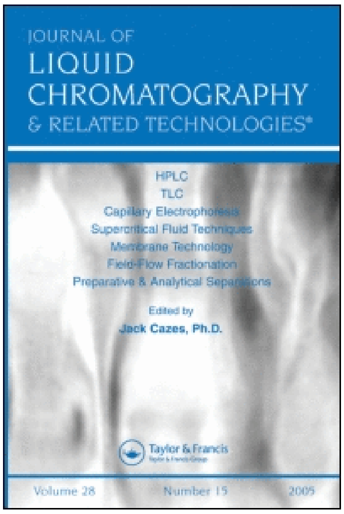

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TLC-FTIR OF COLOR PIGMENTS OF CHESTNUT SAWDUST

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ABSTRACT

The separation of color pigments of chestnut sawdust was carried out on various direct (silica, alumina, and diatomaceous earth) and reversed-phase (RP) (silica, alumina and diatomaceous earth impregnated with paraffin oil) TLC layers, and the plates were evaluated by a dual wavelength TLC scanner. The mean fraction was tentatively identified by on-line and off-line TLC-FTIR. The titration curve and strong binding of pigments to alumina indicated that each pigment fraction is of acidic character. Pigments showed anomalous retention behavior under RP-TLC conditions; retention decreased with increasing concentration of organic modifier in the mobile phase.

The anomaly was tentatively explained by the suppression of dissociation of the highly polar groups of pigments, resulting in enhanced apparent lipophilicity. Multi-step gradient elution sepa-

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rated pigments on both silica and impregnated silica layers, the separation being better under RP conditions. Only the presence of a carbonyl group in the main fraction was verified by on-line FTIR, because the method requires a higher concentration of solutes than the TLC scanner did. Off-line FTIR spectra suggested that each pigment fraction contains -OH and $-COO^-$ groups, and that they are probably tannic acid like compounds.

INTRODUCTION

Various thin-layer chromatography (TLC) methods have been frequently used for the separation and quantification of natural pigments in complicated matrices, such as foods and food products, plant extracts, etc. The quantity and composition of color pigments influence consumer choice, and have a marked impact on the commercial value of the product. Besides the commercial importance, the measurement of the composition of pigments may help the prediction of the stability of the product,[1,2] and facilitates the determination of the origin of the product (authenticity test).[3,4]

The considerable importance of the exact knowledge of the pigment composition promoted the development of a high number of liquid chromatographic methods suitable for their separation. Earlier results have been reviewed many times.[5,6] Because of the advantageous application characteristics (i.e., easy to use, inexpensive, possibility for parallel determination, no need of complicated instrumentation), TLC has been a method of choice for the chromatographic analysis of pigments. Earlier results in the TLC separation of natural pigments in general[7] and, specifically in plants,[8] have also been reviewed. However, the traditional TLC methods using visual or ultraviolet detection are not suitable for the exact identification of solutes. The co-development of well-known standards may facilitate the identification, but it is theoretically not correct, and it does not exclude the possibility of erroneous identification.

Much effort has been devoted to enhance the discriminative power of TLC.[9] Thus, the successful application of hyphenated techniques, such as TLC-MS,[10,11] TLC-Ramman spectroscopy,[12,13] TLC-solid-phase NMR,[14] etc., have been reported. Because of the high power of identification, various TLC-FTIR techniques have also found application in the analysis of a wide variety of compounds.[15] TLC-FTIR can be carried out in on-line and off-line experimental designs. The on-line method is easier to perform,[16] however, sometimes the infrared absorbance of the stationary phase interferes with the spectrum of the analyte, making identification impossible.[17] In these cases, the solute has to be extracted from the stationary phase, and an IR-transparent media has to be used for the measurement.

The separation capacity of traditional TLC can be considerably enhanced by the employment of multi-step gradient elution,[18,19] and the optimization of two-step[20] and multistep gradients[21] further improved the efficacy of the method.

The objectives of our work were the development of new normal- and reversed-phase (RP) TLC methods for the separation and quantitative determination of the color pigments of chestnut sawdust, the development of gradient elution systems suitable for the separation, and the application of on-line and off-line TLC-FTIR for the tentative identification of the main pigment fraction.

EXPERIMENTAL

One gram of chestnut sawdust was extracted with 5 mL of boiling distilled water. After cooling to room temperature, the mixture was centrifuged and the supernatant was used for the further experiments without any more pretreatment. The efficacy of the extraction was determined by drying an aliquot of the supernatant at 105°C to constant weight. The acidic character of the supernatant was assessed by measuring the original pH of the solution and the titration curve (10 mL of supernatant titrated with 1 M NaOH in steps of 0.1 mL); pH was monitored by a PHM 240 pH/ion Meter (Radiometer, Copenhagen, Denmark).

The visible spectrum of the aqueous extract was determined after ten-fold dilution from 340 to 600 nm with a Jasco (Tokyo, Japan V-570 UV/VIS/NIR) spectrophotometer. DC-Alufolien silica gel 60 F_{254}, aluminium oxide 60 F_{254}, and kieselguhr F_{254} (diatomaceous earth) plates were purchased from Merck (Darmstadt, Germany). The plates were used as received for adsorption TLC, and they were impregnated by overnight predevelopment in *n*-hexane-paraffin oil (95:5) for reversed-phase TLC.

Various mixtures of *n*-hexane, acetone, acetonitrile, ethyl acetate, tetrahydrofuran (THF), dioxane, methanol, ethanol, 1- and 2-propanol, ethylcellosolve, and water were used for adsorption and RP-TLC separation of pigments. Each solvent was of HPLC quality. Plates were developed in sandwich chambers (22 x 22 x 3 cm) at room temperature, the distance of development being approximately 17 cm. Because of the possible sensitivity of pigments to light, development was carried out in the dark.

After development, the plates were dried at ambient temperature and evaluated visually. The plates showing acceptable separation of pigments were evaluated by a Shimadzu (Kyoto, Japan) CS-930 dual wavelength TLC scanner at 340 nm. As the best separations were achieved on silica and impregnated silica layers with aqueous solutions of 2-propanol, THF, and dioxane, their effect was studied in detail. The R_F value of the less retained pigment fraction was measured on both silica and impregnated silica supports in mixtures of water - 2-propanol, THF, and dioxane with the concentration of organic modifier varying from 10 to 90 % (v/v), and the R_M value was calculated by the equation:

$$R_M = \log(1/R_F - 1) \qquad (1)$$

In order to determine the elution strength of the organic modifiers, linear relationships were calculated between the R_M value and the concentration of the organic modifier in the mobile phase:

$$R_M = R_{M0} + b.C \qquad (2)$$

where R_{M0} is the hypothetical R_M value extrapolated to zero concentration of organic modifier, b is the change of R_M values caused by 1% change of the concentration of organic modifier (related to the elution strength), and C is the concentration of the organic modifier in the mobile phase. Calculation was carried out separately for silica and impregnated silica and for each organic modifier (altogether 6 equations).

As pigments were not adequately separated under normal TLC conditions, multistep gradient elution was employed for their separation using silica and impregnated silica stationary phases and aqueous solutions of 2-propanol, THF, and dioxane at various volume ratios. Developments resulting in good separations of pigments were run in triplicate, and the mean and relative standard deviation of the retention values (R_F and peak area) were calculated (intra-day reproducibility). The inter-day reproducibility was calculated from three parallel determinations carried out for four consecutive working days (altogether, 12 measurements). The reproducibility values were compared by using the "F" probe.

The in-situ FTIR spectra of the main pigment fractions and the background spectra of layers were determined by a Nicolet Magna 750 FTIR spectrometer equipped with a diffuse reflectance accessory (resolution, 4 cm^{-1}; scan, 1024). The background spectra of the plate were extracted from those of the pigment fraction. In order to compare the background spectrum, the spectrum of silica was extracted from that of impregnated silica.

Off-line TLC-FTIR investigation of the mean pigment fractions was carried out by scraping off the fraction and putting it into a glass tube of 100 x 10 mm size. It was washed continuously with 40 mL of water - THF (1:1) at a flow rate of 0.5 mL/min. The eluate was lyophilized, and the FTIR spectra were determined by the same spectrometer using a KBr pellet of 4 mm diameter. The spectra were identified using an FTIR spectra library.[22]

RESULTS AND DISCUSSION

The color of chestnut sawdust remained brown even after aqueous extraction. This result indicates that a considerable amount of pigments remain bonded to the water-insoluble part of the chestnut sawdust, and all the following results are valid only for the water-soluble pigment fractions. It has to be emphasized, that any extrapolation of the results may lead to serious misinterpretation concerning the total pigment composition of chestnut sawdust.

The efficacy of extraction was 5.04%, proving again that a considerable part of pigments and other components were insoluble under the experimental conditions. The initial pH of the supernatant was 2.98, showing the strongly acidic character of the aqueous solution. The titration curve indicated that the supernatant contains strong acids and the molecular mass of the hypothetical acid containing one acidic group in the molecule is higher than 500. The molecular mass of pigments containing more than one acidic group can be considerably higher.

The spectra of the water-soluble pigments of chestnut sawdust showed an increasing absorbance towards the UV region without a maximum in the wavelength range under investigation (data not shown). This finding indicates, that the compounds accounting for the brown color of chestnut are not color pigments in the traditional meaning of the word. They are unknown molecules without a wavelength maximum in the visible range, but they have considerable absorbance in the visible range too.

Although pigments showed acceptable mobility on both impregnated and unimpregnated kieselguhr (diato-maceous earth) stationary phases, they were not well separated in any mobile phase system. Interestingly, pigments were very strongly retained on alumina and on impregnated alumina layers in each mobile phase. This result entirely supports the previous conclusions drawn from the titration curve. The strong binding of each pigment fraction to the basic alumina proves again that pigments are of acidic character, and the impregnated alumina partially retains its basic character even after impregnation. Only aqueous mobile phases can move the pigments in the adsorption separation mode, also indicating the highly hydrophilic nature of pigments. Unexpectedly, the retention behavior of the less-retained pigment fraction was highly similar on silica and on impregnated silica layers. The retention decreased with increasing concentration of organic modifier in the mobile phase on both layers.

It is normal retention behavior on silica where water is the strongest eluent and abnormal on reversed-phase plates. This anomaly can be tentatively explained by the supposition that pigments contain highly polar dissociable substituents (according to the previous results: acidic substructures). As the dissociation of the polar substituents can be suppressed in the presence of organic modi-

Table 1. Relationship Between the R_M Values of the Less Retained Pigment Fraction on Silica and Impregnated Silica Layers and the Concentration of 2-Propanol, Tetrahydrofuran (THF) and Dioxane (C)[a]

Parameter	Silica	Impreg. Silica	2-Propanol-THF	Dioxane-2-Propanol	THF	Dioxane
R_{MO}	−2.19	−5.19	−0.81	−1.85	−4.77	−3.58
$b.10^2$	3.64	6.50	1.90	3.21	6.01	5.23
$s_b.10^3$	3.35	7.31	2.88	1.89	2.61	2.17
r_{calc}	0.9344	0.9720	0.9393	0.9172	0.9227	0.9058

[a] $R_M = R_{M0} + b.C.$

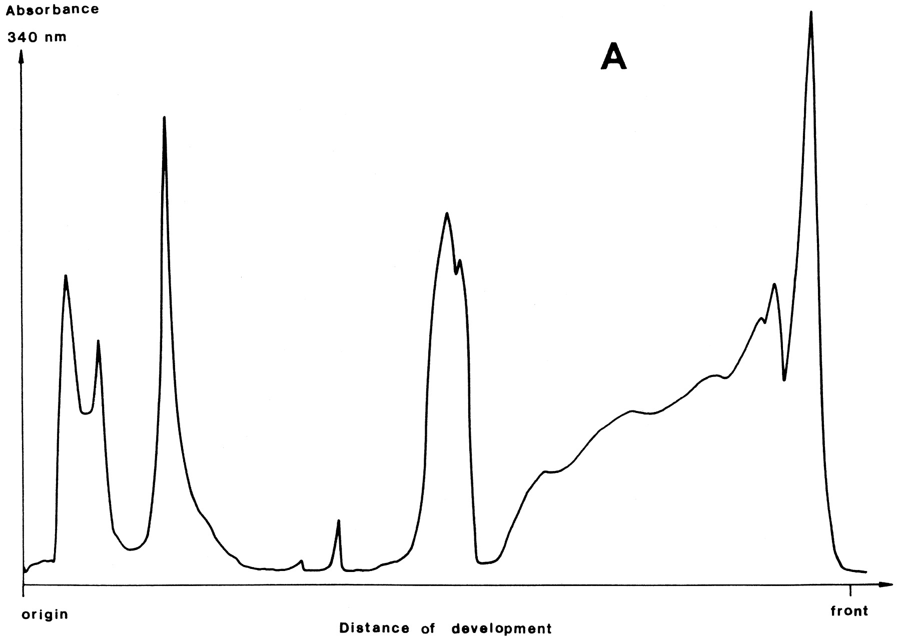
fier (lower dielectric constant), the apparent lipophilicity of solutes increases, resulting in higher retention. The parameters of eq.2 are compiled in Table 1.

The parameters in Table 1 entirely support our previous qualitative conclusions. The regression coefficients (b values) are equally positive for silica and impregnated silica, proving the anomalous retention behavior of this pigment fraction under reversed-phase conditions. Considerable differences were found between the elution strength of organic modifiers, THF being the strongest mobile phase additive.

It was observed that in each mobile phase, some pigment fractions either remained on the origin or moved with the mobile phase front. This finding proves that there are marked differences among the retention capacities of pigments, therefore, they cannot be separated in one run and in one mobile phase. Furthermore, this result suggests that the pigments can be separated by high-performance liquid chromatography only by using gradient elution.

Densitograms of color pigments of chestnut sawdust on silica and impregnated silica at 340 nm detection wavelength are shown in Fig.1. The multi-step gradient development consisted of water-THF (1:1) for 3 cm, water-THF (3:7) for 8 cm, then water-THF (1:4) to the end of development. The pigment composition is similar, but not identical, on silica and impregnated silica plates. The separation of pigments under both adsorption and RP conditions indicates that pigments markedly differ not only in their adsorption capacity but also in their lipophilicity. Pigments are separated in three large fractions: the first near to the origin, the second at the middle of the chromatogram, and the third near to the mobile phase front. The separation was better on RP-TLC plates; pigments inside the three mean groups were further separated from each other.

However, the peaks of pigment fractions were wider than expected from the separation capacity of TLC. This discrepancy may be due to the fact that pigments are relatively large molecules having adsorption centers of different bind-



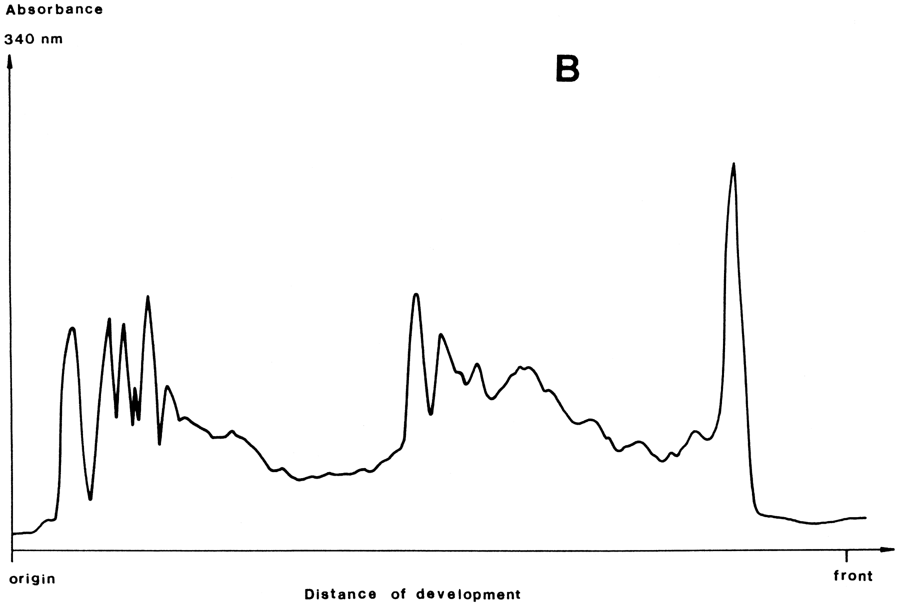


Figure 1. Densitogram of color pigments of chestnut sawdust on silica (A) and impregnated silica (B) layers at 340 nm. Multistep gradient development: water-THF 1:1 (v/v) for 3 cm; water-THF 3:7 (v/v) for 8 cm; then water-THF 1:4 (v/v) to the end of development.

ing strength in the same molecule. This finding is in good agreement with the conclusions drawn from the titration curve. The intra-day and inter-day reproducibilities of R_F values and peak areas of the three main fractions are compiled in Table 2. The TLC and RP-TLC systems described in Fig.1 were employed.

No significant differences were found between the intra-day and inter-day repoducibilities of R_F values and peak areas, proving the similar stability and reproducibility of both the TLC and RP-TLC systems. However, the standard deviations are higher than those generally found in TLC. This discrepancy can be tentatively explained by the supposition that multi-gradient development impairs the reproducibility of TLC and RP-TLC probably due to the multi-fold manual steps included in the analysis.

Interestingly, the amount of pigments sufficient for evaluation by the TLC scanner was not enough for the assessment by on-line TLC-FTIR. Only the presence of a carbonyl group in the main fractions was unambigously established. This finding indicates the TLC-FTIR has a lower sensitivity than TLC scanning, and the plates have to be slightly overloaded to obtain a good FTIR spectrum. It has to be emphasized that this conclusion is valid only for the separation and identification conditions used in these experiments and cannot be extrapolated to other TLC-FTIR on-line systems.

The off-line FTIR spectrum of the pigment fraction at the middle of chromatogram is shown in Fig.2 (stationary phase: silica; multiple step gradient elution as in Fig.1). The spectrum indicates that a considerable amount of hydroxyl and carboxyl groups are present in the samples. This finding is in accordance

Table 2. Intra-Day and Inter-Day Reproducibilities of R_F Values and Peak Areas of Main Pigment Fractions Separated with Multistep Gradient Elution. For TLC and RP-TLC Conditions, See Fig. 1

Fraction No.	System	Intra-Day Reprod. (%)	Inter-Day Reprod. (%)
R_F Values			
1	TLC	7.41	7.39
2	TLC	6.43	6.05
3	TLC	8.82	8.66
1	RP-HPLC	7.76	7.61
2	RP-HPLC	6.96	6.47
3	RP-HPLC	8.27	8.19
Peak Areas			
1	TLC	8.83	8.34
2	RP-TLC	6.70	6.05
3	RP-TLC	8.61	8.12

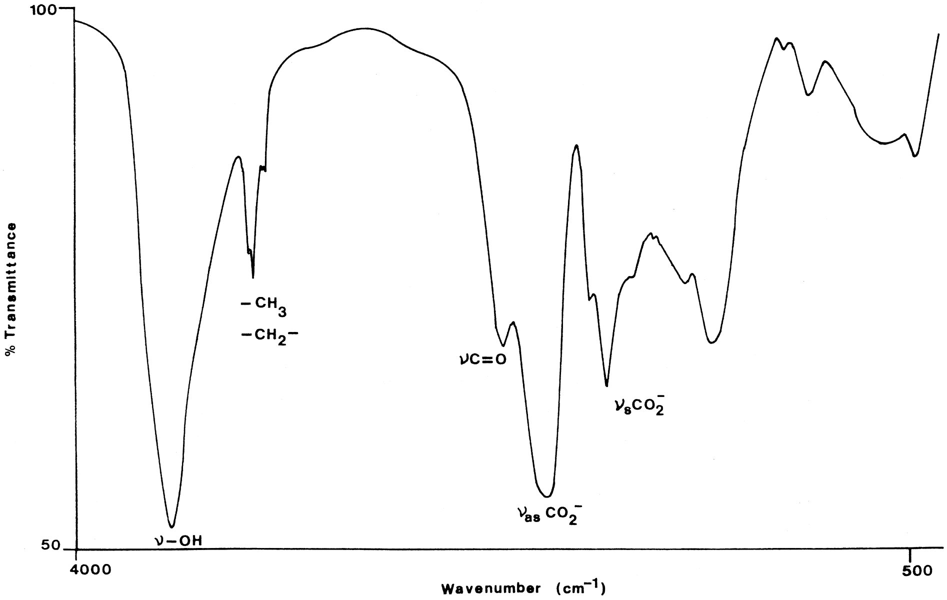


Figure 2. Off-line FTIR spectrum of the pigment fraction at the middle of chromatogram. Stationary phase: silica. Multistep gradient development as in Fig.1.

with the previous results concerning the highly hydrophilic character of the pigment. The off-line FTIR spectrum of the strongly retained main pigment fraction is shown in Fig.3 (stationary phase: silica impregnated with paraffin oil; multistep gradient elution as in Fig.1). According to the FTIR spectrum, the pigment fraction contains a considerable number of hydrophobic alkyl carbons. This finding suggests that some paraffin oil can be coextracted with the pigment and can enhance the intensity of the alkyl vibrations. Except for this part, the spectra are highly similar with an overwhelming majority of -OH and COO- vibrations. Similarly to the densitograms, the widths of some vibrations on the FTIR spectra are unusually large indicating, again, the polymeric character of the solute. Unfortunately, the spectra did not match with any spectra in the library at minimally 90% level. It can be assumed that the color pigments of chestnut sawdust are complex, very hydrophilic compounds with high molecular mass, and many hydroxyl and carboxyl groups in the same molecule. These structural characteristics suggest that these pigments are tannic acid derivatives or tannic acid-like compounds. The exact elucidation of their structures needs further investigation.

It can be concluded, from the data, that the water-soluble color pigments of chestnut sawdust can be successfully separated by both TLC and RP-TLC tech-

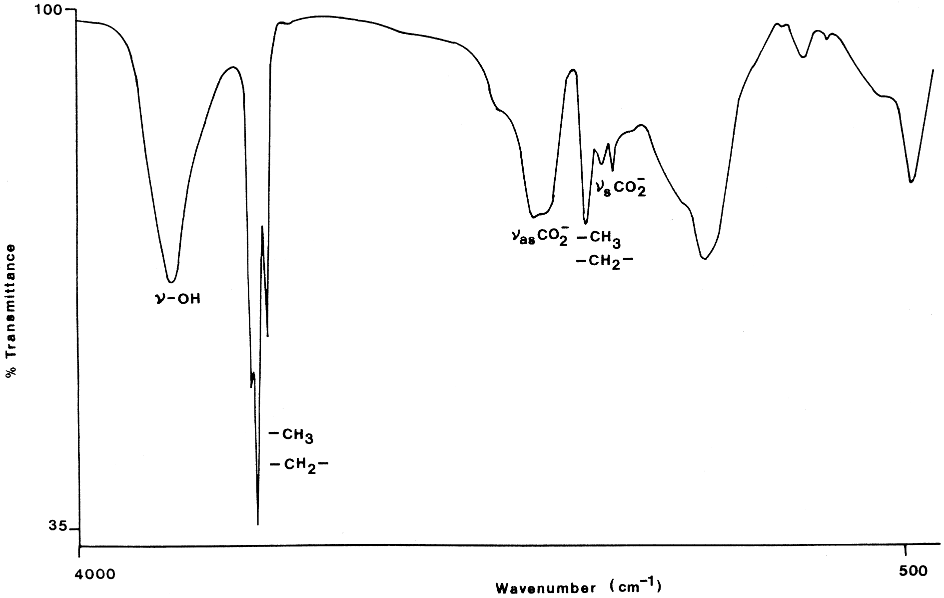


Figure 3. Off-line FTIR spectrum of the strongly retained pigment fraction. Stationary phase: silica impregnated with paraffin oil. Multistep gradient development as in Fig. 1.

niques using multi-step gradient elution. On-line TLC-FTIR showed a lower sensitivity than visible TLC scanning. Off-line TLC-FTIR indicated that each pigment contains hydroxyl and carboxyl groups, which make it probable that they are compounds with high molecular mass similar to tannic acid.

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