogenic acid, caffeic acid, caftaric acid, chicoric acid	(HP)TLC silica gel $60F_{254}$	Ethyl acetate-ethylmethyl ketone-formic acid-water 15 : 9 : 3 : 3	NP reagent UV light (366 nm)	Echinacea	[25]
Quercetin, biapigenin, quercitrin, isoquercitrin, hyperoside, rutin, quercetin-glycoside, caffeic acid, chlorogenic acid	TLC silica gel F_{254}	Ethyl acetate-formic acid-acetic acid-water 100 : 11 : 11 : 26, v/v Ethyl acetate-formic acid-water 8 : 1 : 1, v/v	NP/PEG UV light (365 nm)	Hypericum	[26]

(*continued*)

Table 2. Continued

Analysed compounds	Stationary phase	Mobile phase	Detection	Sample	Ref.
Vitexin, isovitexin, isoorietin	(HP)TLC RP-18W F_{254}	Tetrahydrofuran-water-phosphoric acid 40 : 60 : 1, v/v	2% methanolic $AlCl_3$ UV light (254 nm)	Cucumis sativus	[15]
Chalcone, apigenin, diosmetic, luteolin, myrecitin, kaempferol, qurcitin, rhamnetin, eriodictyol, hesperetin, neringenin, genistein	TLC silica gel F_{254}	Ethyl acetate-chloroform 60 : 40, v/v	1% NP + 5% PEG UV light (365 nm)	*Crataegus monogyna, Barosma betulina Citrus aurantium, Tilia cordata, Ginkgo biliba, Sophora japonica*	[27]
Cyanidin, delphinidin, malvidin	TLC silica gel F_{254}	Ethyl acetate-methyl ethyl ketone-formic acid-2M HCl 65 : 10 : 6 : 9, v/v	Daylight	*Centaurea cyanus, Papaver rhoeas, Ribes nigrum, Vaccinium myrtillus, Vitis vinifera, Hibiscus sabdariffa, Malva sylvestris*	[27]
Cyanidin-3-malonylglucoside, cyanidin-3-glucoside, petunidin-3-glucoside, malvidid-3-glucoside, cyanidin	2D TLC DC-cellulose	I–n-amyl alcohol-acetic acid-water 2 : 1.1 : 1 II–formic acid-hydrochloric acid-water 10 : 1 : 3, v/v	Daylight	*Rudbeckia hirta*	[15]
Anthocyanins	TLC corn or rice starch	n-butanol-glacial acetic acid-water-benzene 50 : 20 : 10 : 0.5, v/v	0.05% methanolic rhodamine B UV light (254 nm)	Fruit juices	[28]

Anthocyanins	TLC silica gel 60	2-propanol-acetone-water 70 : 10 : 20	UV light	*Coleus, Prunus cerasifera* and *Rhus hirta* leaves	[29]
Cyanidine, pelargonine, pelargonidine, malvinine	TLC silica gel 60	Stepwise gradient ethyl acetate-2-propanol- acetic acid-water	a–4% aqueous KOH b–5% ethanolic $AlCl_3$ c–5% aqueous Na_2CO_3	Fruit and flower petals	[30]
Ethyl gallate, methyl galate, galic acid, m-digallic acid, catechol, ellagic acid	TLC silica gel	benzene-acetic acid 1 : 1, v/v	a–UV light b–$FeCl_3$ or Folin's reagent	*Acacia nilotica*, *Acacia farnesiana*	[31]
Ellagitannins, gallotannins, flavonoid glycoside	Tannins 1–TLC silica gel 2–TLC RP-18 3–TLC cellulose Flavonoids 1–(HP)TLC silica gel CN 2–(HP)TLC sil Diol 3–(HP)TLC sil RP-18	Tannins 1–diisopropyl ether-acetone-98% formic acid 5 : 4 : 1, v/v and ethyl acetate-acetone-glacial acetic acid 7 : 2 : 1, v/v 2–water-methanol-98% formic acid 69 : 30 : 1, v/v 3–water-glacial acetic acid 47 : 3, v/v Flavonoids 1–n-hexane- acetone 1 : 1, v/v 2–diisopropyl ether-acetone-water-98% formic acid 12 : 6 : 1:1, v/v water-methanol-98% formic acid 49 : 50 : 1, v/v	a–UV light 254 nm and 366 nm Tannins b–1% methanolic $FeCl_3$ + bis-diazotized sulfanilamide Flavonoids a–1% methanolic $AlCl_3$	Erodium	[32]

(*continued*)

Table 2. Continued

Analysed compounds	Stationary phase	Mobile phase	Detection	Sample	Ref.
Delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside	TLC cellulose	Formic acid-HCl-water 1 : 1 : 2, v/v or n-butanol-acetic acid-water 4 : 1 : 5, v/v	UV light 254 and 366 nm daylight	Glycine max Merr	[33]
Cyanidin-3-glucoside, peonidin-3-glucoside, coumaroyl esther of cyanidin-3-glucoside	TLC silica gel	Chloroform-formic acid-acetic acid 9 : 1 : 1, v/v benzene-ethyl acetate 17 : 1, v/v	Bromcresol green	Podalyria and Virgilia	[34]
Malonylated malvidin glycoside, cyanidin-3-glucoside, cyanidin-3-rutinoside	TLC microcrystalline cellulose	Butanol-2 N HCl 1 : 1, v/v or acetone-HCl-water 15 : 3 : 82, v/v	Daylight	Cephaelis cynomorium, Enterpe, Lavatera, Pinanga	[35]

[a]Methanolic diphenylboryloxyethylamine (NP).
[b]Ethanolic polyethyleneglycol 4000 (PEG).

The samples can be applied onto the plate as spots or bands by contact spotting and spray-on application. The chromatographic separation is dependent on the type and saturation of the developing chamber and developing distance. The detection of compounds can be made by examination of the chromatogram prior to, and/or after, derivatization, under UV and visible light. Chemical derivatization can be performed without difficulties, either by spraying with, or dipping into solution, of specific or non-specific reagents. A heating step is usually necessary after derivatization.

Some recommendations for chromatographic conditions have been made in the case of method standardization:[36]

Stationary Phase

(HP)TLC silica gel 60 F_{254}, predeveloped with methanol, dried at 120°C for 20 min, cooled to room temperature, and equilibrated with the relative humidity of the laboratory.

Sample Application

Band application by a spray-on technique, 8 mm bands, 10 mm apart, 8 mm from the lower edge of the plate, first application position 20 mm from left edge of the plate, not less than 2 μL volume per band.

Development

Saturated twin-trough chamber, 5 or 10 mL of developing solvent in the front trough of a 10 × 10 cm or 20 × 10 cm chamber. The chamber is saturated for 20 min prior to plate development. Developing distance is 60 mm from the lower edge of the plate.

Derivatization

Derivatization by rapid and short immersion in a suitable reagent, followed by heating of the derivatized plate for 5 min at 110°C, or until colored spots appear.

Documentation

Documentation is done with a video or digital system, under UV light at 254 nm and 366 nm prior to derivatization, and under 366 nm and visible light after derivatization.

Evaluation

Scanning in the absorbance mode with a slit 6 × 0.3 mm, scanning distance 5–65 mm, at 254 nm, using a deuterium lamp. When the target compound fluoresces, scanning in the fluorescence mode with a mercury lamp at 366–400 nm. Recording spectra from 200 to 400 nm for each detected peak. Performing quantitative evaluation at absorption maximum of target compound.

SCREENING AND DETERMINATION OF ANTIOXIDANT ACTIVITY

There has been considerable interest in recent years in finding new natural antioxidants for application in foods, pharmaceuticals, and cosmetics. Use of natural antioxidants receives much attention because they are natural, non-synthetic, products and their appreciation by consumers is very favorable.[37] Mostly complex crude plant extracts are screened for antioxidant activity or to distinguish the components of plant extracts with antioxidant character or radical-scavenging properties. The antioxidant activity of plant constituents is of interest in order to determine the individual antioxidant capacity of target compounds and to verify if the antioxidant capacity of a compound remains constant or not, if it is a single substance or in a mixture. It is well known that the antioxidants could be have a synergistic, additive, or opposite effect when they are in mixtures.

Regarding the determination of antioxidant capacity, the spectrophotometrical methods represent the state-of-art. The activity screening involves the reaction between sample and a free stable radical, when the antioxidants reduce the radical. In order to identify the most promising natural antioxidants, expressed as radical scavenging capacity, in tests of antioxidant activity, the application of (HP)TLC using different detection reagents have been reported.[38–43]

In the screening of antioxidant capacity of polyphenols, the most often used reagent is 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH is a stable purple free radical, having an unpaired electron, and absorbs strongly in the visible region ($\lambda = 517$). DPPH can be used for both hydrophilic and lipophilic antioxidants. In contact with an antioxidant, its extensive conjugation is disrupted because the electron becomes paired and the absorption disappears, and the compound turns yellow. The resulting decolorisation is stoichiometric with respect to the number of electrons captured. The main reaction would be:[44]

$$\mathrm{DPPH}\bullet + \mathrm{PheOH} \rightarrow \mathrm{DPPHH} + \mathrm{PheO}\bullet \quad (1)$$

$$\mathrm{DPPH}\bullet + \mathrm{DPPH}\bullet \rightarrow \mathrm{DPPH} - \mathrm{DPPH} \quad (2)$$

$$\mathrm{DPPH}\bullet + \mathrm{PheO}\bullet \rightarrow \mathrm{DPPH} - \mathrm{PheO} \quad (3)$$

$$\mathrm{PheO}\bullet + \mathrm{PheO}\bullet \rightarrow \mathrm{PheO} - \mathrm{PheO} \quad (4)$$

The reactions 2–4 are theoretical termination reactions. Equation (2) is forbidden due to steric hindrance. Equation (3) will compete with Equation (4), and may be forbidden, depending on molecular PheOH and aromatic ring substituent volumes.

The parameter which is widely used as measure of antioxidant capacity is the concentration of polyphenol needed to decrease the initial substrate concentration by 50% (EC_{50}).[6,45,46] The lower the value of EC_{50}, the higher will be the antioxidant power.

For antioxidant capacity screening, after the chromatographic separation, the plates are sprayed with, or dipped for 5s, in a methanolic solution of DPPH. The radical scavenging activity is quantitatively determined by measuring the areas of the bright yellow spots on the purple background.

An illustrative example is in the TLC screening of antioxidant activity of pure anthocyanins: malvidin-3-glucoside, cyaniding-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside and petunidin-3-glucoside.[40] TLC was performed on (HP)TLC silica gel 60 plates eluted with ethyl acetate-formic acid-twice distilled water, 85 : 10 : 15 (v/v) in an unsaturated glass twin-trough chamber. After separation, the plates were dried in air and then were evaluated by spraying with DPPH solution. By statistical testing, a high correlation between the TLC and spectrophotometric methods was determined.

Another reagent used for the TLC detection of antioxidants is β-carotene.[38,39,41] The chromatographic plates were sprayed with a β-carotene solution in chloroform and then exposed to UV light at 254 nm for 15 min. β-Carotene undergoes bleaching, except in places where antioxidants prevent its degradation. Antioxidants appear as orange spots on the pale background. Moreover, the progress of oxidation could be monitored densitometrically using a substance with known antioxidant capacity as reference. The antioxidant capacity is determined by subtracting the blank from the values of the sample and reference. The ratio of the slope for the sample and reference function describes the antioxidant capacity. The main disadvantage of this method is that it gave results that are sometimes difficult to interpret visually, due to the poor contrast between spots and background.

The antioxidative activities could be determined as the amount of sample required for the inhibition of the peroxidation of linoleic acid by 50%.[42] The ethanolic solution of linoleic acid and the test sample mixture was applied to the (HP)TLC plate, followed by a peroxidation at 80^0C for 20 min. The lipid peroxides produced by spraying with α-methylindole were detected and were measured densitometrically at 557 nm.

Qualitative analysis of antioxidant capacity was performed by TLC coupled with UV spectroscopy.[43] The plant extract (*Tilia Platyphyllos*) was treated with 4-hydroxy-2,2,6,6-tetramethyl-piperidin-N-oxyl (Tempol), a free stable nitroxidic radical. The extract and the treated extract were applied to the TLC Sil G F_{254} plate and eluted with ethyl acetate-methylethyl ketone-formic acid-water, 50:30:10:10 v/v. The densitogram and the *in situ* UV spectra were recorded at 254 nm. Comparing the densitograms of

samples, it can be observed that the suitable spots of each compound decreased in intensity. The *in situ* UV spectra of each compounds from *Tilia Platyphyllos* are also compared and, from treated extract. From these UV spectra, it was noted that the absorbances of compounds decreased in intensity, or the location of the absorbance maximum is changed. The results are proof of antioxidant capacity of studied plant.

CONCLUSIONS

(HP)TLC is the method of choice for the analysis of plant materials, to separate the components from extracts, and for the screening of plant extracts. It is an inexpensive and effective technique. The commercialization of a large selection of stationary phases and the automation of chromatographic equipment make (HP)TLC a modern technique.

Nowadays, the analysis and identification of natural antioxidants with practical applications in food, medicine, and cosmetics, and the determination of antioxidant capacities of target compounds become more attractive and of great interest. (HP)TLC is a valuable technique for chemical and antioxidant screening of plant material.

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