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

Tibor Cserháti* and **Esther Forgács**
Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences, P.O. Box 17, 1525 Budapest, Hungary

Manuel Candeias, Louis Vilas-Boas, and **Rosario Bronze**
Estacao Nacional de Investigacao Agraria, Oeiras, Portugal

Isabell Spranger
Estacao Vitivinicola Nacional, Dois Portos, Portugal

Abstract

The soluble color pigments of raisin are separated by reversed-phase thin-layer chromatography (TLC), and the capacity of TLC–Fourier transform infrared (FTIR) with both on-line and off-line coupling is assessed for the identification of the main fraction. TLC has also been used as a pilot technique for the development of a gradient elution method for the separation of pigments by high-performance liquid chromatography (HPLC). On-line TLC–FTIR cannot be used for identification because of the strong adsorbance of the stationary phase. Off-line TLC–FTIR combined with the retention behavior of the main pigment fraction indicates that it is a polymer, caramel-like compound composed of erythrose and fructose monomers. Baseline separation of pigments is achieved by HPLC using TLC as a pilot method.

Introduction

The quantity and composition of color pigments in foods and food products exert a marked influence on the consumer acceptance and, consequently, the commercial value of the products. It has been established many times that one of the main properties employed for the evaluation of the product quality is the color; that is, an adequate color considerably enhances the marketability. Spectroscopic methods measuring either the absorbance of pigment solutions or the adsorbance of the color of product surfaces on one or more wavelengths in the visible range are reliable tools for the accurate determination of the quantity of pigments (1,2). The greatest disadvantage of the spectroscopic methods is that they do not contain any useful information on the concentration of the various pigment fractions, and they are not suitable for the identification of the individual pigments. Because the stability of the pigments against hydrolysis, oxidation, and other environmental and technological conditions (3,4) shows marked differences, the exact determination of the pigment composition may help in the prediction of the shelf-life of products and the assessment of the influence of technological steps on the pigment fractions, resulting in more consumer-friendly processing methods. Furthermore, the qualitative determination and identification of the pigments may contribute to the establishment of the provenance of the product (5,6).

The advantageous application parameters of thin-layer chromatography (TLC), such as ease of use, inexpensiveness, no need for complicated instrumentation, multifold possibilities of detection, etc., have made it a preferred method for the separation and, to a lesser extent, quantitative analysis and identification of natural pigments. Previous results in the employment of TLC for the separation of natural color pigments in general (7) and specifically in plants (8) have been reviewed. The information content of TLC can be considerably enhanced by using various hyphenated techniques (9). Due to its high capacity of discrimination, Fourier transform infrared (FTIR) has also been successfully coupled with TLC (10,11). The application of on-line TLC–FTIR coupling for the analysis of drugs (12,13), adenosine in biological samples (14), and environmental pollutants in waste water (15) was previously reported.

* Author to whom correspondence should be addresed.

A possible drawback of the on-line TLC–FTIR coupling is the strong absorbance of infrared light by the supports, which can interfere with the IR spectra of the compounds. This disadvantage can be overcome by the extraction of compounds from the support followed by the determination of the IR spectra in an IR-transparent medium. This method is more time consuming and less elegant than the on-line coupling; moreover, the probability of introducing impurities during the extraction process is higher. The use of on-line or off-line TLC–FTIR methods always depends on the characteristics of the TLC/solute molecule system under investigation.

TLC can be used not only for the rapid separation of compounds but also for the prediction of their retention behavior in high-performance liquid chromatography (HPLC) (16,17). The application of TLC as a pilot method for HPLC makes possible the detection of compounds in the sample that show very low mobility or do not move in the mobile phase. These compounds can cause baseline drifting and can contaminate the column.

The objectives of the present study were the development of a TLC and HPLC method for the separation of the color pigments of raisins and the application of on-line and off-line TLC–FTIR coupling for the tentative identification of the main pigment fraction. To the best of our knowledge, the color pigments of raisins have never been studied in detail.

Experimental

Materials

Raisins were prepared from grapes (provenance Algarve, Portugal) by sun drying. Grape varieties were as follows for samples: numbers 1–4, "Rubi Seedless"; number 5, "D. Maria"; numbers 6 and 8, "Sultana" (seedless); and number 7, "Centenial" (seedless).

Extraction of color pigments

Preliminary investigations

One gram of raisin was thoroughly ground with 2 g of acid-washed sand, and 8.5 mL of extracting solvent was added to the sample under continuous mixing. Water, methanol, acetone, acetonitrile, terahydrofuran, and dioxane and the mixtures of water–organic components in 1:3, 1:1, and 3:1 (v/v) ratios were employed as extracting solvents. After extraction, the samples were centrifuged, and the visible spectra of the supernatant was determined from 400 to 700 nm with a Jasco (Tokyo, Japan) V-570 UV/VIS/NIR spectrophotometer.

Determination of extraction time and efficiency

Because pure methanol was found to be the best solvent, it was used for the following investigations. Samples were prepared with methanol as described previously and were filled in a glass tube of 100 × 10-mm i.d. The column was washed with methanol at a flow rate of 0.2–0.4 mL/min, and 8 fractions of 1 mL each were collected. The visible spectra of fractions were determined as described previously. The efficiency of the extraction was determined by measuring the spectra of the solid sample before and after the methanol extraction in reflectance mode and then extracting the spectra taken after extraction from the spectra taken before extraction. Each experiment was run in quadruplicate, and the mean and relative standard deviation of the various extraction steps were calculated.

Reversed-phase TLC

DC-Alufolien Kieselgel 60 F_{254} and DC Alufolien Aluminiumoxid 60 F_{254} plates (Merck, Darmstadt, Germany) were impregnated by overnight predevelopment in 95:5 (v/v) *n*-hexane–paraffin oil. Alugram RP-18/UV_{254} plates (Macherey-Nagel, Düren, Germany) were used as received. Extracts of raisins were spotted onto the plates, and the plates were developed with water containing various concentrations of methanol, acetone, tetrahydrofuran (THF), and dioxane. Plates were developed in sandwich chambers (22 × 22 × 3 cm) at ambient temperature, the distance of development being approximately 17 cm. Because of the possible sensitivity of pigments to light, development was performed in the dark. After development, the plates were dried at room temperature and evaluated visually, and the plates showing good separation were evaluated by means of a Shimadzu (Kyoto, Japan) CS-930 dual wavelength TLC scanner at 340 and 670 nm, the characteristic wavelengths of yellow and red pigments. Developments were run in triplicate, and the mean and relative standard deviation of the R_F values were calculated separately for each fraction and each TLC system. Because the separation capacity of the various plates was similar, further investigations were carried out on Alugram RP-18/UV_{254} plates developed with 3:7 (v/v) water–THF as mobile phase. The use of ready-made reversed-phase plates made the time-consuming impregnation step unnecessary.

TLC–FTIR on-line coupling

The main pigment fraction was measured on the plate by a Nicolet 170SX FTIR spectrometer (Nicolet Instrument Co., Madison, WI) equipped with a diffuse reflectance accessory prepared in our institute. The spectra of the plate and the main pigment fraction were measured (resolution, 4 cm^{-1}; scan, 1024) and the background spectra of the plate was extracted from that of the pigment.

TLC–FTIR off-line coupling

The main pigment fraction was scraped off and put into a glass tube 100 × 10 mm in size. It was washed continually with 20 mL of methanol at a flow rate of 0.5 mL/min. The eluate was concentrated in a vacuum and then redissolved in 20 µL of methanol, and the FTIR spectra were determined by a Nicolet Magna 750 FTIR spectrometer using a KBr pellet of 4-mm diameter (resolution, 4 cm^{-1}; scan, 128). The spectra were identified using an FTIR spectra library (18). The spectra of D-erythrose, showing the highest similarity with the spectra of the main pigment fraction, were extracted from the pigment spectra, and the difference spectra were again identified using the same library. FTIR measurements and the interpretation of FTIR spectra were carried out by Dr. Sándor Holly (Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences, Budapest, Hungary).

Reversed-phase HPLC

Separations were carried out with an ISCO (Lincoln, NB) model 2360 pump, a Waters (Milford, MA) photodiode-array detector, an NEC PowerMate SX/16 computer with PDA program, and a Valco (Houston, TX) injector with a 20-µL sample loop. Pigments were separated on an analytical column (250 × 4-mm i.d.) packed with octadecylsilica (Lichrospher, 5-µm particle size, Merck) in our laboratory using a Shandon (Pittsburgh, PA) analytical HPLC packing pump. A precolumn (35 × 4 mm i.d.) was filled with the same stationary phase. Columns were not thermostated, separations were performed at room temperature (20–22°C). Because TLC indicated that only a THF organic modifier can elute each pigment fraction under reversed-phase conditions, a gradient elution using water and THF was employed for the separation. The steps of gradient elution are shown in Table I. The flow rate was 1 mL/min, and the detection wavelength was set from 300 to 600 nm.

Table I. Steps of Gradient Elution for the Separation of Color Pigments of Raisin by Reversed-Phase HPLC

Time (min)	Water volume (%)	Tetrahydrofuran volume (%)
0	100	0
5	100	0
25	50	50
45	50	50
55	5	95
80	5	95

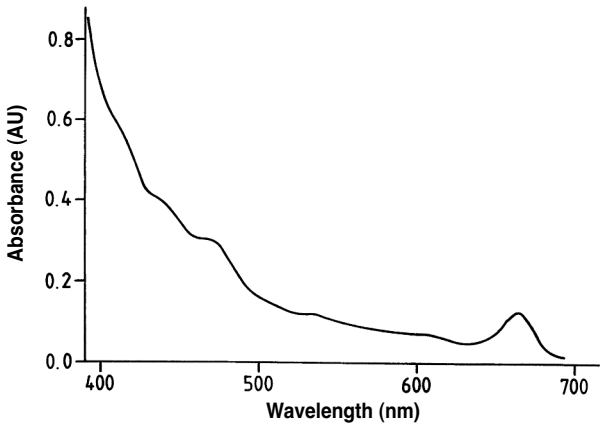

Figure 1. Visible spectrum of methanol extract (raisin number 3, fraction 4).

Results and Discussion

The relative standard deviation of the various extraction steps was high, ranging from 23 to 34%. The low repeatability of the extraction can be tentatively explained by the uneven

Table II. Relative Percent Quantity of Color Pigments in Raisins Extracted with Methanol*

Number of raisin	Pigment (%)
1	100
2	99.17
3	87.38
4	76.48
5	48.61
6	30.38
7	24.96
8	12.62

* Absorbance at 400 nm. Numbers refer to raisins in Experimental section.

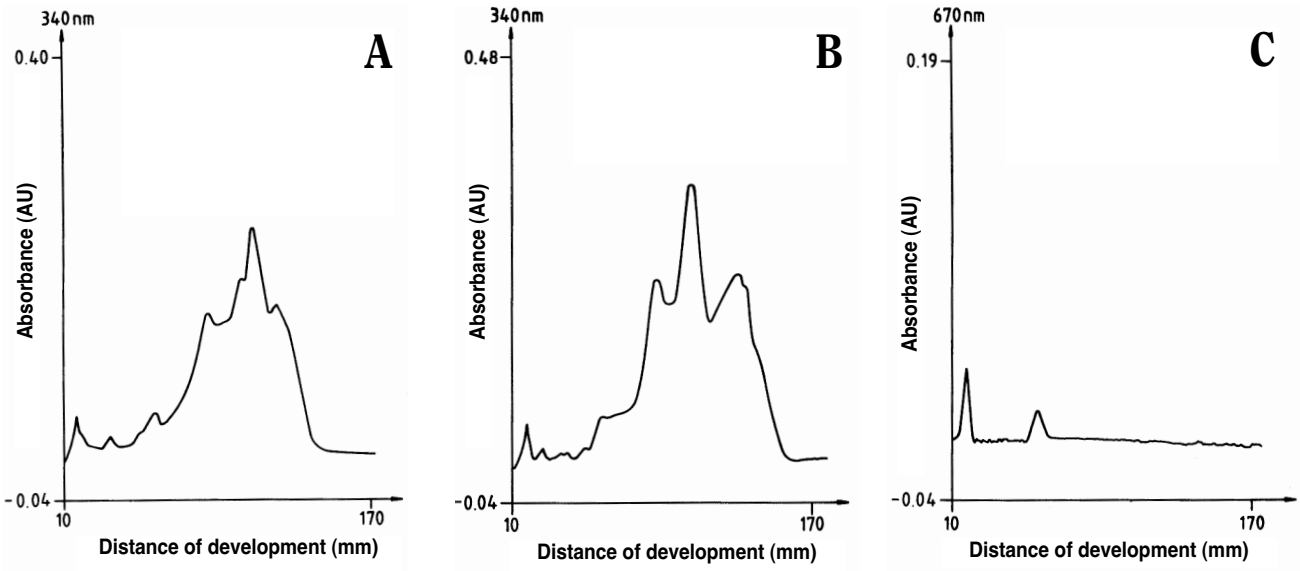

Figure 2. Densitograms of color pigments of raisins. Alugram RP-18/UV_{254} plates; mobile phase water–THF (3:7): raisin 8, detection wavelength 340 nm (A); raisin 3, detection wavelength 340 nm (B); raisin 8, detection wavelength 670 nm (C).

efficiency of the grinding and the inherent inhomogeneity of the raisin samples. The extraction efficiency of water and water–organic solvent mixtures were markedly lower than that of pure organic solvents. The differences among the performance of organic solvent were relatively low. Because of the relatively high quantity of extracted pigments and the transparency, methanol was chosen as the solvent for the further investigations of the extract.

When the time of extraction is rapid, the monotonous decrease of absorbance can be expected by washing the sample with methanol. The results contradict this supposition; the highest concentration of pigments was found in the second, third, and even fourth fraction, depending on the type of raisin. This fact indicates that pigments are more or less strongly bonded to insoluble cell constituents in raisin; their dissolution is fairly slow and the dissolution rate depends considerably on the binding characteristics of the accompanying matrix. A typical visible spectrum of a fraction is shown in Figure 1. The spectrum indicates that the quantity of yellow pigments is considerably higher than that of red ones. This finding was equally true for raisins prepared from red and white grapes, indicating that during the drying, red pigments are lost or bonded covalently to insoluble cell fractions. The spectrum further suggests that anthocyanins, the main color pigments of grapes, are not present in the soluble pigment fraction of raisins. The quantity of extracted pigments are compiled in Table II. Large differences were observed among the quantity of soluble pigments, indicating that the pigment concentration markedly depends on the type of grapes used for the production of raisins. However, it must be emphasized that this conclusion is valid only for the soluble pigment fraction, and it is not necessarily true for the total pigment concentration, which can be measured only in the reflectance mode.

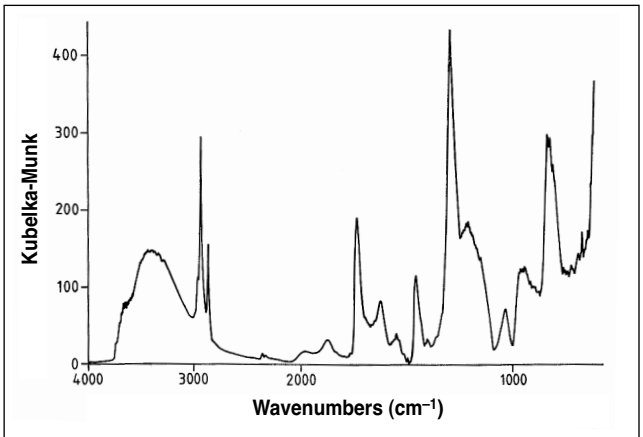

Figure 3. Kubelka–Munk spectrum of Alugram RP-18/UV_{254} plate.

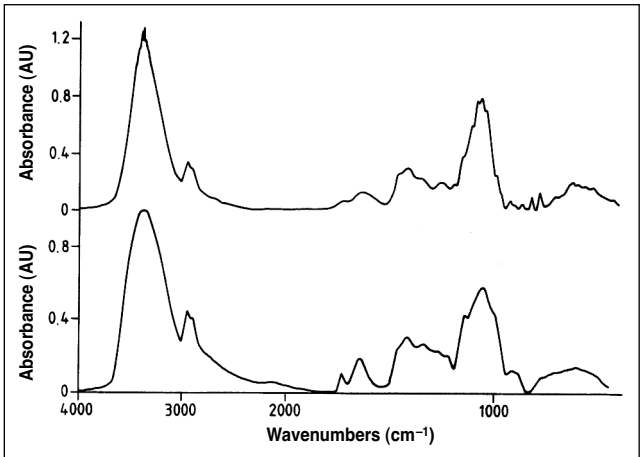

Figure 4. FTIR spectra of the main pigment fraction (upper) and D-Erythrose (lower).

Reversed-phase TLC

The number and R_F value of a pigment fraction depended markedly on the type and concentration of the organic modifier in the mobile phase. Each pigment showed regular retention behavior on each plate: the retention decreased with increasing concentration of organic component in the mobile phase. Except in water–THF mobile phases, some pigment fractions remained on the start in every other TLC system.

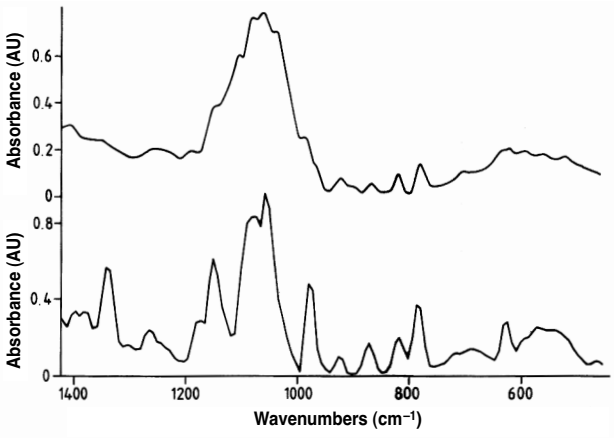

Figure 5. Difference spectra of the main pigment fraction and D-Erythrose (upper) and D-Fructose (lower).

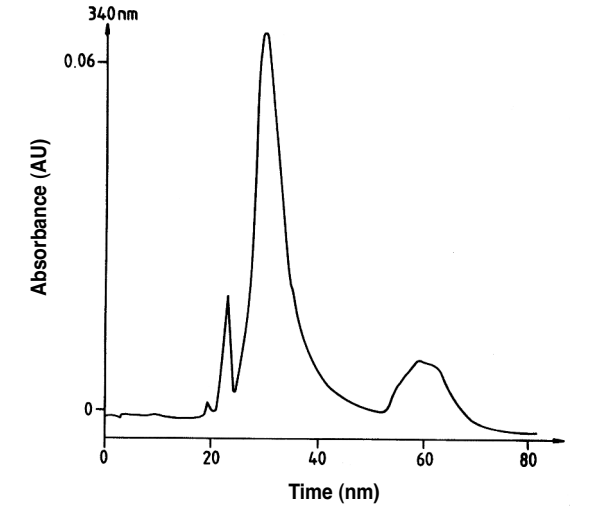

Figure 6. HPLC chromatogram of the color pigments of raisin. For chromatographic conditions, see the Experimental section.

This finding indicates that the solvent strength of THF is specially high for these classes of color pigments. Because THF is a good solvent for polymers, its high elution strength indicates the polymer character of the pigments (19). Some densitograms are shown in Figure 2. The pigment composition of raisins was similar but not identical, the ratio of pigment fractions being markedly different (compare densitograms A and B). The peaks of the main pigment fractions were wider than expected from the separation capacity of TLC. Because small pigment molecules as anthocyanins form well-defined narrow peaks in TLC, the width of the peaks indicates that the pigments are not small molecules; they possibly contain more than one fraction, and the fractions are of a polymeric character. The comparison of densitograms A and C supports our previous conclusion that the quantity of red pigments is relatively low in the soluble pigment fraction of raisin.

TLC–FTIR on-line coupling

The Kubelka–Munk spectra of the Alugram RP-18/UV_{254} plate without pigment fraction is shown in Figure 3, proving that the reversed-phase support is a strong IR adsorbent. Because of the strong background adsorbance, the difference spectra was not clear enough and cannot be used for identification.

TLC–FTIR off-line coupling

The FTIR spectra of the main pigment fraction and that of D-Erythrose are shown in Figure 4, and their difference spectra and that of D-Fructose are shown in Figure 5.

The spectra clearly show that off-line TLC–FTIR can be successfully used for the determination of the IR spectra of the main pigment fraction, whereas on-line TLC–FTIR was not suitable for this purpose. It must be emphasized that the conclusion outlined here is based only on the experimental results, and it is not supported with theoretical considerations; therefore, its generalization may lead to serious misinterpretation of the performance of on-line and off-line TLC–FTIR.

The comparison of spectra suggests that the main pigment fraction probably contains a saccharide such as erythrose, which is the decomposition product of glucose and fructose as monomer building units.

Reversed-phase HPLC

A typical chromatogram is shown in Figure 6. Similar to TLC, pigments are separated in three main fractions. The baseline separation indicates that HPLC can also be successfully used for the analysis of color pigments of raisins. The uncommon peak width again suggests the polymer character of the fractions.

Conclusion

It can be concluded from the data that the majority of color pigments of raisins are insoluble in common solvents. It can be assumed that they are covalently bound to the insoluble cell components. TLC–FTIR off-line coupling can be used for the separation of soluble pigment fraction and the tentative identification of the main pigment fraction. Furthermore, TLC can be employed as a pilot method for the development of a gradient elution method for the HPLC separation of pigments. TLC–FTIR indicated that the main soluble pigment fraction of raisin is a yellow polymer composed of monosaccharides. The process of polymerization is not known. It can be assumed that monosaccharides are oxidized chemically or enzymatically during the drying of grapes, and the caramel-like oxidation products account for the color of raisins. The determination of the pigment composition may help the rapid evaluation of the quality of raisins and the identification of their origin.

Acknowledgments

This work was supported by the Portuguese–Hungarian cooperation grant "Development of New Methods and Their Application for the Assessment of the Effect of Environmental Conditions on the Stability of Colour Pigments in Grapes and Red Wines".

References

1. R.S. Conrad, F.J. Sundstrom, and P.W. Wilson. Evaluation of two methods of pepper fruit colour determination. *Hortscience* **22:** 608–609 (1987).
2. F. Navarro and J. Costa. Note. Evaluation of paprika pepper colour by treistimulus colorimetry. *Rev. Esp. Cienc. Tecnol. Aliment.* **33:** 427–34.
3. P.A. Lunning, T. Ebbonhorst-Seller, J.N. Sorensen, and K. Kaack. Effect of hot-air drying on flavour compounds of bell peppers (*Capsicum annuum*). *J. Sci. Food Agr.* **68:** 355–66 (1995).
4. A. Pribela, J. Piry, J. Karovicova, M. Kovacova, and F. Mihnya. Bitterness of sweet pepper (*Capsicum annuum L.*). Bitter compounds production during heat-treatment of sweet pepper. *Die Nahrung* **39:** 83–89 (1995).
5. M.I. Mingues-Mosquera, M. Jarén-Galán, and J. Garrido-Fernández. Effect of processing of paprika on the main carotenes and esterified xanthophylls present in fresh fruit. *J. Agric. Food Chem.* **41:** 2120–24 (1993).
6. A. Levy, S. Harel, D. Palevitch, B. Akiri, E. Menagem, and J. Kanner. Carotenoid pigments and β-carotene in paprika fruits (*Capsicum spp*) with different genotypes. *J. Agric. Food Chem.* **43:** 362–66 (1995).
7. O.M. Andersen and G.W. Francis. Natural pigments. In *Handbook of Thin-Layer Chromatography,* J. Sherma and B. Fried, Eds. Marcel Dekker, New York, NY, 1996, pp 715–52.
8. J. Pothier. Thin-layer chromatography in plant sciences. In *Practical Thin-Layer Chromatography*. B. Fried and J. Sherma, Eds. CRC Press, Boca Raton, FL, 1996, pp 33–49.
9. T. Cserháti and E. Forgács. Hyphenated techniques in thin-layer chromatography. *J. Am. O. A. C. Intl.* **81:** 329–32 (1998).
10. O.R.Frey, K.-A. Kovar, and V. Hoffmann. Possibilities and limitations of assays by on-line coupling of thin layer chromatography and FTIR spectroscopy. *J. Planar Chromatogr. Mod. TLC* **6:** 93–99 (1993).
11. A. Stahlmann. Ten year report on HPTLC–FTIR online coupling. *J. Planar Chromatogr. Mod. TLC* **12:** 5–12 (1999).
12. A.M. Pfeifer and K.-A. Kovar. Identification of LSD, MBDB, and atropine in real samples with on-line HPTLC–FTIR coupling. *J. Planar Chromatogr. Mod. TLC* **8:** 388–92 (1995).

13. C. Brandt and K.-A. Kovar. Determination of 11-nor-9-tetra-hydrocannabinol-9-carboxylic acid in urine by use of HPTLC–UV/FTIR on-line coupling. *J. Planar Chromatogr. Mod. TLC* **10:** 349–52 (1997).
14. A.M. Pfeifer, G. Tolimann, H.P.T. Ammon, and K.-A. Kovar. Identification of adenosine in biological samples by HPTLC–FTIR online coupling. *J. Planar Chromatogr. Mod. TLC* **9:** 31–34 (1996).
15. H. Engelhardt and P. Engel. Quantitative determination of hydrocarbons in waste water by thin-layer chromatography. *J. Planar Chromatogr. Mod. TLC* **10:** 336–41 (1997).
16. J.K. Rozylo and M. Janicka. Thermodynamic description of liquid–solid chromatography process in the optimization of separation conditions of organic compound mixture. *J. Liq. Chromatogr.* **14:** 3179–12 (1991).
17. J.K. Rozylo and M. Janicka. Different planar techniques for prediction of solute retention in column liquid chromatography. *J. Planar Chromatogr. Mod. TLC* **9:** 418–24 (1996).
18. *The Aldrich Library of FT-IR Spectra*. Edition I. C.J. Pouchert, Ed. 1985.
19. T. Cserháti, E. Forgács, and V. Kiss. High performance liquid chromatographic detection of a strongly retained pigment fraction in *Capsicum annuum*. *Die Nahrung* **39:** 269–74 (1995).

Manuscript accepted February 18, 2000.