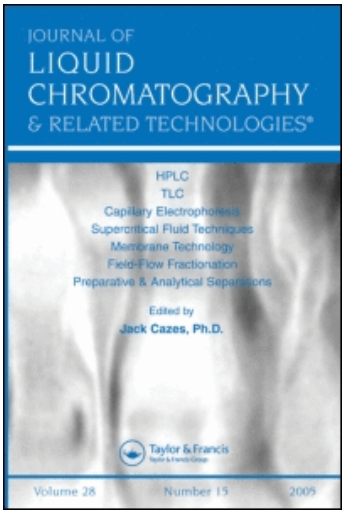

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THIN-LAYER CHROMATOGRAPHY OF NATURAL PIGMENTS: NEW ADVANCES

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THIN-LAYER CHROMATOGRAPHY OF NATURAL PIGMENTS: NEW ADVANCES

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ABSTRACT

The newest achievements in the use of thin-Layer chromatography (TLC) for the separation and quantitative determination of natural pigments in various matrices are assembled. The techniques applied for the analysis of individual pigments and pigment classes (anthocyanins, flavonoids, carotenoids, and other pigment classes) are surveyed, critically evaluated, and the advantages and disadvantages of the individual methods are discussed in detail. Future trends in the separation and identification of pigments by TLC are delineated.

INTRODUCTION

Natural pigments, such as carotenoids, anthocyanins, chlorophylls, and chlorophyll derivatives, etc., are abundant in food and food products, plants, plant extracts, and flowers. It has been many times demonstrated that consumers prefer

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products with natural color compounds compared to the color of synthetic dye additives. Consequently, the quantity and type of pigments influence considerably the buyer's decision and exert a marked impact on the mercantile value. It has been frequently established that an appropriate pigment composition is a prerequisite of marketability.

The exact amount of pigments can be easily measured by various spectroscopic methods.[1,2] Measurements can be performed directly on the sample (e.g., wine, spirit) after extraction or on the surface of the products, using the reflectance mode. Although various spectroscopic techniques are suitable for the exact determination of the total amount of pigments,[3,4] the information obtained by traditional visible spectroscopy is generally not sufficient for the quantitative determination of the individual pigment fractions. It was recognized early that the sensitivity of pigments towards oxidation,[5–7] temperature change, pH, moisture content of the environment,[8] and other storage conditions depends considerably on the chemical structure of the pigment molecule.[9] Therefore, the exact knowledge of the composition of a pigment mixture may improve the reliability of the prediction of the shelf-life of the product and may help the development of adequate technologies for preserving pigment composition. As pigments are characteristics for a given product, their accurate determination may contribute to the identification of the product, to the detection of the unauthorised addition of natural pigments,[10] and to the elucidation of adulteration.[11]

Besides their considerable commercial value, various pigments, especially carotenoids, may function as antioxidants,[12] decreasing, in this manner, disease risks[13] and exhibiting antitumor effects.[14,15] It has been established that the antitumor effect can be closely related to the increase of cell-to-cell communication.[16] Thus, the beneficial effect of pigments on lung and on other epthelial cancers has been demonstrated.[17]

Because of their high separation power and sensitivity, chromatographic methods have been extensively employed for the quantitative determination of individual pigment fractions.[18] As the thermal stability of the majority of pigments is fairly low, and they are generally not volatile, gas-chromatographic methods cannot be employed. The new achievements in the thin-Layer chromatographic (TLC) and high-performance liquid chromatographic (HPLC) analysis of various pigments[19] and carotenoids[20] have been recently reviewed.

The advantageous application parameters (low operation cost, simplicity, numerous possibilities of detection, and simultaneous analysis of a considerable number of samples) of TLC and HPTLC have been frequently exploited in the analysis of pigments. Earlier results, in general,[21] and those with special emphasis on application in plant sciences have been previously reviewed.[22]

Pigments under natural conditions commonly occur as a complex mixture of various compounds containing molecules with different adsorption capacity and hydrophobicity. The marked differences of the physicochemical

characteristics of pigments makes ineffective the use of the simple isocratic development mode. In order to overcome this difficulty, multistep gradient development[23,24] and the optimization of two-step[25] and multistep gradients[26] have been developed and successfully applied in up-to-date TLC analysis.

Pigments are more or less strongly bonded to the other components of the accompanying matrix and they have to be extracted before any chromatographic separation process. This binding exerts a considerable influence on the efficacy of the extraction methods. Consequently, it has to be taken into consideration in the development of a suitable extraction process. The selection of a successful extraction method has to take into consideration the character of the other substances bonded to the pigments and their physicochemical parameters. The overwhelming majority of extraction procedures use the traditional liquid–liquid extraction;[27,28] however, the successfull application of modern extraction techniques such as solid-phase microextraction[29] and microwave-assisted extraction[30] has also been reported.

The objectives of this work are to assemble the newest achievements in the field of the TLC separation of pigments present in various products, to describe and critically evaluate the techniques, to compare the benefits and shortcomings of the separation methods, and to outline the future prospects in this rapidly evolving field of chromatographic analysis.

CAROTENOIDS

Carotenoids are tetraterpenes present in a variety of photosynthetic tissues, and they occur in other organisms too. Unfortunately, the majority of carotenoids in nature are in esterified form. As they form esters with various fatty acids, the number of free and esterified carotenoids in a sample may be fairly high, making their separation difficult.

Carotenoids for TLC analysis are generally extracted with moderately polar organic solvents such as acetone and/or methanol. As the pigments are readily degradable at elevated temperature, in light, and at extreme pH values, these conditions have to be avoided to ensure high efficacy of extraction. Because of the strong binding of pigments to other components, sometimes the extraction step has to be repeated to reach an acceptable recovery of pigments. The combined extracts can be further purified by liquid–liquid extraction and preconcentrated by evaporation when it is necessary.

Earlier TLC methods employed only silica stationary phase and organic mobile phases of various composition for the separation of carotenoids. Because of its good separation characteristics, silica has been used recently for the simultaneous separation of carotenoids and capsaicinoids in fruit of hot pepper (*Capsicum annuum* L.).[31] Solutes were extracted with acetone–petroleum ether

(1 : 1), the extract was washed with water, preconcentrated, and separated with benzene-ethyl-acetate-methanol (75 : 25 : 5). It was found that carotenoids are well separated, the R_F values being 0.98 (β-carotene), 0.64 (cyproxanthin), 0.58 (zeaxanthin), 0.42 (antheraxanthin), 0.35 (violaxanthin), 0.29 (capsanthin), 0.23 (capsorubin), and 0.18 (neoxanthin). Silica stationary phase has also been employed for the determination of carotenoids in tissues of white storks (*Ciconia ciconia*) feeding on introduced crayfish (*Procambus clarkii*).[32] Tissues were extracted with acetone or diethyl ether, and separated by using three different mobile phases (petroleum ether-acetone-diethylamine, 10 : 4 : 1, hexane-acetone, 3 : 1, and benzene–ethyl acetate, (1 : 1). It was found that astaxanthin is the major carotenoid present in the tissues.

The development and commercialization of new chemically bonded TLC stationary phases (octyl-, octadecyl-, cyano-, diol-, and aminopropyl- chemically bonded silica) considerably enhanced the separation power of TLC technique. In order to find the best system for the separation of the color pigments of paprika (*Calsicum annuum*) TLC has been carried ut on a considerable number of TLC supports and mobile phase combinations.[33] Alumina, silica, silica-diatomaceous earth (1 : 1 w/w), diatomaceous earth, cellulose, polyamide, cyano-, diol- and amino- stationary phases have been employed for the experiments. Inorganic stationary phases have been also used in the reversed-phase (RP) separation mode after impregnating, by overnight predevelopment in *n*-hexane paraffin oil (95 : 5). Mobile phases for both adsorption and RP separation modes consisted of various mixtures of *n*-hexane, carbon tetrachloride, chloroform, acetonitrile, acetone, tetrahydrofuran, pyridine, acetic acid, methanol, 1-propanol, and dioxane.

The results clearly indicated that the considerable differences among the retention parameters of the individual pigment fractions make infeasible their baseline separation using isocratic development. The best separations have been achieved in adsorption TLC on alumina stationary phase with *n*-hexane chloroform mixtures and in reversed-phase TLC on impregnated diatomaceous earth applying mixtures of water with acetone or tetrahydrofuran. A theoretical study measured the retention of a considerable number of carotenoid standards on silica stationary phase using three different mobile phases (1 = petroleum ether-acetone, 6 : 4; 2 = petroleum ether-*tert*-butanol, 8 : 2; 3 = methanol-benzene-ethyl acetate, (5 : 70 : 25). The respective R_F values of carotenoids in the three systems were: β-carotene (0.93, 0.92, 0.93); α-cryptoxanthin (0.76, 0.83, 0.80); β-cryptoxanthin (0.75, 0.83, 0.80); zeaxanthin (0.46, 0.72, 0.51); lutein (0.46, 0.73, 0.52); nigroxanthin (0.52, 0.78, 0.63); α-carotene monoepoxide (0.92, 0.92, 0.91); β-carotene monoepoxide (0.92, 0.90, 0.91); β-carotene diepoxide (0.91, 0.90, 0.91); lutein epoxide (0.46, 0.58, 0.47); antheraxanthin (0.45, 0.55, 0.47); violaxanthin (0.41, 0.46, 0.44); cycloviolaxanthin (0.58, 0.74, 0.77); Cucurbitaxanthin A (0.50; 0.72; 0.63); capsanthin 3,6-epoxide (0.43, 0.61, 0.55); capsanthin (0.38, 0.60, 0.44); capsanthin 5,6-epoxide (0.35, 0.47, 0.39); capsorubin (0.32,

0.42, 0.38); capsanthol (6′R) (0.25, 0.26, 0.23); Capsanthol (6′S) (0.38, 0.68, 0.49); 5,6-diepikarpoxanthin (0.35, 0.36, 0.39); 6-epikarpoxanthin (0.26, 0.38, 0.24); 5,6-diepilatoxanthin (0.36, 0.45, 0.33); and 5,6-diepicapsokarpoxanthin (0.28, 0.30, 0.30). The results indicate that carotenoids, even in unesterified form, cannot be separated in one run using the isocratic separation mode. The pigments of chili (*Capsicum fluorescence*) powders have also been separated[35] by employing the same TLC stationary and mobile phases as in Ref. 33, and the powders have been tentatively classified according to the pigment composition by principal component analysis (PCA).[36] Similarly to the pigment of paprika powder, the best separation of the pigments of chili powders has been achieved on impregnated diatomaceous earth stationary phase with acetone–water (17 : 3) mobile phase. PCA indicated that the TLC profiles of chili powders of various origin can be used for the facilitation of the authenticity test of these products.

Chromarod TLC-FID (Iatroscan) has been also employed for the separation and quantitative determination of glycoglycerolipids, carotenoids, and chlorophyll *a* in spinach, the flagellate *Isochrysis galbana*, and the toxic dinoflagellate *Gymnodium* sp.[37] Spinach samples and cultures of microalgae were mixed with 2-propanol and then sonicated with chloroform–methanol (2 : 1). Extracts were concentrated at the origin of acid-cleaned (50% HNO_3) Chromarods-SIII by developing twice in acetone, and conditioning for 5 min over a saturated solution of NaCl. Separation was performed firstly in chlorofom–acetone (3 : 2) to detect monogalactosyl diacylglycerol, and then the rods were developed in acetone–formic acid (49 : 1) for 30 min. The scan times (min) of various pigments were: chlorophyll *a* (0.04 0.07), fucoxanthin (0.12 0.14), chlorophyll *c* (0.27 0.30), and a light orange pigment (0.39 ± 0.01). It was concluded from the data that the method is suitable for the polar lipids (monogalactosyl dieacylglycerol, digalactosyl diacylglycerol, and sulphoquinovosyl diacylglycerol) and pigments (chlorophylls and carotenoids).

FLAVONOIDS

Flavonoids are benzopyrene derivatives which are widely distributed in plants and frequently present as glycosides or aglycones. Because of their marked importance in human health care (anti-inflammatory, anti-allergic, anti-tumor, anti-viral, anti-diabetes, vaso-protective, and radical-scavenging activities), the quantity and composition of flavonoid pigments in medicinal plants have been extensively investigated. The exact knowledge of the chemical structure of flavonoids is of paramount importance in biochemical, pharmacological, phytochemical, and chemotaxonomical studies.

A considerable number of TLC and HPTLC techniques have been developed and successfully applied for the separation of one or more flavonoids

from each other and from other components of the accompanying matrix. Thus, a two-dimensional TLC method has been employed for the determination of ononin in the roots and aerial parts of *Ononis arvensis* L.[38] Dried and crushed plant samples were extracted with methanol, and the extract was filtered and used for TLC. Development was carried out in the first and second dimensions with the mobile phases 3% formic acid and *n*-butanol-acetic acid–water (4 : 1 : 5), respectively. After development, the ononin spots were detected at 254 nm, scratched from the layer, dissolved in methanol, and the concentration of ononin was measured by spectrophotometry at 260 nm. The averages of ononin concentration were $0.498 \pm 0.045\%$ and $0.153 \pm 0.0278\%$ in the aerial parts and roots of *O. arvensis*, respectively. Because of the simplicity and precision, the method has been proposed for the quantitative determination of ononin in plant extracts.

Another two-dimensional method was employed for the study of the pigment composition of white carnation (*Dianthus caryophyllus* L.) cultivars.[39] Pigments from petals were extracted with methanol, partitioned between water and ethyl acetate, and both extracts were analyzed on cellulose plates using *n*-butanol-acetic acid–water (6 : 1 : 2) and 2% acetic acid as first and second mobile phases, respectively. Naringenin and kaempferol derivatives were identified in the petals.

An HPTLC procedure has been employed for the measurement of the amount of kaempferol and quercetin in the extract of *Ginkgo biloba* leaves.[40] Dried leaves were pulverized and extracted with methanol under reflux for 30 min. Glycosides were hydrolysed with 25% of HCl under reflux for 60 min. After cooling, the sample was neutralized by ammonia solution (25%) and concentrated under a nitrogen stream. Silica HPTLC plates were prewashed with chloroform–methanol (1 : 1) and air dried. The mobile phase consisted of toluene–acetone–methanol–formic acid (46 : 8 : 5 : 1). Flavonoids were separated by incremental multiple development, and the plates were evaluated by a TLC scanner at 254 nm. It has been established that kaempferol and quercetin were well separated from each other and from the other matrix components. The relative standard deviation was low (1.37 and 1.40% for kaempferol and quercetin, respectively), the recovery varied between 94.11 and 97.06%. Good quadratic relationships were found between the amounts of kaempferol and quercetin in the spots and the peak areas; the coefficient of regression was 0.9999 for both compounds. It has been stated that this simple, selective, precise, and accurate HPTLC method can be employed for the standardization of Ginkgo preparations.

A different HPTLC procedure was developed for the quantitative analysis of flavonoids in the extract of *Passiflora coerulea* L.[41] Flavonoids were extracted under reflux with different mixtures of water–methanol (20, 40, 60, 80, and 100%) for 15 min and the procedure was repeated three times. The collected

extracts were used for HPTLC separation. Silica plates were pre-washed with methanol and treated with 0.1 M aqueous K_2HPO_4 solution. The best separation of flavonoid C-glycosides was achieved with the mobile phase ethyl acetate–formic acid–water (9 : 1 : 1) using multiple development (three developments to a distance of 5 cm). Flavonoids were detected by fluorescence reagents and evaluated by a TLC scanner. It was established that the best yields were obtained by 60 and 80 vol.% methanol for both isoorientin ($1.21 \pm 0.03\%$) and total flavonoids ($7.38 \pm 0.25\%$). The optimized method separated 10 flavonoids, most of which have not yet been identified. Oppositely to the analysis of kaempferol and quercetin, linear relationships were found between the quantity of analytes and the detector response, with the linear range of 57–171 ng for isoorientin and 114–456 for total flavonoids. It was stated that the method is rapid, simple, and suitable for the fingerprinting of the flavonoid content of *Passiflora* species.

Silica stationary phase was employed for the study of flavonolignan production from *Silybum marianum* transformed and untransformed root cultures.[42] Dried samples were extracted with petroleum ether, the extract was washed with methanol, and the methanol phase was concentrated and used for TLC. Solutes were developed with chloroform–acetone–acetic acid (9 : 2 : 1). The fractions separated by TLC have were redissolved and further analyzed with HPLC. It was found that untransformed root cultures contained silybin, isosilybin, silychristin, and silydianin.

HPTLC silica plates were employed for the detection of adulteration of extracts of *Maytenus ilicifolia* and *Maytenus aquifolium* with *Sorocea bomplandii*.[43] Flavonoid extraction was performed with boiling methanol, and flavonoids were separated with ethyl acetate–formic acid–water (6 : 1 : 1). The predominance of highly glycosylated quercetin and kaempferol derivatives has been established. The method was proposed for the authenticity test of the extract.

The flavonoid glycosides and aglycones of the extracts of the leaves of *Vaccinium myrtillus* L. and *Vaccinium vitisideae* L. have also been separated by HPTLC performed on silica stationary phase.[44] Dried and powdered leaves were extracted three times with 80% methanol under reflux (1 hour for each extraction procedure). The combined extracts were evaporated at 40°C in vacuum, and the viscous residue was dissolved in hot water and then extracted with petroleum ether once and five times with ethyl acetate. The ethyl acetate fraction was evaporated to dryness, redissolved in methanol, and used for HPTLC. Different elution steps have been used for the separation of various flavonoids, each mobile phase containing toluene-hexane (7 : 3) + 0.1 mL of formic acid (component A). Quercetin was separated by stepwise gradient elution using a three step gradient, the developement distance being always 3 cm: step 1 = component A-ethyl acetate–methanol (60 : 30 : 10); step 2 = the same components as in step 1 in the ratio 50 : 25 : 25; step 3 = the same as step 2. Multiple gradient elution

(development distance 9 cm) was employed for the separation of hyperin, isoquercitrin, and avicularin, the plates being dried after each separation step. The compositions of mobile phases for hyperin were: step 1 = component A-ethyl acetate-2-propanol (55 : 25 : 20); step 2 = the same components as in step one in ratio 50 : 40 : 10; step 3 = the same components as in step one in ratio 60 : 30 : 10. Isoquercitrin and avicularin were separated in two steps: step 1 = identical with step three used for the determination of hyperin; step 2 = component A-ethyl-acetate-2-propanol 50 : 25 : 25. Plates were evaluated by a densitometer at 254 nm. Good linear correlations were found between the content of flavonoid in the spot and the peak area, the coefficient of correlation varying between 0.994 and 0.998. Detection limits were 80, 170, 130, and 70 ng per spot for quercitrin, isoquercetin, hyperin, and avicularin, respectively. The quantity of flavonoids (μg/g dry leaves) found in *V. myrtillus* L. were: hyperin, 450; isoquercitrin, 134; and in *V. vitisidaea* L were: hyperin, 380; avicularin 460; quercetin, 290.

HPTLC silica plates have been employed for the measurement of flavonoids in some members of the genus *Wrightia*.[45] Dried and powdered leaves were extracted with chloroform, dried, reextracted with methanol, and used for HPTLC and HPLC analysis. Solutes were separated with chloroform–methanol (95 : 5 and 98 : 2) and ethyl acetate–formic acid–acetic acid–water (100 : 11 : 11 : 27). The presence of indigotin, indirubin, tryptanthrin, isatin, anthranilate, and rutin was verified.

Flavones and flavone glycosides have also been separated and identified in the extracts of *Phillyrea latifolia* L. using RP-TLC carried out on octadecyl-bonded silica (C_{18}) stationary phase.[46] The fat content of leaves was removed by chloroform extraction for 24 h, then the solid residue was extracted with ethanol–water (80 : 20). The extract was concentrated and extracted again with *n*-hexane. Flavones were extracted at the end by ethyl acetate. Preparative separation of flavones was performed on silica (mobile phase: ethyl acetate–formic acid–water, 6 : 1 : 1) and on C_{18} layers (mobile phase: methanol–water-acetic acid, 25 : 25 : 3). The spots were scraped from the plates, redissolved in methanol–water (1 : 1) and identified by their UV spectra and by mass spectrometry. The present of luteolin-4′-O-glucoside, apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin, and luteolin was established.

The relationship between the retention of 18 flavonoids and the composition of the mobile phase has been studied in detail.[47] Flavonoids involved in the investigations were: 3-hydroxyflavone; 5-hydroxyflavone; 7-hydroxyflavone; 5-hydroxy-8-methoxyflavone; 5,7-dihydroxyflavone; 4′,5,7-trihydroxyflavone; 4′,5-dihydroxy-7-methoxyflavone; 5,6,7-trihydroxyflavone; 3,5,7-trihydroxy-flavone; 3,5-dihydroxy-4,7-dimethoxyflavone; 3,5,7,4′-tetrahydroxyflavone; 3,3′,4′,5-tetrahydroxy-7-methoxyflavone; 3,5,7,3′,4′-pentahydroxyflavone; 3,7,3′,4′,5′-penta-hydroxyflavone; 3,5,7,2′,4′-pentahydroxyflavone; 5,7-dihydroxyflava-none; 5,7,4′-trihydroxyflavanone; and pinostrobin chalcone. R_F values of model

compounds have been measured on silica stationary phase using heptane, benzene or dichloromethane as the weaker component and ethyl acetate or methyl ethyl ketone as the stronger component of the mobile phases. The R_M values were calculated by $R_M = \log (1/R_F\ 1)$ and were plotted vs. the logarithm of the percentage of the stronger component in the mobile phase. It was found that the relationship is linear in the majority of cases, and that it depends on the chemical structure of the model compounds, mainly on the number of hydroxyl groups in the flavonoid molecule. The retention was equally influenced by the chemical character of the weaker and stronger components of the mobile phase. It was suggested that the data may facilitate the optimization of the separation of this set of flavonoids.

Similar investigations were carried out with another set of flavonoids.[48] kaempferol, kaempferol-3-glucoside, kaempferol-3-robinobioside-7-rhamnoside, kaempferol-3-*p*-coumaroyloglucoside, quercetin, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-arabinoside, quercetin-3-rhamnoglucoside, isorhamnetin-3-glucoside, isorhamnetin-3-glucoside-7-rhamonglucoside, myricetin, acacetin, apigenin, apigenin-7-glucoside, luteoiln, luteolin-7-glucoside, luteolin-6-C-glucoside, rhoifolin, naringenin, hesperetin, and hesperetin-7-rhamoglucoside. R_F values were determined on silica, diol, aminopropyl, cyano, and C_{18} HPTLC layers. Various mixtures of ethyl acetate, methanol, dichloromethane, and water were employed as mobile phases for normal phase and RP separations. It was found that the selectivity difference between the silica and diol stationary phases is relatively low, while the selectivity of the aminopropyl layer is markedly different. As in the previous publication,[47] the data were proposed for the optimization of the TLC separation of flavonoids.

The TLC behavior of flavonoids together with other classes of pigments has also been extensively investigated. Thus, the retention parameters of both coumarins and flavonoids have been determined in RP-HPTLC systems using binary mobile phases.[49] The R_M values of the following test substances have been determined: 4-hydroxycoumarin, umbelliferon, 4-methylesculation, isopinpinellin, esculin, flavone, α-naphthoflavone, kaempferol, quercetin, isoquercitrin, robinetin, robinin, myricetin, luteolin-7-O-glucoside, rutin, herperetin, hesperidin, naringin, pelargonidin chloride, polargonin chloride, and malvin chloride. The mobile phases consisted of 0.01 M aqueous phosphate buffer (pH 4) and methanol, 2-propanol, acetonitrile, dioxane, or tetrahydrofuran as organic modifiers. Linear correlations were calculated between the R_M value of test substances and the concentration of organic modifier in the mobile phase. The results emphasized the importance of intermolecular interactions (hydrogen bonds) in the retention and demonstrated the good separation power of RP-HPTLC for coumarins and flavonoids. The significant linear relationships indicated that the data can be employed for the prediction of the R_F value of solutes and can facilitate the optimization of their separation. The same set of

model pigments have been applied for the study of their retention behavior on polyamide and alumina layers using unbuffered mobile phases composed of the same components as in the previous paper.[50] Also in these cases, the relationships between the R_M value of test substances and the concentration of organic modifier in the mobile phase were highly significant. The data proved that the selectivity of alumina is higher than that of polyamide for the separation of this group of pigments. The investigations have been extended for the evaluation of the retention capacity of florisil and silica layers using the same series of pigments.[51] Also in these cases, the R_M value depended linearly on the concentration of stronger component in the mobile phase, and both the character of the stationary phase and that of the mobile phase components influenced considerably the selectivity of retention. The importance of hydrogen bond formation was also verified in this instance.

Flavonoids, anthocyanins, phenolic acids, and penta- and tetracyclic triterpenes have been analyzed in the callus cultures of different plant species.[52] Flavonoids (vitexin, isovitexin, and isoorientin) were extracted from the pulverized callus of *Cucumis sativus* L. successively with chloroform and methanol. The methanol extract was concentrated and used for TLC measurements. Anthocyanins were extracted from the cultures of *Rudbeckia hirta* L. with methanol–HCl (100 : 8.6). After extraction, the liquid phase was evaporated to dryness and the residue redissolved in methanol. Triterpenoids were extracted from the cultures of *Arctostaphylos uva orsi, Vaccinium corymbosum* var. Bluecrop, *C. sativus*, and *Bryonia dioica* Jacq. with chloroform in a Soxhlet apparatus and then concentrated under low pressure. Callus culture of *Rudbeckia hirta* L. was prepurified by subsequent extraction with petroleum ether and chloroform in a Soxhlet apparatus, and then phenolic acids were extracted with methanol. The optimal conditions of separation were different for each class of metabolites. Anthocyanins (cyanidin-3-malonylglucoside, cyanidin-3-monoglucoside, petunidin-3-monoglucoside, malvidin-3-monoglucoside, and cyanidin) were separated on cellulose layers using two-dimensional development. Mobile phases were *n*-amyl alcohol–acetic acid–water (2 : 1.1 : 1) for the first direction and formic acid–hydrochloric acid–water (10 : 1 : 3) for the second direction. The best separation of phenolic acids (gallic acid, protocatechuic acid, elagic acid, chlorogenic acid, *p*-coumaric acid, synapic acid, and *o*-coumaric acid) was achieved on silica stationary phase using also two-dimensional development. Mobile phases consisted of benzene–acetic acid–water (6 : 7 : 3) and sodium formate–formic acid–water (10 : 1 : 200) for the first and second developments, respectively. Pentacyclic triterpenes showed the most effective separation on silica plates using three subsequent developments with chloroform. Tetracyclic triterpenes were best separated also on silica layers, but with a slightly modified mobile phase (chloroform–methanol, 9.5 : 05). Altogether, 14 triterpenoids were detected, among them α-amyrin, ursolic acid, and cucurbitacines. It was

concluded from the results that TLC can be successfully employed for the analysis of complicated plant extracts with a minimal purification of the samples.

A rapid and simple method has been developed for the TLC separation of flavonoid and xanthone aglycones with monohydroxyphenyl and *o*-dihydroxyphenyl moieties.[53] Plant materials were extracted with hot ethanol, evaporated to dryness, and defatted with chloroform. Glycosides were separated on polyamide column with water–ethanol (70 : 30) and then aglycones were eluted with 70–90% ethanol and evaporated to dryness. This fraction was separated by TLC carried out on silica stationary phase. Mobile phases consisted of chloroform–acetone–formic acid (76 : 16.5 : 2.5) or toluene–ethyl acetate–formic acid (5 : 4 : 1). The following aglycones were detected in the plant samples: kaempferol, 3,8′-biapigenin, quercetin, myricetin, and norathyrol (*Hypericum aucheri* Jaub. et Spach); kaempferol, gentisein, quercetin, myricetin, and norathyrol (*Hypericum annlatum* Moris); kaempferol, 3,8′-biapigenin, quercetin, and norathyrol (*Hypericum maculatum* Crantz); kaempferol quercetin, and myricetin (*Melaleuca leucodendron* L.); kaempferol, and quercetin (*Rosa damascena* Mill.); and apigenin, genistein, and luteolin (*Stella media* L. Vill.). The method was proposed for the analysis of small amounts of aglycones in phytochemical research as well as an industrial scale procedure.

The efficacy of 10 TLC systems for the separation of flavonoid and phenolic acids has been compared.[54] Extracts were prepared by refluxing the air-dried and powered flowers of *Sambucus nigra* with methanol for 30 min and filtered. The filtrate was concentrated and redissolved in methanol. Separation were performed on silica layers using 10 different mobile phases composed of ethyl acetate, formic acid, acetic acid, methyl ethyl ketone, 1-butanol, methanol, and water in various ratios. It was established that the best separation of identified (ferulic acid, caffeic acid, and chlorogenic acid) and unidentified phenolic acids, and identified (isoquercitrin, hyperoside, and rutin) and unidentified flavonoids was obtained with the mobile phases ethyl acetate–methanol–formic acid–water (100 : 13.5 : 2.5 : 10) or ethyl acetate–formic acid–water (8 : 1 : 1). A similar procedure has been employed for the evaluation of the quality of TLC separations of flavonoid consituents of *Chamomila recutia* L. Rauschert.[55]

Cellulose layers also found application in the TLC analysis of flavonoids. They were successfully employed for the mesurement of the flavonoid composition of three genotypes of dry bean (*Phaseolus vulgaris*).[56] Dried and pulverized seedcoats were extracted with hexane, ethyl acetate, methanol, and methanol–water (1 : 1). The mobile phase consisted of 1-butanol-acetic acid–water (4 : 1 : 5). It was found that flavonoid polymers and astragalin are present in the seedcoats.

The enantiomeric separation of a wide variety of compounds including flavonoids has been performed on microcrystalline cellulose triacetate (MCTA) layers.[57] Plates were prepared by mixing 3 g of silica with 15 mL of distilled

water and then adding 9 g of MCTA and 35 mL of ethanol. Layers of 250 μm thickness have been used for the separation with the mobile phase consisting of ethanol–water (80 : 20). The respective resolution parameters (α and R_S) of flavonoids were as follows: taxifolin (1.17, 1.3); hesperetin (1.24, 1.5); naringenin (1.30, 1.6); flavanone (1.12, 0.4); 6-methoxy-flavanonne (1.17, 0.8), and 6-hydroxyflavanone (1.14, 0.8). It has been established that the chemical structure of flavonoids and the number and position of substituents exert a considerable influence on their enantioseparation.

The separation of flavonoids (catechin, kaempferol, and rutin) from the ethyl acetate-soluble fraction of the methanolic extract of *Phyllanthus emblica* L. leaves was performed in both the direct and RP separation mode, and the plates were evaluated by densitometry and video-documentation.[58] Direct-phase separation was carried out on silica HPTLC plates with toluene-ethanol-formic acid-*n*-hexane (60 : 18 : 4 : 18) mobile phase. RP-TLC of samples was performed on RP-18 plates, the mobile phase consisting of methanol–water (1 : 1) containing 1% of *o*-phosphoric acid. The results indicated that flavonoids can be effectively separated by direct and RP-TLC, and densitometry and video documentation can be equally applied for zone analysis.

TLC carried out on silica layers has also been applied in the identification process of two novel flavans from *Cyperus conglomeratus*.[59]

OTHER PIGMENTS AND PIGMENT CLASSES

Besides carotenoids and flavonoids, a considerable number of other pigments of scientific or industrial importance have been investigated by various TLC methods. Thus, the anthocyanin composition of the flowers of *Delphinium* species has been analyzed on cellulose layers. Pigments were extracted with 70% acetonitrile containing 3% trifluoro-acetic acid, the extract was hydrolyzed with 2 M HCl, and anthocyanidins were separated with water–HCl–acetic acid (10 : 3 : 3) mobile phase. It was established that flowers contain pelargonidin, cyanidin, and delphinidin.[60] Preparative silica plates have been used for the isolation of curcumin from turmeric. Turmeric was extracted with dichloromethane, the extract was evaporated to dryness, and the residue redissolved in hexane. Plates were developed three times with dichloromethane–methanol (88 : 1). The R_F value of curcumin was 0.52.[61]

Oxidized chlorophylls and metallochlorophyllic complexes of copper were analyzed in green table olives using silica stationary phase. Pigments were preconcentrated by partition between *N*,*N*-dimethylformamide and hexane. Separations were performed with the mobile phase petroleum ether–acetone–diethyl amine (10 : 4 : 1) or petroleum ether–acetone–pyridine (10 : 4 : 2.5). The

method proved the presence of Cu-15-formylpheophytin, 15^1-OH-lactone-pheophytin, and Cu-15-glyoxylic acid pheophytin in the olives.[62]

The mobility of crown ether derivatives of *meso*-Tetraphenylporphyrin has been measured on silica, alumina, and cellulose stationary phases using several mobile phases.[63] Ethyl acetate, methanol, their mixtures, acetonitrile, 2-butanol, 2-propanol, and 2-butanone were applied as mobile phases for silica; 2-butanone, cyclohexane, their mixtures, dichloromethane, ethyl acetate, their mixtures, 1,1-dichloroethane, and nitromethane were the mobile phases for alumina stationary phase. Model compounds were separated on cellulose with methanol and mixtures of cyclohexane, 2-butanone, acetone, and ethyl acetate. It has been found that the separation of compound pairs depended on the the character of the stationary and mobile phase as well as on the chemical structures of compounds to be separated. The methods have been proposed for monitoring of the synthesis of porphyrins and for control of the end products. The separation of tetraphenylporphyrin derivatives was also carried out by RP-TLC, and the results were compared with those obtained on silica stationary phase.[64] Mobile phase were acetone–methanol and dichloromethane–methanol mixtures for RP and direct phase TLC, respectively. It was found that topological indexes are suitable for description of the retention behavior of tetraphenylporphyrin derivatives in direct and RP-TLC.

The pigments of chestnut sawdust were separated on direct and RP-TLC stationary phases, and the main fraction was tentatively identified by on-line and off-line Fourier transform infrared spectrometry (FTIR).[65] Pigments were extracted with boiling distilled water, and the filtered extract was employed for TLC analyses. Normal phase separations were carried out on silica, alumina, and diatomaceous earth stationary phases, while the same stationary phases impregnated with paraffin oil were employed for RP-TLC. Mobile phases consisted of *n*-hexane, acetone, acetonitrile, ethyl acetate, tetrahydrofuran (THF), dioxane, methanol, ethanol, 1- and 2-propanol, ethylcellosolve, and water in various ratios. The best separations were obtained on silica and RP-silica layers using 2-propanol, THF, dioxane, and water mixtures and multistep gradient elution. FTIR investigations indicated that the main pigment fraction contains a high amount of hydroxyl and carboxyl groups, but the exact chemical structure of pigment could not be determined. It was concluded from the data that the main pigment in chestnut sawdust is an acidic polymer and its chemical structure is probably similar to those of tannic acid derivatives.

The formation of indigo by recombinant mammalian cytochrome P450 was verified by TLC.[66] Pigment was produced by incubating bacterial membranes in 0.10 M potassium phosphate buffer in the presence of 5 mM indole. After incubation, cultures were centrifuged and pigments were extracted three times with chloroform from the pellets. The combined extracts were concentrated and spotted onto silica plates together with authentic indigo standard.

Chloroform–methanol (50 : 1) served as the mobile phase. Results indicated that indole can be formed as the decomposition product of tryptophan, and then hydroxylated by the P450 system to 3-hydroxyindole, which is the precursor of indigo. It was suggested that mammalian P450 enzymes can be employed for the production of indigo.

The soluble pigments of raisins were separated and tentatively identified by RP-TLC-FTIR.[67] The extraction efficiency of water, methanol, acetone, acetonitrile, THF, and dioxane were compared and methanol was chosen as the most effective solvent. The same solvents have been employed for the RP-TLC separation of methanol-soluble pigment fractions of raisins. On-line and off-line TLC-FTIR indicated that the main pigment fraction is a yellow polymer composed of monosaccharides.

A similar TLC-FTIR technique has been used for the analysis of the pigments of *Trichoderma harzianum*.[68] The results indicated that the main pigment fraction is an oxidation polymer originating from monomer compounds containing polar substructures and double bonds in the alkyl chain, which are liable for oxidation during the aerobic fermentation process.

Silica plates have further been employed for the study of the formation of haemozoin/ß-haematin[69] and the depolymerization of malarial hemozoin.[70] Separations were carried out with methanol–acetic acid–water (8 : 1.5 : 0.5). Normal phase TLC (silica and cellulose layers, mobile phase *n*-propanol-1% ammonia, 2 : 1) has been also used for the study of mutations affecting xanthophore pigmentation in the zebrafish, *Danio rerio*.[71] For the elucidation of the effect of jasmonates and exogenous polysaccharides on production of alkannin pigments in suspension cultures of *Alkanna tinctoria*,[72] and for the measurement of the pigment content of granules in the central nervous system of the silkworm, *Bombyx mori*.[73]

FUTURE TRENDS

Sample preparation is a crucial step in the TLC analysis of pigments. The traditional liquid–solid and liquid–liquid extraction methods are time consuming and need a considerable quantity of organic solvents, enhancing, in this manner, environmental loading by chemicals. The more widespread use of modern extraction processes, such as solid phase extraction, microwave assisted extraction, pressurized liquid extraction,[74] may result in enhanced efficiency of extraction and decreased extraction time and solvent consumption.

Much effort has been devoted to increase the power of TLC[75] by the application of a wide variety of hyphenated techniques, such as TLC-MS,[76,77] TLC-Raman spectrometry,[78,79] TLC-solid-phase NMR spectrometry,[80] TLC-FTIR,[81] etc. As these methods highly facilitate the identification of unknown

components, even in complicated matrices, their more general application can be expected in the future.

REFERENCES

1. Conrad, R.S.; Sundstrom, F.J.; Wilson, P.V. Evaluation of Two Methods of Pepper Fruit Colour Determination. Hortscience **1987**, *22*, 608–609.
2. Navarro, F.; Costa, J. Evaluation of Paprika Pepper Colour by Triestimulus Colorimetry. Rev. Esp. Cienc. Tecnol. Aliment. **1993**, *33*, 427–434.
3. Minguez-Mosquera, M.I.; Jaren-Galan, M.; Garrido-Fernández, J. Color Quality of Paprika. J. Agr. Food Chem. **1992**, *40*, 2384–2388.
4. Nambiar, V.; Seshadri, S. A study on β-Carotene Content of Some Green Leafy Vegetables of Western India by High Performance Liquid Chromatography. J. Food Sci. Technol. **1998**, *35*, 365–367.
5. Martinez-Sanchez, F.; Nunez, M.; Amaros, A.; Gimenez, J.L.; Alcaraz, C.F. Effect of Titanium Leaf Spray Treatments on Ascorbic Acid Levels of *Capsicum annuum* L. Fruits. J. Plant Nutr. **1993**, *16*, 975–981.
6. Carneval, M.; Martinez-Sanchez, F.; Alcaraz, C.F. Improvement of Fruit Colour of Paprika Combined Treatments of Ti(IV) and Humic Acid. Acta Aliment. **1995**, *24*, 321–329.
7. Jaren-Galan, M.; Minguez-Mosquera, M. β-Carotene and Capsanthin Co-Oxidation by Lipoxygenase. Kinetic and Thermodynamic Aspects of the Reaction. J. Agric. Food Chem. **1997**, *45*, 4814–4820.
8. Malchev, E.; Ioncheva, N.; Tanchev, S.; Kalpachieva, H. Quantitative Changes in Carotenoids During Storage of Dried Pepper. Nahrung **1982**, *26*, 415–423.
9. Biacs, P.A.; Czinkotai, B.; Hoschke, A. Factors Affecting Stability of Colored Substances in Paprika Powders. J. Agric. Food Chem. **1992**, *40*, 363–367.
10. Tricard, C.; Cazabeil, J.M.; Medina, B. Identification de Colorants Naturels Ajoutes aux Aliments. Sci. Aliment. **1998**, *18*, 25–40.
11. Wrolstadt, R.E.; Wightman, J.D.; Durst, R.W. Use of Anthocyanin Pigment Analyses for Detecting Adulteration in Fruit Juices. In *Methods to Detect Adulteration in Fruit Juices*; Nagy, J., Wade, P., Eds.; Agscience Inc.: Auburndale, FI, 1995; 260–286.
12. Sies, H.; Stahl, W.; Sundquist, A.R. Antioxidant Functions of Vitamins. Vitamins E and C, β-Carotene, and Other Carotenoids. Ann. N. Y. Acad. Sci. **1992**, *669*, 7–20.
13. Graham, S.; Haughey, B.; Marshall, J.; Brasure, J.; Zielezny, M.; Freudenheim, J.; West, D.; Nolan, J.; Wilkinson, G. Diet in the Epidemology of Gastric Cancer. Nutr. Cancer **1990**, *13*, 19–34.

14. Poppel, V.G. Carotenoids and Cancer: An Update With Emphasis on Human Intervention Studies. Eur. J. Cancer **1993**, *29*, 1335–1344.
15. Micozzi, M.S.; Beecher, G.R.; Taylor, P.R.; Khachik, F. Carotenoid Analyses of Selected Raw and Cooked Foods Associated with a Lower Risk for Cancer. J. Natl. Cancer Inst. **1990**, *82*, 282–285.
16. Zhang, L.-X.; Cooney, R.V.; Bertram, J.S. Carotenoids Enhance Gap Functional Communication and Inhibit Lipid Peroxidation in C3H/10T1/2 Cells; Relationship to Their Cancer Chemopreventive Action. Carcinogenesis **1991**, *12*, 2109–2114.
17. Harris, R.W.C.; Key, T.J.; Silcocks, P.B.; Wald, N.J. A Case Control Study of Dietary Carotene in Men with Lung Cancer and in Men with Other Epthelial Cancers. Nutr. Cancer **1991**, *15*, 63–68.
18. Cserhati, T.; Forgacs, E. *Chromatography in Food Sciences and Technology*; Technomic Publishing Co. Inc.: Lancaster, Basel, 1999; 324–469.
19. Cserhati, T.; Forgacs, E.; Morais, H.M.; Mota, T. Liquid Chromatography of Natural Pigments. Biomed. Chromatogr. **2000**, *14*, 281–286.
20. Mercadante, A.Z. Chromatographic Separation of Carotenoids. Arch. Latinoam. Nutr. **1999**, *49*, 52S–57S.
21. Andersen, O.M.; Francis, G.W. Natural Pigments. In *Handbook of Thin-Layer Chromatography*; Sherma, J., Fried, B., Eds.; Marcel Dekker, Inc.: New York, 1996; 715–752.
22. Pothier, J. Thin-Layer Chromatography in Plant Sciences. In *Practical Thin-Layer Chromatography*; Sherma, J., Fried, B., Eds.; CRC Press: Boca Raton, 1996; 33–49.
23. Matysik, G.; Giryn, H. Gradient Thin-Layer Chromatography and Densitometry. Determination of Alternaria Mycotoxin. Chromatographia **1996**, *42*, 555–558.
24. Matysik, G.; Modified Programmed Multiple Gradient Development (MGD) in the Analysis of Complex Plant Extracts. Chromatographia **1996**, *43*, 39–43.
25. Wang, Q.S.; Yan, B.W.; Zhang, L.J. Computer-Assisted Optimization of Two Step Development High-Performance TLC. J. Chromatogr. Sci. **1996**, *34*, 202–205.
26. Markowski, W.J. Computer-Assisted Optimization of Gradient Multiple Development Thin-Layer Chromatography. III. Multiple-Stage Development Over a Constant Distance. J. Chromatogr. A **1996**, *726*, 185–192.
27. Bohm, V. Effect of Subambient Temperature on RP-HPLC of β-Carotene Isomers. Chromatographia **1999**, *50*, 282–286.
28. Lacker, T.; Strohschein, S.; Albert, K. Separation and Identification of Various Carotenoids by C_{30} Reversed-Phase High-Performance Liquid Chromatography Coupled to UV and Atmospheric Pressure Chemical

Ionization Mass Spectrometric Detection. J. Chromatogr. A **1999**, *854*, 37–44.

29. Emenhiser, K.; Simunovic, N.; Sander, L.C.; Schwartz, S.J. Separation of Geometrical Carotenoid Isomers in Biological Extracts Using a Polymeric C_{30} Column in Reversed-Phase Liquid Chromatography. J. Agric. Food Chem. **1996**, *44*, 3887–3893.
30. Csiktusnadi Kiss, G.A.; Forgacs, E.; Cserhati, T.; Mota, T.; Morais, H.; Ramos, C. Optimisation of the Microwave-Assisted Extraction of Pigments from Paprika (*Capsicum annuum* L.) Powders. J. Chromatogr. A **2000**, *889*, 41–49.
31. Perucka, I.; Oleszek, W. Extraction and Determination of Capsaicinoids in Fruit of Hot Pepper (*Capsicum annuum* L.) by Spectrophotometry and High-Performance Liquid Chromatography. Food Chem. **2000**, *71*, 287–291.
32. Negro, J.J.; Garrido-Fernández, J. Astaxanthin is the Major Carotenoid in Tissues of White Storks (*Ciconia ciconia*) Feeding on Introduced Crayfish (*Procambus clarkii*). Comp. Biochem. Pysiol. Part B **2000**, *126*, 347–352.
33. Cserhati, T.; Forgacs, E.; Hollo, J. Separation of Color Pigments of *Capsicum annuum* by Adsorption and Reversed Phase Thin Layer Chromatography. J. Planar Chromatogr. Mod. TLC **1993**, *6*, 472–475.
34. Deli, J. Thin-Layer Chromatography of Carotenoids. J. Planar Chromatogr. Mod. TLC **1998**, *11*, 311–312.
35. Cserhati, T.; Forgacs, E.; Morais, H.; Mota, T. Classification of Chili Powders by Thin-Layer Chromatography and Principal Component Analysis. J. Biochem. Biophys. Meth. **2000**, *45*, 221–229.
36. Mardia, K.V.; Kent, J.T.; Bibby, J.M. *Multivariate Analysis*; Academic Press: London, 1979; 213–254.
37. Parrish, C.C.; Bodennec, G.; Gentien, P. Determination of Glycoglycerolipids by Chromarod Thin-Layer Chromatography with Iatroscan Flame Ionization Detection. J. Chromatogr. A **1996**, *741*, 91–97.
38. Spilkova, J.; Dusek, J.; Solich, P.; Stranska, J.; Ruzickova, K. Application of Two-Dimensional Chromatography for Determination of Ononin in the Roots and Aerial Parts of *Ononis arvensis* L. J. Planar Chromatogr. Mod. TLC **1996**, *9*, 299–302.
39. Onozaki, T.; Mato, M.; Shibata, M; Ikeda, H. Differences in Flower Color and Pigment Composition of White Carnation (*Dianthus caryphyllus* L.) Cultivars. Sci. Horticult. **1999**, *82*, 103–111.
40. Jamshidi, A.; Adjvadi, M.; Husain, S.W. Determination of Kaempferol and Quercetin in an Extract of *Ginkgo biloba* Leaves by High-Performance Thin-Layer Chromatography. J. Planar Chromatogr. Mod. TLC **2000**, *13*, 57–59.

41. Pastene, E.; Montes, M.; Vega, M. New HPTLC Method for Quantitative Analysis of Flavonoids of *Passiflora coerulea* L. J. Planar Chromatogr. Mod. TLC **1997**, *10*, 362–367.
42. Alikaridis, F.; Papadakis, D.; Pantelia, K.; Kephalas, T. Flavonolignan Production from *Silybum marianum* Transformed and Untransformed Root Cultures. Fitoterapia **2000**, *71*, 379–384.
43. Vilegas, J.H.Y.; Lancas, F.M.; Wauters, J.N.; Angenot, L. Characterization of Adulteration of "Espinheira santa" (*Maytenus ilicifolia* and *Maytenus aquifolium*, Celastraceae) Hydroalcoholic Extract with *Sorocea bomplandii* (moraceae) by High-Performance Thin Layer Chromatography. Phytochem. Anal. **1998**, *9*, 263–266.
44. Smolarz, H.D.; Matysik, G.; Wojciak-Kosior, M. High-performance Thin-Layer Chromatographic and Densitometric Determination of Flavonoids in *Vaccinium myrtillus* L. and *Vaccinium vitis-ideae* L. J. Planar Chromatogr. Mod. TLC **2000**, *13*, 101–101.
45. Muruganandam, A.V.; Bhattacharya, S.K.; Ghosal, S. Indole and Flavanoid Constituents of *Wrightia tinctoria, W. tomentosa* and *W.coccinea.* Ind. J. Chem. **2000**, *39B*, 125–131.
46. Pieroni, A.; Heimler, D.; Huang, Y. A TLC Method for Separation and Identification of Flavones and Flavone Glycosides from Biflavones in Vegetable Extract. J. Planar Chromatogr. Mod. TLC **1998**, *11*, 230–232.
47. Maciejewicz, W.; Soczewinski, E. Chemometric Characterization of TLC Systems of the Type Silica-Binary Non-Aqueous Mobile Phase in the Analysis of Flavonoids. Chromatographia **2000**, *51*, 473–477.
48. Matysik, G.; Soczewinski, E.; Wojciak-Kosior, M.; Wojtasik, E. Optimization of Chromatographic Systems for the High-Performance Thin-Layer Chromatography of Flavonoids. Chromatographia **2000**, *52*, 357–362.
49. Bieganowska, M.L.; Petruczynik, A.; Zobel, A.M. Retention Parameters of Coumarins and Flavonoids in Binary Reversed-Phase HPTLC Systems. J. Planar Chromatogr. Mod. TLC **1996**, *9*, 273–279.
50. Bieganowska, M.L.; Petruczynik, A. Retention Behavior of Coumarins and Flavonoids on Polyamide and Alumina Layers. Part I. J. Planar Chromatogr. Mod. TLC **1999**, *12*, 135–139.
51. Petruczynik, A.; Bieganowska, M.L. Retention Parameters of Coumarins and Flavonoids on Florisil and Silica Layers. Part II. J. Planar Chromatogr. Mod. TLC **1998**, *11*, 267–271.
52. Cisowski, W.; Dembinska-Migas, W.; Krauze-Baranowska, M.; Luczkewicz, M.; Migas, P.; Matysik, G.; Soczewinski, E. Application of Planar Chromatography to the Analysis of Secondary Metabolites in Callus

Cultures of Different Plant Species. J. Planar Chromatogr. Mod. TLC **1998**, *11*, 441–446.
53. Kitanov, G. A Method for the Separation of Flavonoid and Xanthone Aglycones with Monohydroxyphenyl and *o*-Hydroxyphenyl Moiety. Acta Pharmaceut. Zagreb **2000**, *50*, 69–73.
54. Males, Z.; Medic-Saric, M.; Investigation of the Flavonoids and Phenolic Acids of Sambuci flos by Thin-Layer Chromatography. J. Planar Chromatogr. Mod. TLC **1999**, *12*, 345–349.
55. Medic-Saric, M.; Stanic, G.; Bosniak, I. The Use of Information Theory and Numerical Taxonomy Methods for Evaluating the Quality of Thin-Layer Chromatographic Separations of Flavonoid Constituents of Matricariae flos. Pharmazie **2001**, *56*, 156–159.
56. Beninger, C.W.; Hosfield, G.L. Flavonoid Composition of Three Genotypes of Dry Bean (*Phaseolus vulgaris*) Differing in Seedcoat Color. J. Amer. Soc. Hort. Sci. **1999**, 514–518.
57. Lepri, L.; Del Bubba, M.; Masi, F. Reversed-Phase Planar Chromatography of Enantiomeric Compounds on Microcrystalline Cellulose Triacetate (MCTA). J. Planar Chromatogr. Mod. TLC **1997**, *10*, 108–113.
58. Summanen, J.; Yrjönen, T.; Hiltunene, R.; Vuorela, H. Influence of Densitometer and Video Documentation Settings in the Detection of Plant Phenolics by TLC. J. Planar Chromatogr. Mod. TLC **1998**, *11*, 421–427.
59. Abdel-Mogib, M.; Basaif, S.A.; Ezmirly, S.T. Two Novel Flavans from *Cyperus conglomeratus.* Phamazie **2000**, *55*, 69–695.
60. Honda, K.; Tsutsui, K.; Hosokawa, K. Analysis of the Flower Pigments of Some *Delphinium* Species and their Interspecific Hybrids Produced via Ovule Culture. Sci. Horticult. **1999**, *82*, 125–134.
61. Anderson, A.M.; Mitchell, M.S.; Mohan, R.S. Isolation of Curcumin from Turmeric. J. Chem. Educ. **2000**, *77*, 359–360.
62. Gandul-Rojas, B.; Gallardo-Guerrero, L.; Minguez-Mosquera, M.I. Identification of Oxidized Chlorophylls and Metallochlorophyllic Complexes of Copper in Table Olives (cv Gordal) with Green Staining Alteration. J. Food Protect. **1999**, *62*, 1172–1177.
63. Kus, P. Thin-Layer Chromatographic Mobility of Crown Ether Derivatives of *meso*-Tetraphenylporphyrin. J. Planar Chromatogr. Mod. TLC **1996**, *9*, 435–438.
64. Podgorna, M.; Kus, P. Separation of Tetraphenylporphyrin Derivatives by Adsorption and Partition Thin-Layer Chromatography. J. Planar Chromatogr. Mod. TLC **2000**, *13*, 166–170.
65. Cserhati, T.; Forgacs, E.; Morais, M.H.; Ramos, A.C. TLC-FTIR of Color Pigments of Chestnut Sawdust. J. Liq. Chrom. & Rel. Technol. **2001**, *24*, 1435–1445.

66. Gillam, E.M.J.; Aguinaldo, A.M.A.; Notley, L.M.; Kim, D.; Mundkowski, R.G.; Volkov, A.A.; Arnold, F.H.; Soucek, P.; DeVoss, J.J.; Guengericht, F.P. Formation of Indigo by Recombinant Mammalian Cytochrome P450. Biochem. Biophys. Res. Comm. **1999**, *265*, 469–472.
67. Cserhati, T.; Forgacs, E.; Candeias, M.; Vilas-Boas, L.; Bronze, R.; Spranger, I. Separation and Tentative Identification of the Main Pigment Fraction of Raisins by Thin-Layer Chromatography Fourier Transform Infrared and High-Performance Liquid Chromatography-Ultraviolet Detection. J. Chromatogr. Sci. **2000**, *38*, 145–150.
68. Csiktusnadi-Kiss, G.; Forgacs, E.; Cserhati, T.; Vizcaino, J.A. Colour Pigments of *Trichoderma harzianum.* Preliminary Investigations with Thin-Layer Chromatography-Fourier Transfrom Infrared Spectroscopy and High-Performance Liquid Chromatography with Diode Array and Mass Spectrometric Detection. J. Chromatogr. A **2000**, *896*, 61–68.
69. Pandey, A.V.; Tekwani, B.L. Formation of Haemozoin/β-Haematin Under Physiological Conditions is not Spontaneous. FEBS Lett. **1996**, *393*, 189–192.
70. Pandey, A.V.; Tekwani, B.L. Depolymerization of Malarial Hemozoin: A Novel Reaction Initiated by Blood Schizontocidal Antimalarials. FEBS Lett. **1997**, *402*, 236–240.
71. Odenthal, J.; Rossnagel, K.; Haffter, P.; Kesh, R.N.; Vogelsang, E.; Brand, M.; van Eeden, F.J.M.; Furutani-Seiki, M.; Granato, M.; Hammerschmidt, M.; Heisenberg, C.-P.; Jiang, Y.-J.; Kane, D.A.; Mullins, M.C.; Nüsslein-Volhard, C. Mutations Affecting Xanthophore Pigmentation in the Zebrafish, *Danio rerio.* Development **1996**, *123*, 391–398.
72. Urbanek, H.; Bergier, K.; Saniewski, M.; Patykowski, J. Effect of Jasmonates and Exogenous Polysaccharides on Production of Alkannin Pigments in Suspension Cultures of *Alkanna tinctoria.* Plant Cell Reports **1996**, *15*, 637–641.
73. Sawada, H.; Nakagoshi, M.; Mase, K.; Yamamoto, T. Occurrence of Ommochrome-containing Granules in the Central Nervous System of the Silkworm, *Bombyx mori.* Comp. Biochem. Physiol. Part B **2000**, *125*, 421–428.
74. Schmidt, A.-C.; Reisser, W.; Mattusch, J.; Popp, P.; Wennrich, R. Evaluation of Extraction Procedures for the Ion Chromatographic Determination of Arsenic Species in Plant Materials. J. Chromatogr. A **2000**, *889*, 83–91.
75. Cserhati, T.; Forgacs, E. Hyphenated Techniques in Thin-Layer Chromatography. J. AOAC Intl. **1998**, *81*, 329–332.
76. Somsen, G.W.; Morden, W.; Wilson, I.D. Planar Chromatography Coupled with Spectroscopic Technique. J. Chromatogr.A **1995**, *703*, 613–665.

77. Ludanyi, K.; Gomory, A.; Klebovich, I.; Monostory, K.; Vereczkey, L.; Ujszaszy, K. Application of TLC-FAB Mass Spectrometry in Metabolism Research. J. Planar Chromatogr. Mod. TLC **1997**, *10*, 90–96.
78. Laserna, J.J. Combining Fingerprinting Capability with Trace Analytical Detection: Surface Enhanced Raman Spectrometry. Anal. Chim. Acta **1993**, *283*, 607–622.
79. Somsen, G.W.; ter Riet, P.G.J.H.; Gooijer, C.; Velthorst, N.H.; Brinkmann, Ua.Th. Characterization of Aminotriphenylmethane Dyes by TLC Coupled with Surface Enhanced Resonance Raman Spectroscopy. J. Planar Chromatogr. Mod. TLC **1997**, *10*, 10–17.
80. Wilson, I.D.; Spraul, M.; Humpfer, E.J. Thin-Layer Chromatography Combined with High-resolution Solid-phase NMR for Compound Identification Without Substance Elution: Preliminary Results. J. Planar Chromatogr. Mod. TLC **1997**, *10*, 217–219.
81. Stahlmann, A. Ten-Years Report on HPTLC-FTIR Online Coupling. J. Planar Chromatogr. Mod. TLC **1999**, *12*, 5–12.

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