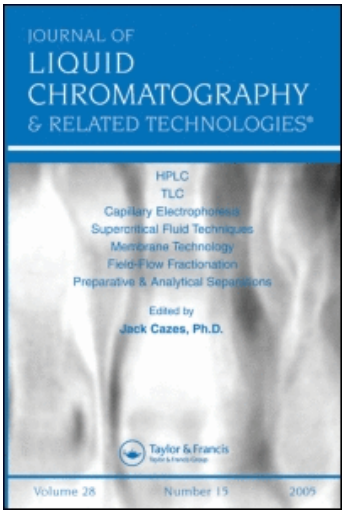

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EVALUATION OF PURE COUMARINS USING TLC-DENSITOMETER, SPECTRO-PHOTOMETER, AND HPLC WITH PHOTODIODE ARRAY DETECTOR

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ABSTRACT

A comparison of the UV spectra of twenty seven pure coumarins was carried out using TLC-densitometer, spectrophotometer and high performance liquid chromatography (HPLC) with photodiode array detector (DAD). The data obtained from TLC-densitometer and HPLC in reference to those of the spectrophotometer showed that the dependencies were statistically highly significant. Regardless of the structure of the coumarin, a common λ_{max} and/or λ_{min} can be detected by the different UV off/on-lines. A shift in the

absorption bands measured by TLC-UV and HPLC was noticed when compared to those measured by the spectrophotometer and the intensities of the absorption bands were not the same among the different UV off/on-lines.

INTRODUCTION

Coumarins are natural compounds widely distributed in plants and possessing a variety of biological activities[1-3] The most obvious physical property of most natural coumarins is the fluorescence they display in the UV light. This feature has been employed widely for their detection by using the different UV off/on-lines.

The basic planar chromatographic technique, thin layer chromatography (TLC), is a useful technique for analytical and preparative work, as well as for mobile phase optimization.[4-6] The "PRISMA" system developed by Nyiredy et al., simplifies the optimization process in planar and column chromatography.[7-8] Quantitative evaluation by TLC using direct photometric scanning, is a method which has not yet made much progress, even though it has been carried out for nearly two decades.[9] There are many optically interfering hazards present in the TLC technique, which for ordinary chromatographic purposes are not usually noticed *e.g.* air bubbles, specks of dust, quality of the sorbent, light absorbing or fluorescing impurities in the substrate, or chromatographic solvent, *etc.* The densitometric application of absorptiometry theory of Kubelka and Munk, based on a one dimensional approximation to transfer of radiation in scattering media, has been studied earlier.[10-11] It is possible to calculate the concentrations in TLC when the data are processed by computer.[12] However, it has been established from previous studies[13] that the Kubelka-Munk theory is valid only in a particular region of low concentration.

High performance liquid chromatography (HPLC) proved to be a rapid and sensitive method for the detection of phototoxic psoralens in citrus oil.[14-16] The application of HPLC to the determination of the strong photosensitizer bergapten in perfumes and suntan cosmetics has also been reported.[17-18] Of special interest is the HPLC determination of 8-methoxypsoralen plasma levels in conjunction with photochemotherapy in the treatment of psoriasis.[19-21]

Over the last few years, HPLC has proven useful for the investigation of furanocoumarins in plant materials and has been used, increasingly, for coumarin separations and determinations.[2,5,22-23]

Spectrophotometric methods of analysis have been worked out for many types of organic compounds. Psoberan is a mixture of the two furanocoumarins, psoralen and bergapten, isolated from the common fig tree. It is an active photosensitizer, recomended for treating vitiligo.[24] Analysis of Psoberan in a powder or medicinal forms, in production stages and in plant raw material, were performed using spectrophotometric methods. The levels of the photoactive furanocoumarin in the milk sap of the fig plant, were also determined using UV spectrophotometer.[25]

In the course of current strategy used for the isolation of coumarins from the plant material, quality control tests for the extracted fractions and the isolated pure compounds are always needed. To date, the UV spectra of coumarins could be obtained by using different chromatographic and spectroscopic UV off/on-lines *e.g.* TLC-densitometer, spectrophotometer and high performance liquid chromatography (HPLC), with reference to photodiode array detector (DAD). However, not much work has been carried out on the spectra obtained from the various UV off/on lines and a comparison for such UV off/on lines among each other is needed. The aim of the present study was to compare the UV spectra of standard coumarins, belonging to the main structural types of coumarins, using TLC-densitometer, and HPLC with DAD in reference to the spectrophotometer, in order to evaluate the different spectroscopic UV off/on lines commonly used for the detection of coumarins.

EXPERIMENTAL

Chemicals

The coumarins, imperatorin, isoimperatorin, osthol, oxypeucedanin, phellopterin, and psoralen were isolated and identified from *Angelica archangelica* L. at the Pharmacognosy Division, Department of Pharmacy, University of Helsinki. Angelicin, herniarin, isopimpinellin, methyl umbelliferone, umbelliferone, and xanthotoxin were obtained from Roth (Karlsruhe, Germany), ostruthol from Serva (Heidelberg, Germany), scopoletin from Sigma (St. Louis, U.S.A.) and 5-methoxypsoralen from Fluka (Switzerland).

Isobergapten, pimpinellin, and sphondin were isolated and identified from *Heracleum sphondylium* L. at the Department of Pharmacy, ETH Zürich, Switzerland. Anomalin, athamantin, cis-epoxypteryxin, ostruthin, peucedanin, pteryxin, peuarenarine, peuarenine, and xanthalin were isolated and identified

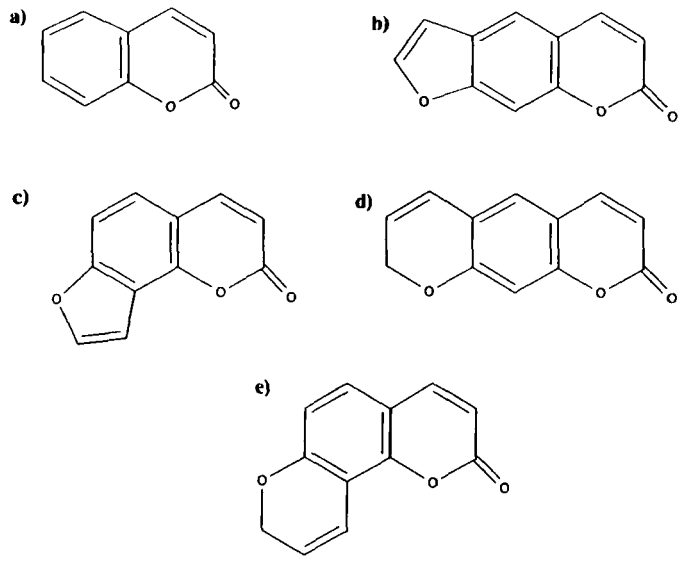


Figure 1. The main structural types of coumarins. a) simple coumarin; b) linear furanocoumarin; c) angular furanocoumarin; d) linear pyranocoumarin; e) angular pyranocoumarin

from *Peucedanum arenarium* W. & K. at the Department of Comparative Phytochemistry, University of Vienna. The *n*-hexane was of technical grade (Oy Exxon Chemicals Ab; Espoo, Finland) and was filtered before use. All other solvents were of HPLC grade (Rathburn, UK). Water was obtained through Alpha-Q (Millipore).

Volumetric flasks 25, 20, and 10 mL were used to prepare the coumarins in chloroform solutions. The concentration of the twenty-seven pure compounds, belonging to the main structural types of coumarins (Figure 1), used for TLC, HPLC, and spectrophotometer evaluations are listed in Table 1.

The coumarins were applied in the form of spots (30 µL) on 10 cm x 20 cm Kieselgel F_{254} TLC plates (Merck, Germany) using a Linomat IV TLC spotter (Camag, Switzerland). The TLC separations were performed in ascending, one-dimensional mode, in 21 cm x 22 cm unsaturated chambers (Camag; Muttenz, Switzerland) at ambient temperature. The solvent volume was 10 mL and the migration distance of the solvents was 8.5 cm. Visual inspection of the TLC plates was done under a UV lamp (Camag, Switzerland) at 254 nm and 366 nm.

Table 1

Concentration of the Standard Coumarins, Belonging to the Main Structural Types, used for TLC, HPLC, and Spectrophotometer Evaluations

Coumarin	Conc. in mg/mL		
	TLC	HPLC	Spectrophotometer
Simple coumarins			
1. Umbelliferone	$2.5x10^{-3}$	1	$2.5x10^{-3}$
2. Herniarin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
3. Scopoletin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
4. Ostruthin	$2.5x10^{-3}$	1	$2.5x10^{-4}$
5. Methyl umbelliferone	$2.5x10^{-3}$	1	$2.5x10^{-4}$
6. Osthol	$4.2x10^{-3}$	1	$4.2x10^{-3}$
Linear furanocoumarins			
7. 5-methoxypsoralen	$2.5x10^{-3}$	1	$2.5x10^{-4}$
8. Isopimpinellin	$3.0x10^{-3}$	1	$2.5x10^{-3}$
9. Imperatorin	$3.2x10^{-3}$	1	$2.5x10^{-3}$
10. Peucedanin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
11. Phellopterin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
12. Oxypeucedanin	$2.8x10^{-3}$	0.056	$2.8x10^{-3}$
13. Isoimperatorin	$3.5x10^{-3}$		$3.5x10^{-3}$
14. Psoralen	$2.5x10^{-3}$	1	$2.5x10^{-3}$
15. Ostruthol	$2.5x10^{-3}$		$2.5x10^{-3}$
16. Xanthotoxin	$<2.5x10^{-3}$	1	$<2.5x10^{-3}$
Angular furanocoumarins			
17. Angelicin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
18. Athamantin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
19. Pimpinellin	$4.0x10^{-3}$		$4.0x10^{-3}$
20. Sphondin	$<2.5x10^{-3}$		$<2.5x10^{-3}$
21. Isobergapten	$<2.5x10^{-3}$		$<2.5x10^{-3}$
Linear pyranocoumarins			
22. Xanthalin	$2.5x10^{-3}$	1	$2.5x10^{-3}$
23. Peuarenarine	$2.5x10^{-3}$	1	$2.5x10^{-3}$
24. Peuarenine	$2.5x10^{-3}$	1	$2.5x10^{-3}$
Angular pyranocoumarins			
25. Cis-epoxypteryxin	$2.5x10^{-3}$		$2.5x10^{-3}$
26. Anomalin	$2.5.x10^{-3}$		$2.5x10^{-3}$
27. Pteryxin	$8.0x10^{-3}$		$8.0x10^{-3}$

The ultraviolet spectra were obtained using a dual wavelength flying-spot scanner CS-9000 (Shimadzu Corporation, Japan). The resolution window was 0.10 nm and the wavelength ranged between 200 nm and 370 nm.

A Waters Assoc. (Milford, Mass. U.S.A.) HPLC system equipped with 600E Multi-Solvent Delivery System was used for this study. The column for HPLC separation was an ELSIsphere 80 C (RP-18; 150 mm x 4 mm; Ø 7 µm, Estonia). The separation was performed at ambient temperature. A mobile phase which consisted of tetrahydrofuran (THF), acetonitrile, methanol, and water was selected (see Results and Discussion section). For all of the investigated coumarins, 10 µL were injected into the column, except in case of oxypeucedanin, 200 µl were injected. Isocratic elution was carried out at a flow rate of 1 mL / min. For UV spectra measurements, a DAD detector (Waters Assoc., Milford, Mass. U.S.A.) was used. The resolution window was kept at 1.3 nm. Wavelength ranged between 200 nm and 380 nm.

The UV spectra of coumarins were also recorded with a Philips PU 8740 UV-vis spectrophotometer of 2 mm resolution window, using one centimeter quartz sample cell at ambient temperature. Wavelength ranged between 200 nm and 400 nm.

Statistical Analysis

Calculation of the correlations and the regression analysis were performed with Stat/View SE+ Graphics™ software on a Macintosh SE Computer.

RESULTS AND DISCUSSION

The coumarin character is strongly indicated by its UV absorption. This phenomena is usually utilized for quality control tests of the extracted fractions from the plant material and for chromatographic analysis of a complex mixture of coumarins. Peak purity has long been a validation problem for the analyst. It often requires at least two analyses, if not more, under various chromatographic conditions. A wide range of chromatographic and spectroscopic UV off/on-lines are now available and used for recording the UV spectra of coumarins.

Thin layer chromatography (TLC), is a useful technique for both analytical and preparative work. TLC is also suitable for the development of the mobile phase of a preparative and analytical column chromatography.[4-6,26-27]

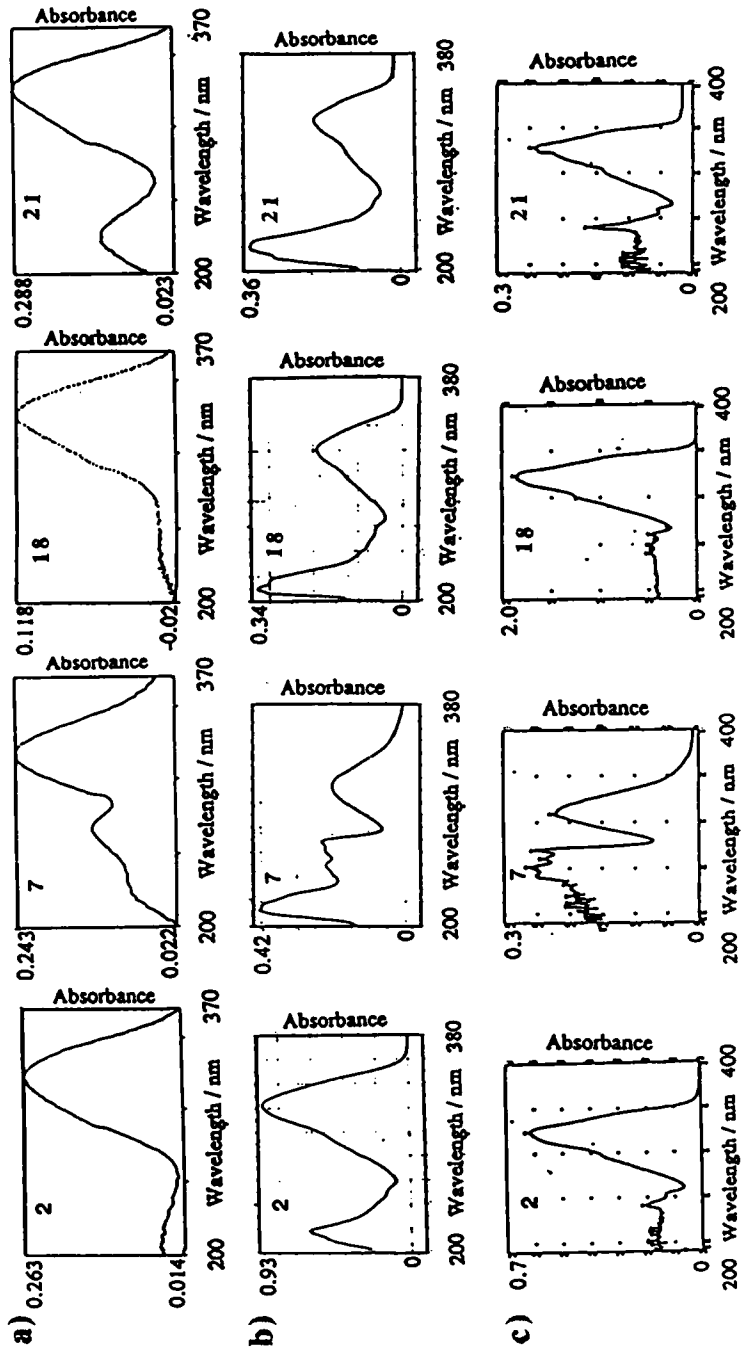
Silica is the most widely used stationary phase in planar chromatography and has an excellent separation power. The "PRISMA" optimization process always starts with this stationary phase.[7-8] Normal phase thin layer chromatographic (NP-TLC) plates were, therefore, used in this study to evaluate the coumarin spectra obtained by the densitometer. Dioxan (S_i=4.8), ethanol (S_i=4.3), and diethylether (S_i=2.8) in n-hexane (S_i=0) were selected, according to the "PRISMA" model, to give the best separation of twenty seven coumarins in unsaturated chambers with normal TLC plates. The selectivity point (P_S) of 333 and the solvent strength (S_i) was adjusted with *n*-hexane to 0.8, resulting in a mobile phase consisting of 5.6% dioxan, 6.3% ethanol, 9.7% diethyl ether, and 78.4% *n*-hexane.

Modern HPLC with DAD was used in this study to determine the UV spectra of coumarins. The handling of the numerous samples for analysis was facilitated by an automatic injection system. For the evaluation of a mobile phase with optimal selectivity to the pure coumarins, the optimisation design " PRISMA" was applied.[28] Following the usual optimization systems for modern HPLC[29-30], an optimal selectivity was reached by a four solvent mixture of THF (S_i=4.5), acetonitrile (S_i=3.2), methanol (S_i=2.6), and water (S_i=0). The mobile phase characterised by S_i=2.6 and P_S of 181 (6% THF, 65% acetonitrile, 10% methanol and 19% water) showed the best elution for the investigated coumarins.

Spectrophotometers are widely applicable to record the UV spectra of many compounds and numerous UV absorption spectra for distinguishing coumarins are existing.[31]

In this study, the UV spectra of the investigated coumarins obtained by spectrophotometer were used as references to compare with the corresponding spectra obtained by the TLC-densitometer and HPLC. The repeatability of the analysis varied between 0.00-0.18% and 0.00-0.10% for umbelliferone (compound 1), and between 0.02-0.25% and 0.04-0.16% for 5-methoxypsoralen (compound 7) with spectrophotometric and HPLC-DAD studies respectively.

Examples of the UV spectra of some compounds, belonging to the main structural types of coumarins, recorded by the different UV off/on-lines are illustrated in Figure 2. Generally, the UV spectra of each coumarin are characterized by certain absorption bands, some of which are shown in Figure 3. The spectra of each compound demonstrated a common λ_{max} and/or λ_{min}, regardless of its structural type. The different spectra showed at least one absorption band at the region of 300-330 nm. In some cases, the intensities of the absorption bands were changed among the different UV off/on-lines.



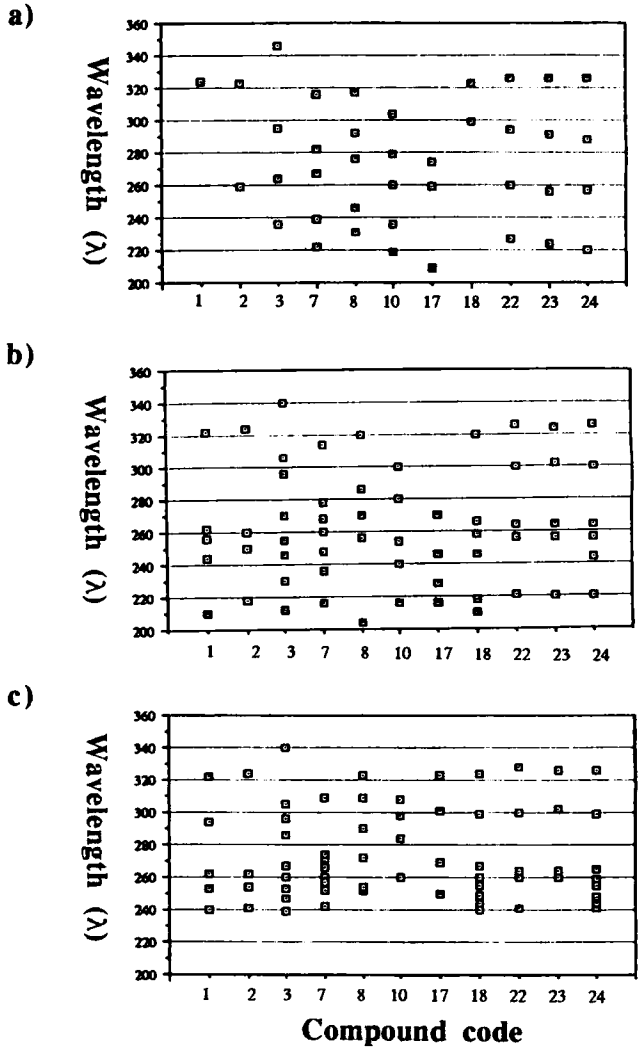


Figure 3. Comparison of the absorption bands of eleven compounds representing the main structural types of coumarins recorded by: a) TLC-densitometer; b) HPLC-DAD; c) spectrophotometer. Compounds coded as in Table 1.

Figure 2. (**left**) Examples of the UV spectra of some compounds belonging to the main structural types of coumarins, recorded by: a) TLC- densitometer; b) HPLC-DAD; c) spectrophotometer. Compounds coded as in Table 1.

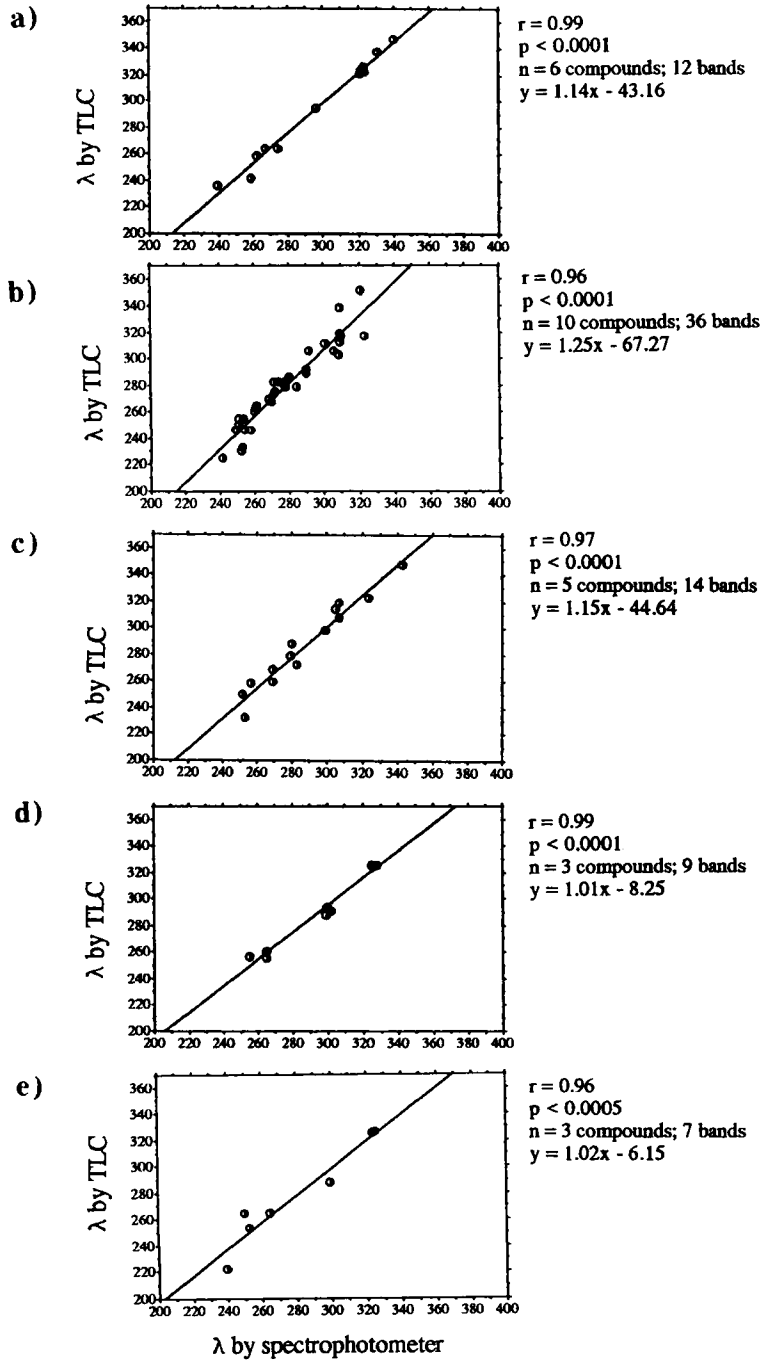


Table 2

Shifts in the Absorption Bands ± SD of Coumarins Measured by TLC-Densitometer and HPLC with DAD in Comparison to the UV Spectra Recorded by Spectrophotometer

Coumarin Type	Shift in the Absorption Bands ± SD	
	TLC Densitometer	HPLC
Simple coumarins (n=6)*	1.7 ± 6.7	1.4 ± 3.5
Linear furanocoumarins (n=10)	-2.2 ± 10.5	3.5 ± 9.6
Angular furanocoumarins (n=5)	0.4 ± 8.5	6.2 ± 7.8
Linear pyranocoumarins (n=3)	4.5 ± 4.8	1.4 ± 1.7
Angular pyranocoumarins (n=3)	1.2 ± 10.3	

*n = number of coumarins; number of bands see Figure 4.

For instance, 5-methoxypsoralen (compound 7) showed a λ_{max} at 308.8 nm of 84.6%, a λ_{max} at 316.2 nm of 100% and a λ_{max} at 314.0 nm of 48.8% responses by the spectrophotometer, the TLC-densitometer and the HPLC-DAD respectively. The UV spectra recorded by the spectrophotometer and HPLC-DAD were rather similar and demonstrated higher spectral resolution when compared to the spectra measured by TLC-densitometer (Figure 3).

HPLC with DAD can provide spectral resolution in addition to temporal resolution. It can detect UV light in discrete increments and present it to the analyst as a three-dimensional chromatogram. Such a chromatogram allows for simultaneous detection at all the wavelengths throughout the UV spectrum.[32]

Regression analysis of the data of TLC-densitometer and HPLC-DAD in reference to the spectrophotometer are shown in Figure 4 and Figure 5. The measurements by TLC showed more deviation from the regression line than those of the HPLC-DAD. However, the results obtained by each of HPLC-DAD and TLC-densitometer showed that the dependencies were statistically highly significant.

Figure 4. (**left**) Scattergram of wavelength measurements (λ_{max} and λ_{min}) by TLC-densitometer in reference to the spectrophotometer for the different structural classes of the investigated coumarin: a) simple coumarins; b) linear furanocoumarins; c) angular furanocoumarins; d) pyranocoumarins. Compounds coded as in Table 1.

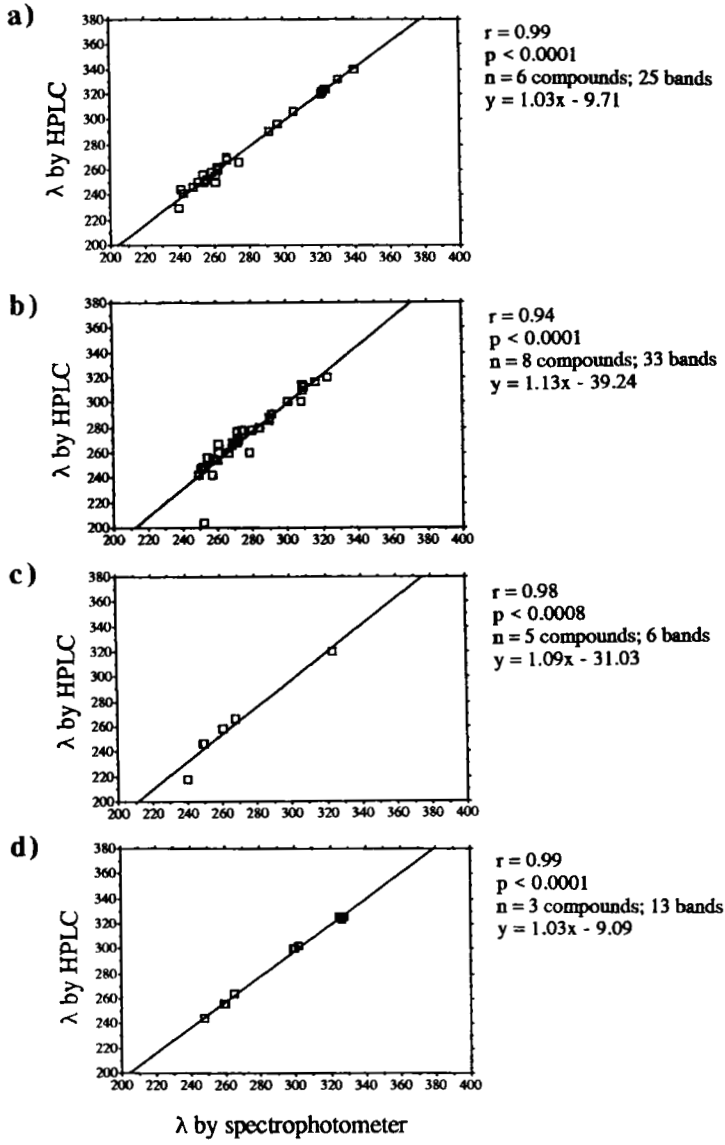


Figure 5. Scattergram of wavelength measurements (λ_{max} and λ_{min}) by HPLC-DAD in reference to the spectrophotometer for the different structural classes of the investigated coumarin: a) simple coumarins; b) linear furanocoumarins; c) angular furanocoumarins; d) pyranocoumarins. Compounds coded as in Table 1.

For each structural category of the investigated coumarins, there was a shift in the absorption bands measured by the TLC-densitometer and HPLC when compared to those obtained by the spectrophotometer (Table 2).

The difference in the UV spectra obtained by the various UV off/on-lines for a single compound may be attributed to the photomode whether it is reflection e.g. in TLC-densitometer or absorbance e.g. in spectrophotometer and HPLC-DAD. Another reason for the difference can be the interaction between the analyte and the mobile phase or the stationary phase. In addition, the individual sensitivity of the detector is subjected to pH, ionic strength, and solvent effect which can be a reason for the presence or absence of certain bands, e.g. in case of the spectra recorded by HPLC, a λ_{max} was observed at 200-220 nm.

The data obtained from the different UV off/on-line techniques is very useful for both quantification and tentative semiidentification. However, special attention should be paid to the shifts which occur for the maxima and the minima of the UV spectra in order to avoid false conclusions about the results.

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