The Analysis of Thin Layer Chromatography Plates by Near-Infrared FT-Raman*

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Chemists in pharmaceutical research are now synthesizing compounds on a much smaller scale than ever before. This is because of expense, safety, pollution control and more sensitive biological screens. Analysts must confirm structures using micro-techniques because exploratory synthetic research is designed to provide new compounds in very limited amounts.

Synthetic reactions can be conveniently and effectively monitored by chemists using thin layer chromatography (TLC). Compounds, for example, starting material, mixture and product from a reaction, are spotted on silica coated glass slides. These slides are placed in a container with an appropriate solvent. The solvent is drawn up the slide by capillary action, and compounds are partitioned between the solvent and the silica gel to extents that differ and are structurally dependent. Compounds move up the plates at characteristically differing rates thereby separating mixtures of compounds. The movement ceases when the plates are removed from the solvent container and spots, which correspond to particular compounds, can be detected by UV light—plates are pre-coated with F254 dye to enable spots to be observed. The end point of the process is determined when the spot corresponding to the starting material is no longer present in the reaction mixture. The desired product is then isolated from the reaction and sent for analysis and biological activity testing.

Considerable work has been done on the analysis of TLC plates with FT-IR. In the experience of the authors, direct measurements using FT-IR have had very limited success since the glass substrate and silica coating absorb strongly and mask the spectrum of the small amount of compound in the TLC spot. Several different approaches are discussed in a more complete report of this work submitted for publication in Spectrochimica Acta.[1] Direct measurement of spectra has met with limited success,[2] although some quantitative analysis is possible for limited applications where the bands of interest lie in suitable regions of the spectrum.[3] NIR diffuse reflectance measurements have been used, since the glass and silica substrate of the plates is transparent in this region,[4] but the data analysis reported may be too complicated for routine laboratory use.

Other possible approaches using FT-IR include the construction of specialist plates designed for IR analysis—requiring a coating and substrate that are IR transparent[5]—or removal of the chromatographed sample from the plate after processing.[6,7]

None of these techniques offer a perfect solution to the problem and several of the authors discuss the limitations of these methods of analysis. For in situ measurements using FT-IR, only narrow windows in the spectral region are available for analysis because of the strongly absorbing nature of the plates themselves. Limiting the useful spectral regions makes the searching of spectral libraries more complicated and less reliable. Problems may also arise due to changes of penetration depth in regions that are strongly absorbing. Techniques that rely on specially designed plates would increase the cost of the analysis and lose the "ruggedness" of the glass substrate systems which are already widely accepted. The alternatives, systems which do not allow measurement in situ, have considerable disadvantages associated with them. The complexity and time consumption of the analysis would considerably increase, and a subjective decision would need to be made as to which area(s) to remove from the plate. The removal, elution and redeposition of the chemicals increases the risk of contamination involved and, for some materials, may result in further reaction.

NIR FT-Raman has several inherent advantages over both conventional Raman and FT-IR, discussed in-depth in the literature.[8] Excitation with a Nd:YAG laser at 1064 nm and collection of the Raman scattered light in the near-infrared region of the spectrum, means that sample holders and cells may be constructed easily and cheaply from glass. For this application, absorptions of the TLC plates from the silica coating and the glass substrate are avoided and the full Raman spectral range may be collected without interference. The information collected still derives from the fundamental vibrational modes of the molecule, and spectral analysis uses the group frequency techniques familiar to IR spectroscopists. The plates may be placed directly in the instrument and measurement is in situ.

Four compounds in common use were selected by the Ciba Geigy pharmaceutical lab for assessing the feasibility of using FT-Raman to study TLC plates. These were (a) Benzoylacetonitrile, (b) 3-Benzoylpyridine, (c) 3,3-Diphenylpropanol and (d) 5Br, 2OH Benzylalcohol. The compounds were deliberately chosen to have similar structures in order to provide a better test of whether the spectra recorded could be successfully matched to library data.

FT-Raman spectra were recorded using a Nicolet 910 instrument. A Nd:YAG laser operating at 1064 nm was used for excitation and provides up to 1 W at the sample. Scattered light was collected using a gold coated paraboloid in an 180 degree configuration. The high purity Ge detector gives sensitivity to around 3500 cm⁻¹ Raman shift when cooled with liquid nitrogen, the low shift cut-off is below 100 cm⁻¹—defined by the Rayleigh rejection filters. The instrument is a dedicated NIR FT-Raman spectrometer, not an attachment to an FT-IR bench, and therefore benefits from an optimized optical path and optical components selected for their high performance in the NIR region using gold coated reflecting optics and a Calcium Fluoride beamsplitter. A high level of instrument performance is essential for recording spectra such as these since the signal strength is very weak.

Two independent sets of TLC plates were spotted and processed for analysis. The processing used standard Merck Chemicals kieselgel 60 F254 TLC plates consisting of a glass substrate, pre-coated with a 0.25 mm silica gel film. The first set were prepared using the normal method employed in the pharmaceutical laboratory for a quick "look and see" type of experiment. No precise concentrations were measured, rather a "small" quantity (approximately 1 mg) of chemical was dissolved in approximately 0.2 ml of dichloromethane solvent. From this solution, 10 microliter drops were used to spot the plates. After chromatographing and drying, a chemical spot on the plate measuring approximately 8 mm in diameter remained—the exact size was sample dependent. The instrument was set to excite and view from a patch approximately 1 mm in diameter, roughly in the center of the 8 mm diameter spots. This means that the amount of sample used for the spectral measurements was extremely small.

The limiting concentration which can be used for TLC analysis is determined by the amount of compound available and the equilibrium point for the silica gel and compound. The equilibrium point is the point at which the compound being analyzed "clogs" the gel coating, producing a smeared trail along the surface rather than moving smoothly as a compact spot. With this in mind, the second set of plates were spotted with higher concentrations in the hope of producing higher quality FT-Raman spectra and/or reducing the measurement time. These higher concentrations were still

below the limit imposed by the chemistry of the plates, suggesting that further optimization of the preparation technique is possible. Experiments to examine the effects of adding more of the solution per spot demonstrated that Raman spectral quality was not improved. Larger spots deposited over a greater area on the plate and did not increase the concentration within the viewed patch.

All FT-Raman spectra were recorded with 700 mW of laser power and 4 cm⁻¹ resolution. Spectra recorded from the first set of plates were accumulated for 20 minutes, those from the second set for 10 minutes.

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When spectra were measured from the TLC plates, the plates themselves gave a distinctive spectrum. An example is shown in Figure 1. The main points of the spectrum are a broad fluorescent background and distinctive narrow bands which are due to the silica gel coating, and broader band structures originating from the glass substrate. Although
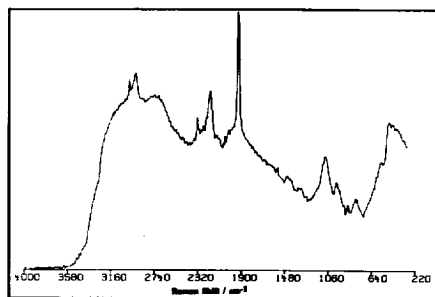
all of these features are of relatively low intensity, they are of the same order as the spectral information which we would like to record. To ensure that the features of the TLC plate would not interfere with the data, background spectra were recorded from the plates and subtracted from the recorded spectra of spots. It was found that for plates prepared within the same batch and measured under the same conditions, it was sufficient to measure a background once and use it for all spectra recorded from that batch of samples.

Figure 2 shows spectra of the two TLC plates prepared in the first batch of compounds. These plates were spotted with a very low concentration solution. Although these spectra are not of particularly high quality—as expected in the circumstance—comparison with the spectra of pure compounds showed they could be easily identifiable.

Most of the compounds in their raw form gave strong, clean Raman spectra. However, 5Br-2OH-Benzylalcohol shown in Figure 3, has a strong fluorescent background that somewhat masks the Raman
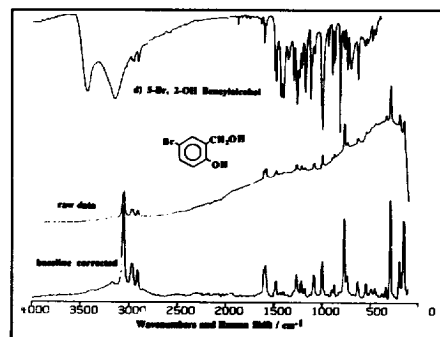

Figure 1 FT-Raman spectrum recorded from a bare area of the TLC plate after chromatography.

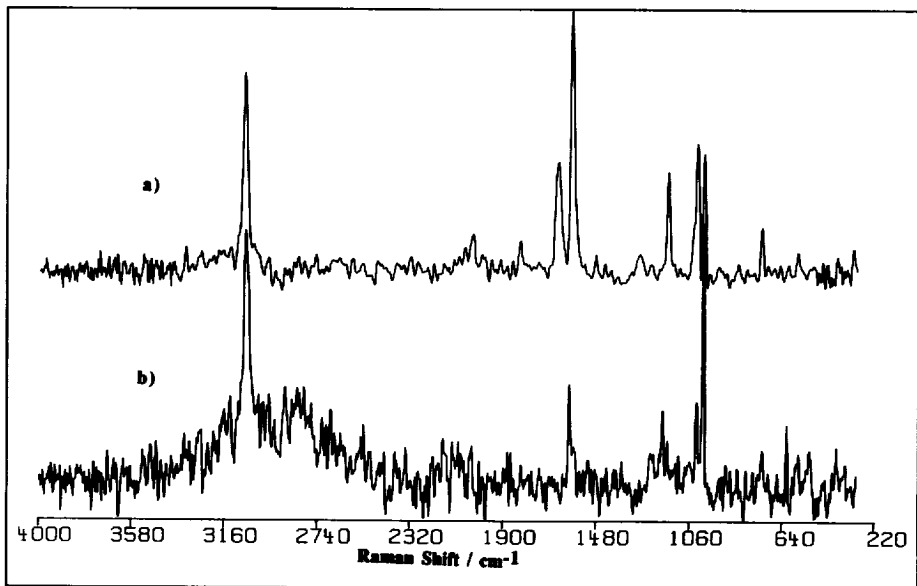**Figure 3** FT-IR, FT-Raman and baseline corrected Raman spectra of 5Br-2OH Benzylalcohol.

Figure 2 FT-Raman spectra of (a) Benzoylpyridine and (b) 3,3-Diphenylpropanol recorded from the low concentration TLC plates. Spectra are shown after subtraction of the TLC plate background.

bands. Although this did not prevent useful spectra being recorded from the raw chemical, it was not possible to record a spectrum of this sample on the TLC plate and subtract the effects of the plate to leave meaningful spectral data.

Figures 4 through 6 show the process of data analysis for the three spectra recorded from the plates spotted with higher concentration solutions. Each figure shows (a) raw data as recorded from the plate, (b) data after subtraction of the bare TLC plate spectrum, (c) deresolved to match the library resolution, and (d) spectrum of raw material used in the library data files. These spectra are clearly of much higher quality than those recorded from the lower concentration plates (Figure 2). Spectral subtraction is performed using the interactive routine available on the instrument workstation. The end point of the subtraction was determined by reducing the strongest band from the background spectrum to zero. The deresolution process for comparing with library spectra is normally performed by the search algorithm and is transparent to the user. The deresolved spectra are included here for clarity.

SPECTRAL LIBRARY SEARCHING

Library searching is a means of comparing an unknown spectrum to a database of pre-recorded spectra as an aid to identification. A search is not infallible since the size of the database is limited and the spectral quality can sometimes be poor. Good library software is normally designed to produce a number of possible matches which the operator must then interpret. While the use of spectral libraries is extensive in FT-IR, it is relatively new to FT-Raman. The reasons for this include the fact that FT-Raman is a relatively new technique and there have been difficulties establishing a universal spectral format.[9] The authors are presently unaware of any FT-Raman libraries that are commercially available, although several are being developed. In principle, NIR FT-Raman may have some advantages over IR for library searching in that spectra do not generally suffer from as much baseline variation. Also Raman does not exhibit broad spectral features caused in the IR by highly polar bonds.

A good search routine will provide some "figure of merit" to indicate which suggested matches best fit the unknown. Library databases can be organized in a number of
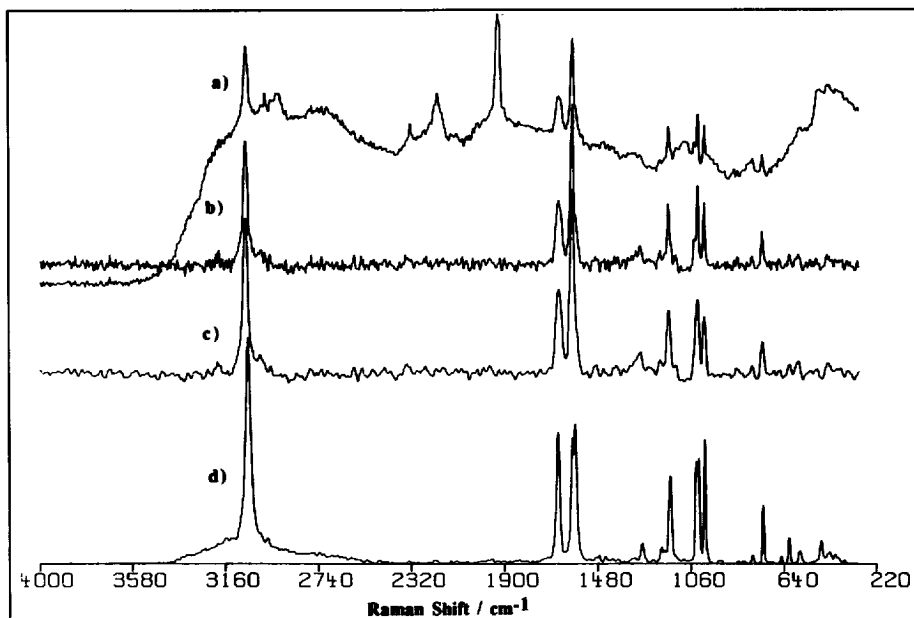
Figure 4 FT-Raman spectra of Benzoylpyridine showing preparation for library searching.
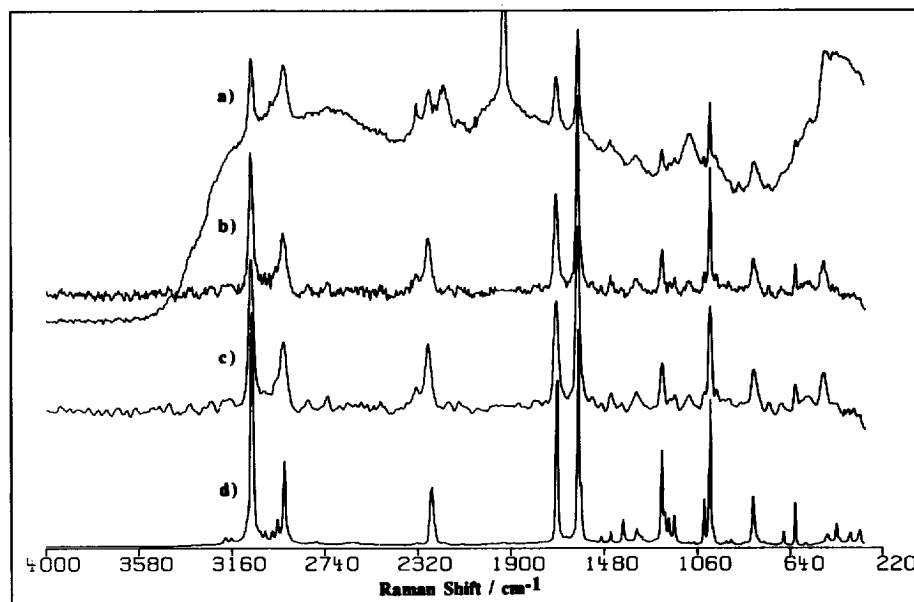
Figure 5 FT-Raman spectra of Benzoylacetonitrile showing preparation for library searching.
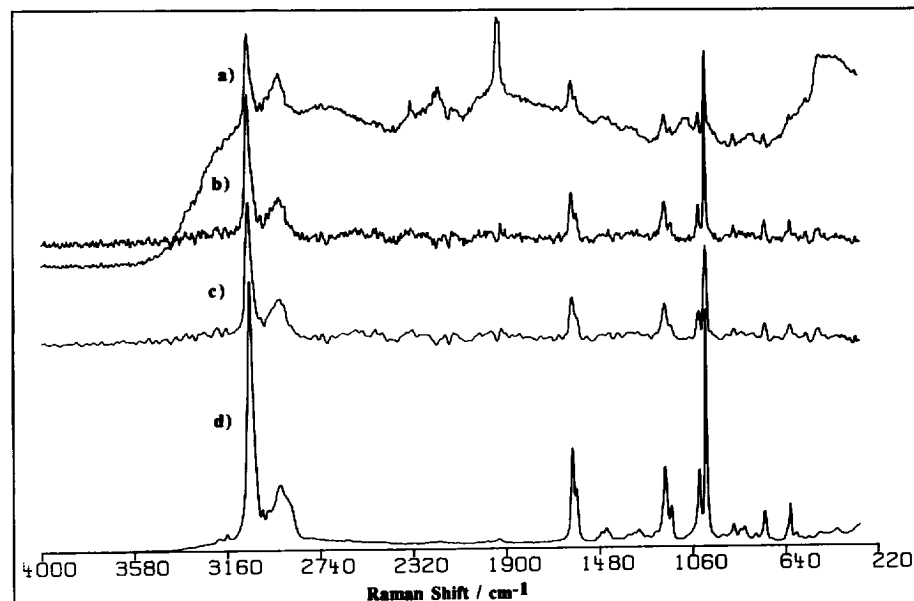
Figure 6 FT-Raman spectra of Diphenylpropanol showing preparation for library searching.

different formats. The simplest of these is as a peak table where the data is stored as a list of the positions of a few of the major peaks from each spectrum. This limited storage is normally used for very large databases since it minimizes both the time required to search the library and the space required to store the information. Obviously, storing only a few points from each spectrum and no information on band intensities can compromise the accuracy of the matched spectra. A "deresolved" format stores a full spectrum for each of the library compounds but at a low resolution—normally 16 cm^{-1} but sometimes as course as 32 cm^{-1}. Using low resolution spectra minimizes the storage space and search time parameters while maintaining a full spectrum for comparison. A third commonly used format is "high resolution" that uses spectra at 8 cm^{-1} or even 4 cm^{-1} resolution. This format is normally restricted to small databases since searching is slow and spectra may exhibit excessive noise associated with the resolution.

The library used for this investigation contains 400 FT-Raman spectra in a deresolved format (16 cm^{-1}). Several search algorithms are available for comparing an unknown with the library data; four were compared in this work and are summarized in Table 1.

Absolute Difference (AB)	Absolute value of a point by point subtraction.	Used for subtle differences in similar spectra. Emphasizes base-line differences.
Least Squares (SQ)	The sum of the squares of a point by point subtraction.	Emphasizes large differences over a number of smaller ones.
Absolute Derivative (AD)	Difference calcu-lated between first derivative of spectral data.	Ignoes DC baseline shifts and de-emphasizes broad features.
Squared Derivative (SD)	Sum of the squares of differences between derivative spectra.	Combines the effects of SQ and AD algorithms.

Table 1 Algorithms used for library searching.

Once again, the search algorithm used is transparent to the user after initial selection and does not increase the complexity of the process. Searching of the library database involves a "subtraction" of the sample spectrum from the reference—the exact form of the subtraction depends on the algorithm selected—and assigning a figure of merit value, called the metric number, to the residual. A low metric number indicates

a low residual and therefore a good match. The workstation sorts the comparisons and displays matches in order of metric number. Interpretations of the metric number output is important because the absolute value of a single number carries little information. Although a low metric number indicates a good match, a high number does not necessarily mean a bad match. Spectra with high noise and/or fluctuating baselines will always cause larger metric numbers even though the peaks may match well. The difference between metric numbers is important. For a good positive identification, the difference between the first and second choice should be considerably larger than that between subsequent suggested matches.

All of the spectra recorded were checked against the 400 spectra library using each of the available algorithms. The most important result to note in this searching procedure is that for all of the comparisons made, the correct spectra were picked as "best fit" for any of the searching algorithms. This is a somewhat surprising result—the normal aim of a search is to provide a number of suggestions from which the operator selects the most likely match. Most applications will also start with considerably higher quality data than is available when studying TLC plates. The data demonstrated that with spectra of the type shown, residual baseline effects are a far more dominant factor in the search procedure than noise. While all of the algorithms picked the correct spectra, the AD algorithm *consistently* gave a definite ID result—indicated by a clear difference in metric number between the first and subsequent picks. This is surprising since these spectra would normally be considered reasonably flat but with poor signal-to-noise ratios due to the weak spectra being recorded. These conclusions were further tested by two other experiments. First the spectra were smoothed using the noise level sensitive algorithm present on the workstation, and secondly small artificial baselines were introduced into the spectra. While smoothing made no significant difference to the accuracy of the library selections, the addition of baselines dramatically reduced the certainty of the first selection.

NIR FT-Raman on a high performance instrument has been demonstrated as a possible technique for the identification of chemicals

in very low concentrations on TLC plates. Using the measured spectra in conjunction with library searching routines has proved an easy and efficient method of spot identi-fication despite the fact that in more routine applications the spectral quality would be considered to be poor. While these tests have been performed on a limited number of compounds, it is thought that analysis should be applicable to a wider range of substances, and work continues in this area. Measurement times used were not excessive and since noise levels were demonstrated not to be the prominent factor affecting library searching routines, it should be possible to use shorter accumulation times while still acquiring adequate data. In addition, it should be noted that even when relatively high concentrations were used for the analysis, the limiting concentration for the chromatography had not been reached. Further optimization of the concentration of sample used could lead to dramatically improved performance, and as a consequence, much lower data accumulation times.

Further work is in progress to investigate factors which could improve the analysis, such as more accurate positioning of the plates in the instrument and automatic location and sampling of multiple chemical spots on a single plate.

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