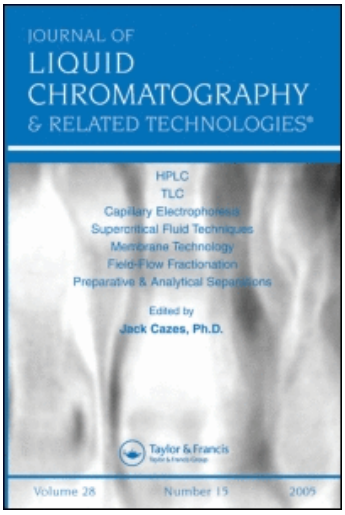


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# Qualitative and Quantitative Analysis by Hyphenated (HP)TLC-FTIR Technique

Claudia Cimpoiu[a]
[a] Faculty of Chemistry and Chemical Engineering, "Babes-Bolyai" University, Cluj-Napoca, Romania

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# Qualitative and Quantitative Analysis by Hyphenated (HP)TLC-FTIR Technique

**Claudia Cimpoiu**
Faculty of Chemistry and Chemical Engineering, "Babes-Bolyai" University, Cluj-Napoca, Romania



**Abstract:** In recent years, much effort has been devoted to the coupling of thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) with spectrometric methods because of the robustness and simplicity of use of (HP)TLC and the need for detection techniques that provide identification and determination of sample constituents. Infrared (IR) is one of the spectroscopic methods that have been coupled with (HP)TLC. IR spectroscopy has a high potential for the elucidation of molecular structures; the characteristic absorption bands can be used for compound specific detection. The (HP)TLC-FTIR coupled method has been widely used in modern laboratories for qualitative and quantitative analysis. The potential and power of this method is demonstrated by its application in various fields of analysis, such as drug analysis, forensic analysis, food analysis, environmental analysis, biological analysis, etc. The hyphenated (HP)TLC-FTIR technique will continue to be developed in the future with the aim of taking full advantage of this method's capabilities.

**Keywords:** Thin layer chromatography, High performance thin layer chromatography, IR spectroscopy, Hyphenated techniques, Qualitative analysis, Quantitative analysis

Address correspondence to Claudia Cimpoiu, Faculty of Chemistry and Chemical Engineering, "Babes-Bolyai" University, 11 Arany Janos, 400028 Cluj-Napoca, Romania. E-mail: ccimpoiu@chem.ubbcluj.ro

## INTRODUCTION

The identification of separated compounds from a mixture, in the absence of standards, is one of the problems in high performance thin layer chromatography (HPTLC). Moreover, quantitative analysis involves prior identification of detected compounds. The selectivity and sensitivity of component detection and identification can be improved by coupling of two or more analytical techniques in HPTLC. For these reasons, in recent years, coupled or "hyphenated" chromatographic techniques are becoming more common in analytical separations. These techniques have the goal of a rapid and efficient chromatographic separation and on-line identification of the separated compounds. The hyphenating of the HPTLC separation technique with spectrometric methods for the analysis of complex mixtures represents the state-of-art in modern analytical laboratories. The increased amount of information obtaining by these hyphenated techniques is often sufficient for identification of the compound structures and for their quantification. (HP)TLC can be coupled with ultraviolet-visible (UV/VIS) and fluorescence spectrometry, infrared spectrometry (IR), Raman spectrometry, photoacoustic spectrometry (PA), and mass spectrometry (MS).[1,2]



Because IR spectrometry has been successfully coupled with liquid chromatography (LC),[3] many attempts have been focused on the coupling of (HP)TLC with IR spectroscopy.[4] Infrared spectroscopy has a high potential for the elucidation of molecular structures and the characteristic absorption bands can be used for compound-specific detection. The HPTLC and FTIR coupling approaches can be divided into two groups, i.e., indirect and direct methods. Indirect coupling involves either the transfer of the substance from a TLC spot to a non-absorbing IR material (KBr or KCl) or in-situ measurement of excised HPTLC spots when the spectra are recorded directly from the plate.[5] The direct methods are based on the direct hyphenated HPTLC-FTIR technique introduced in 1989 by Glauninger and co-workers.[6] Until then, the combination of HPTLC and ultraviolet-visible (UV-VIS) spectroscopy was the only on-line coupling method available in planar chromatography. The information content of UV-VIS spectra is rather poor and rarely enables unambiguous identification of a substance; furthermore, a chromophore is needed for UV detection.

Almost all chemical compounds yield good IR spectra that are more useful for identification of unknown substances and discrimination between closely related substances.[7] The HPTLC-FTIR spectra make possible the detection and quantification of even non-UV absorbing substances on HPTLC plates.[8] These reasons make this hyphenated technique more universally applicable. The direct on-line coupled HPTLC-FTIR offers some advantages relative to other hyphenated techniques (HPTLC-Raman spectroscopy, HPTLC-PA, and HPTLC-MS), such as: the ease of operation and the optimized operational aspects of on-line coupling.

## PRINCIPLES, INSTRUMENTATION, AND DATA PRESENTATION

The principle of the HPTLC-FTIR hyphenated technique depends on scanning the plate with an infrared beam in a diffuse reflectance infrared Fourier transform (DRIFT) device connected to a Bruker IFS 48 FTIR spectrometer (Fig. 1). The plate is fixed onto a computer controlled x-y stage. The special mirror arrangement was constructed to enable DRIFT measurements and to eliminate, to a large degree, the specular (Fresnel) reflectance in the 3600 to 1350 $cm^{-1}$ region. The diffuse reflectance containing the desired spectral information is collected and directed to the mercury, cadmium, and telluride (MCT) detector.

Diffuse reflectance is not a direct method of measurement. The particles of the samples scatter, remit, and absorb most of the radiation and the intensity of the reflected radiation is the same in every direction. The desired spectral information about the sample is contained in the diffusely remitted radiation. A part of the remitted radiation, called Fresnel reflectance, (Fig. 2) does not contain spectral information and leads to distortion and shifting of the bands in the reflectance spectra. This specular reflectance



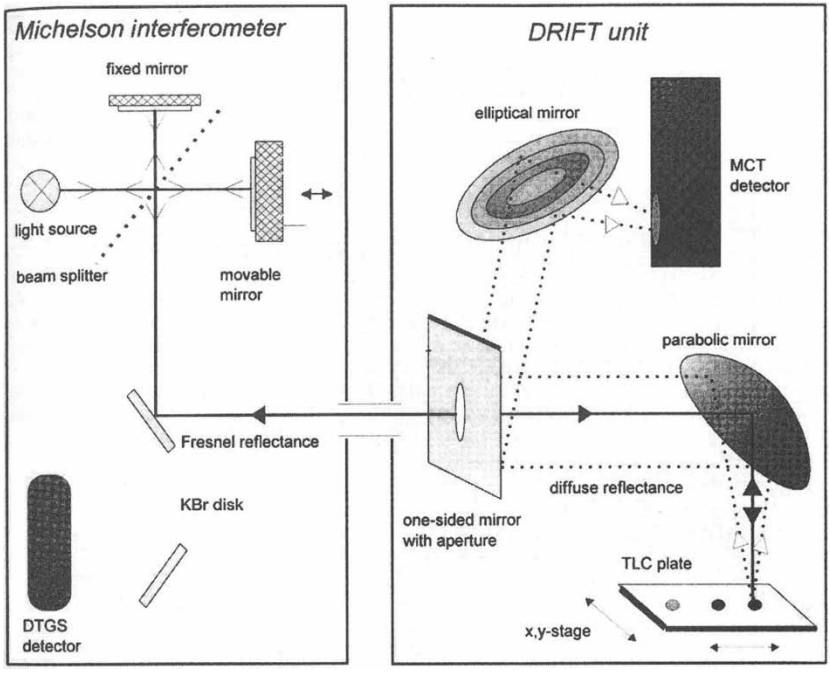


***Figure 1.*** Diagram of the Bruker HPTLC-FTIR unit.

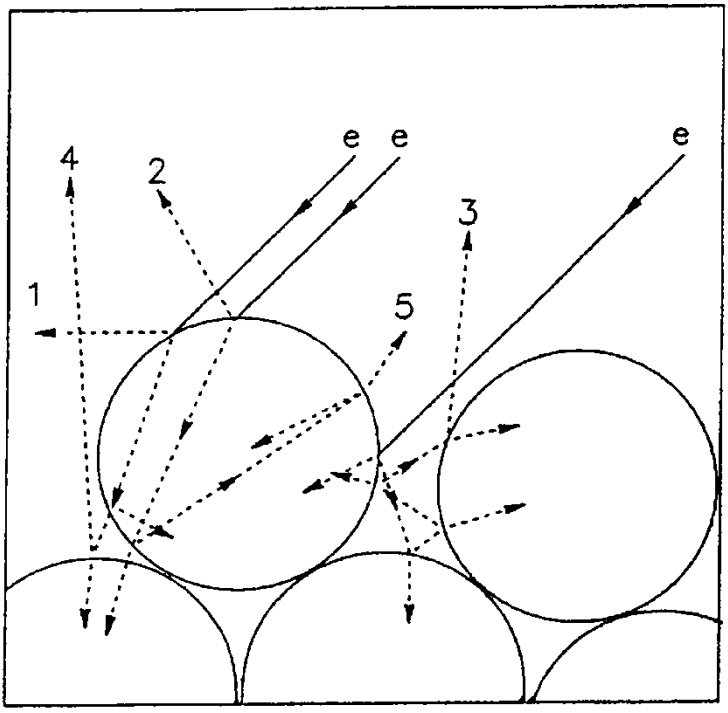


*Figure 2.* Interaction between the radiation and the samples: 1, 2 – directional Fresnel radiation; 3 – scattered Fresnel radiation; 4, 5 – diffuse radiation.



must be minimized in order to obtain the desired quality of spectral information. The diffuse reflectance follows the Lambert cosine law:

$$\frac{df/dI_r}{d\omega} = \frac{CS_0}{\pi \cos\alpha \times \cos\theta} = B\cos\theta \qquad (1)$$

where $I_r$ is the remitted radiation flux in an area f ($cm^2$) and solid angle $\omega$ (sr), $S_0$ is the intensity of irradiation (W $cm^{-2}$), constant C ($<1$) is the fraction of the incident radiation flux which is remitted, $\alpha$ is the angle of incidence, $\theta$ is the angle of observation, and B is the radiation density or surface brightness (W $cm^{-2}$ sr) (Fig. 3).

A simplified solution to this equation is obtained by making several assumptions by the Kubelka and Munk technique and is known as the Kubelka-Munk function.

$$f(R_\infty) = K/S = k' \times c \qquad (2)$$

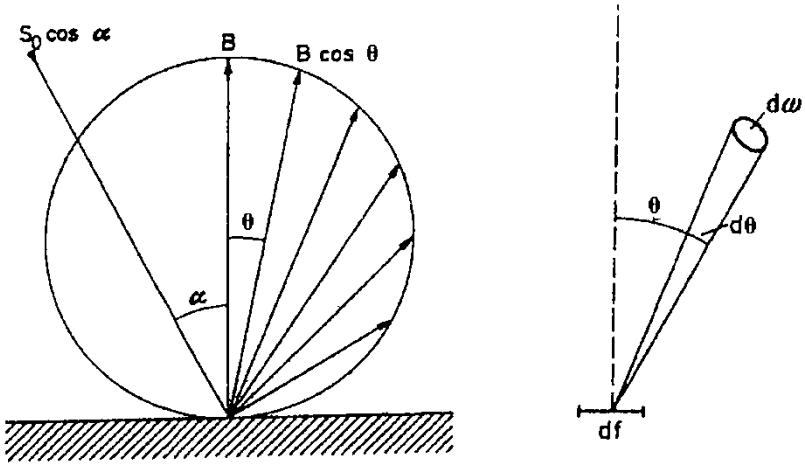


*Figure 3.* Variables used in the Lambert cosine law.



where $R_\infty$ is the ratio of the diffuse reflectance single-beam spectra of sample and reference at infinite thickness of the sample layer, K is the absorption coefficient, S is the scattering coefficient, k′ is a proportionality constant, and c is the concentration of an absorbing compound. This equation allows the correlation between sample concentration and the intensity of scattered light in a manner similar to the Bouguer-Beer law and can predict for spectrometry by transmittance. The Kubelka-Munk equation is applicable to very dilute samples and can be used in the case of spots on HPTLC plates. An advantage of the DRIFT technique comparison with transmission spectrometry is that the signal-to-noise ratio diminishes only with the square root of the concentration.

In the direct coupling HPTLC-FTIR method, some difficulties appear in comparison with the approaches involving the transfer of the substance to a non-absorbing material. One of the most important problems is the absorption by conventional stationary phases, e.g., silica gel, which absorb strongly in the IR range. It is very difficult to obtain reliable spectra in the regions where the layer shows strong IR absorption. At wavelengths where the absorbance and refractive index of the matrix are high, the 'reststrahlen' effect could occur – no diffuse reflectance occurring, almost the entire radiation being reflected from the surface. The silica gel, the most widely used adsorbent in HPTLC, presents absorption bands between 1350 and 1000 $cm^{-1}$ and above 3550 $cm^{-1}$ which are superimposed on the spectra of compounds and only the region between 3550 $cm^{-1}$ and 1350 $cm^{-1}$ can be evaluated.[9] Therefore, measurements in this region are not possible, but it is possible to make measurements up to 1000 $cm^{-1}$ on cellulose. The best results are

obtained when the mixture of silica gel 60 and magnesium tungstate (1:1) is used as stationary phase. This adsorbent improves signal-to-noise ratios and enhances the performance of the diffuse reflectance of the matrix.[10] Another problem is due to the particle size, particle-size distribution, and the layer thickness, which affect the scattering, remitting, and absorbing of the radiation by the matrix. A stationary phase with particle diameter of 10 μm, a narrow particle size distribution, and a layer thickness of 200 μm on glass is found to be ideal in the mid IR range.[5] Finally, the binder or the fluorescence indicator added to the adsorbent and the mobile phase could lead to altered HPTLC-FTIR spectra.[11]

The data generated by HPTLC-FTIR analysis are saved on a hard disk. The interferograms are then converted by use of commercially available GC-IR software, resulting in various ways of information presentations (Fig. 4). The DRIFT spectra can be calculated from the interferograms using Fast Fourier Transformation (FFT).[12] The spectra can be converted to normalized reflectance spectra (Fig. 4A, B), to quasi-absorbency units (Fig. 4C), or to Kubelka-Munk units (Fig. 4D). The quasi-absorbency units and the Kubelka-Munk units are proportional to concentration. By Gram-Schmidt vector orthogonalization, a chromatogram can be created from the interferogram data (Fig. 4F). The resultant chromatogram is a substance-non-specific one in which the total integrated absorption of the sample is measured.[13] This procedure is one of the possibilities to locate the spots on the plates. Another procedure is the use of window chromatograms (Fig. 4E), i.e., the frequency-dependent chromatograms at any wavelengths of interest. The first method can be used to increase selectivity and the second method is universally applicable and independent of wave number.[14]



## QUALITATIVE ANALYSIS

The recorded IR spectra form the basis of compound identification. The IR spectrum of an organic compound provides a unique fingerprint, because the IR spectrum of a polyatomic molecule is based on molecular vibrations, which depend on atomic masses, bond strengths, and intra- and intermolecular interactions. The IR spectrum can be distinguished from the IR absorption patterns of other compounds, including isomers.

The identification can be realized by fitting the reference spectra to sample spectra and visual comparison. The spectra of separated compounds are the spectra extracted at the peak maxima. A reference spectrum is needed for identification of compounds, because false bands, called artifacts, arise due to the irregularities in the stationary phase. The reference spectra must be recorded under the same conditions because, in the case of in-situ measurements, the interaction between adsorbed substance and the layer leads to significant changes of the peak maximum position.

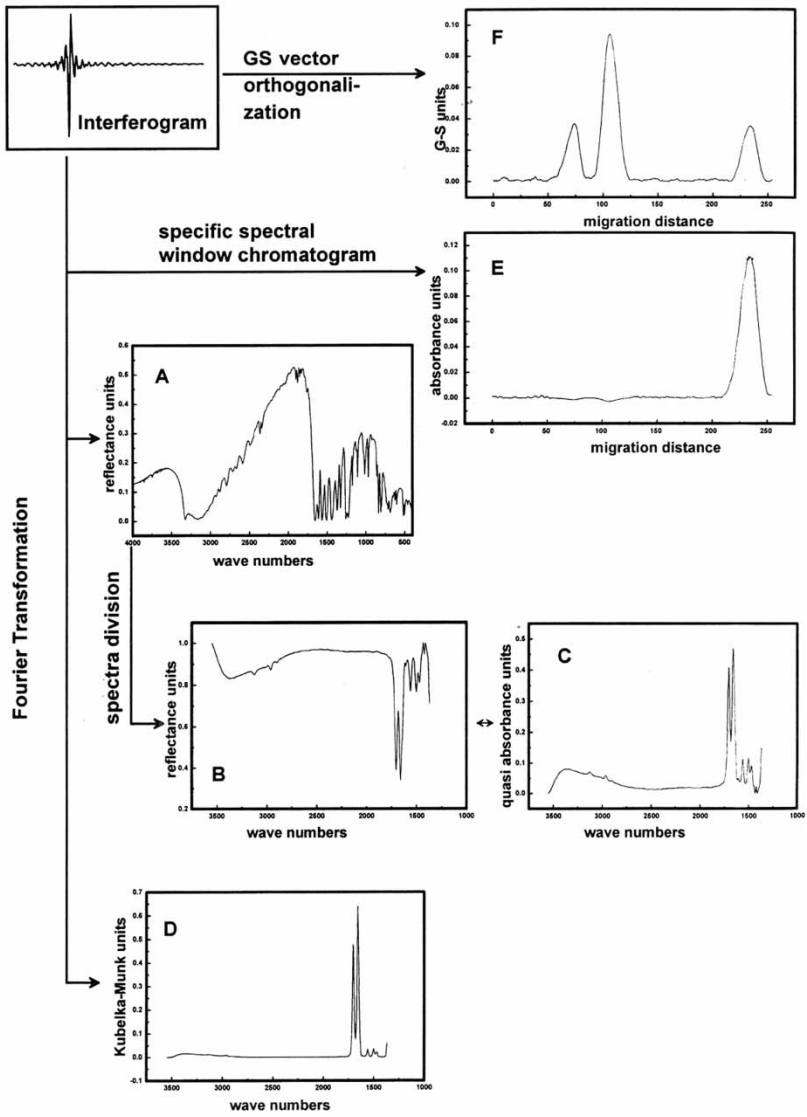


*Figure 4.* Possibilities for data presentation.

The compounds separated by HPTLC can be also identified using an HPTLC-FTIR library. The band position, width, and intensity are automatically compared and the reliability of the results is described in terms of hit quality ($\geq$800). More detailed spectra and an increased hit quality can be obtained by post-run measurement of the located spots with higher

resolution. These methods allow unambiguous identification of separated compounds.

The identification by a direct HPTLC-FTIR hyphenated technique is demonstrated by the applications in the field of drug analysis, e.g., amphetamines, morphine and indole derivatives,[15] benzodiazepines,[16] phtalazine derivatives,[17] food analysis, e.g., pigments in raisins[18] and in chestnut sawdust,[19] forensic analysis,[20,21] and biological analysis.[7,22]

## QUANTITATIVE ANALYSIS

Quantitative analysis with the HPTLC-FTIR technique is generally applied for the substances that do not absorb in the UV/VIS range and when the precision required is not too high. The lack of precision is due to the increase of sample spot broadening with increased migration distance and to the measurement not being exactly at the peak maximum. These problems are due to the circular infrared beam with small diameter.



The determination of compounds is made on the basis of evaluation of the peak areas in the Gram-Schmidt trace or in the window diagram, or by the evaluation of Kubelka-Munk spectra with integration of their strongest bands. The method using the Gram-Schmidt traces indicates the changes in absorbance over the whole spectral region and, therefore, it is suitable and practical for rapid determinations. The evaluation of the peak areas in the window chromatogram is appropriate for the quantification of individual substances. An advantage of this method is a better signal-to-noise ratio, but the disadvantage is the poorer precision. More precise results are obtained using the evaluation of Kubelka-Munk spectra. The limit of identification and determination is 10 times higher than those obtained by densitometry.[23] This method has the disadvantages of the measurement only of the fraction of the substance in the peak maxima and the additional processing step.

In conclusion, none of these methods is perfect and appropriate for all samples. The choice of a method depends on the goals of the analysis. Moreover, in quantitative analysis, a great deal of attention must be given to parameters influencing the application, development, and measurement to avoid the presence of the errors in the final results. Relative standard deviations between 1.3 and 6.1% are achieved for the determination of pure substances, without sample preparation.[24]

Many components from different types of samples have been quantified by the on-line HPTLC-FTIR method. An example of on-line HPTLC-FTIR application is in the determination of edetic acid (EDTA) from environmental samples.[25] The EDTA is determined in surface water samples by on-line coupling of HPTLC and FTIR after the treatment of the water with cobalt(II) acetate and formic acid and the enrichment of the sample by solid phase extraction (SPE). The limit of detection and determination were

determined to be 250 ng and 450 ng, respectively. The method has the advantages of sample analysis without derivatization and the discrimination from related substances.

An another example is in the analysis of hexobarbital, phenobarbital, caffeine, salicylic acid, and ascorbic acid by this hyphenated technique.[26] For the improvement of identification and determination limits and of signal-to-noise ratio, the analysis was performed by automated multiple development (AMD) on water-resistant silica gel $60WRF_{254S}$. The determined identification limits were 55 ng for hexobarbital, 55 ng for phenobarbital, 30 ng for caffeine, 220 ng for salicylic acid, and 240 ng for ascorbic acid.

The potential of the HPTLC-FTIR coupled method has also been demonstrated by its application to forensic chemistry.[20] 11-Nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) was determined in urine by use of HPTLC-UV/FTIR on-line coupling. The sample was enriched by SPE after alkaline hydrolysis of ester glucuronide. Derivatization with an azo dyestuff is not necessary. The detection limits for THC-COOH are $4\,ng\,mL^{-1}$ for UV and $14\,ng\,mL^{-1}$ for IR; therefore, this method enables qualitative and quantitative analysis in the region of the $20\,ng\,mL^{-1}$ cutoff.



## CONCLUSIONS

HPTLC, coupled with FTIR spectroscopy, is a powerful technique for the identification and determination of sample components. This technique is a non-destructive method that offers the advantage of elimination of substance transfer from a TLC spot. Because the IR spectrum is like a fingerprint of the analyzed substance, this method enables unambiguous identification of the substances. With modern FTIR instrumentation, IR spectroscopy is suitable for molecular recognition.

Due to the simplicity and robustness of HPTLC, much attention must be given to further developments of the hyphenated HPTLC-FTIR method, which has a great potential for identification and determination of compounds from complex mixtures. This technique, together with HPTLC-UV could be used for routine analysis in modern analytical laboratories.

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