COMPOUND IDENTIFICATION IN THIN LAYER CHROMATOGRAPHY USING SPECTROMETRIC METHODS

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CONTENTS

Summary	1
1. Introduction.	2
2. Confirmation of Spot Identity Using R_f Values and Colour Reactions	2
3. Thin Layer Chromatography – UV/Vis Spectrometry and Fluorimetry	3
4. Thin Layer Chromatography – Infrared Spectrometry	5
5. Thin Layer Chromatography – Photoacoustic Spectrometry	10
6. Thin Layer Chromatography – Raman Spectrometry	12
7. Thin Layer Chromatography – Mass Spectrometry	14
8. References	20

SUMMARY

Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) can be coupled with spectrometric methods in order to improve the selectivity and sensitivity of component detection and identification. Ultraviolet - visible (UV/Vis), infrared (IR), Raman (RS), photoacoustic (PAS) and mass spectrometry (MS or MS-MS) methods are reviewed emphasising the component identification in modern planar

chromatography. Practical examples of these combined methods, allowing the identification of a large number of compounds, are given.

1. INTRODUCTION

The identification of unknown compounds from a mixture is one of the fundamental problems in analytical chemistry. Quantitative analysis involves previous identification of a detected compound and the plotting of the corresponding calibration curves.

It is well known that chromatographic methods provide insufficient qualitative information in order to obtain a reliable identification of compounds using only their retention values. This problem can be solved by coupling the chromatographic techniques with spectrometric methods resulting in an increased amount of information, usually sufficient to identify the structure of the analysed compound, or at least to identify the compound by comparison with pure, standard substances. In thin layer chromatography (TLC) the identification and the confirmation of a compound structure is obtained by combining the planar chromatographic technique with Ultraviolet-visible (UV/Vis) and fluorescence spectrometry, infrared spectrometry (IRS), Raman spectrometry (RS), photoacoustic spectrometry (PAS) and mass spectrometry (MS).

2. CONFIRMATION OF SPOT IDENTITY USING R_f VALUES AND COLOUR REACTIONS

From the early times of thin-layer chromatography, compound identification was obtained by specific reactions using colour reagents. Unfortunately, the colour reagents are specific for a class of compounds or for a specific functional group and, as a consequence, different intensities and shades of the same colour can be obtained. Jork *et al.* /1/ have published a monographic book regarding the reagents used in thin-layer chromatography for component identification.

An interesting method for the visualisation of a thin-layer chromatogram was reported by Bladec /2-4/ who used liquid crystals. The method consists in transferring the organic substances from the chromatographic plate to a liquid crystals layer and was successfully applied for pesticide detection.

De Zeeuw *et al* /5/ used the R_f values obtained for different pure substances in 5 eluent systems and combined them with colour codes (for 4 colour reactions). The observed colour for an unknown compound was compared with a chart containing 21 different colours that had been coded numerically for a set of 99 basic and neutral drugs. The colour codes /5/ and the corresponding R_f values were expressed as corrected R_f^c . These corrections were made using a standard reference sample /6/. The colour codes and the R_f^c values provides the input for the database. The experimental R_f^c values and colour codes can be arranged as a basic matrix with 9 columns and 99 rows. The first 5 columns contain the R_f^c values and the next 4 columns, respectively, the numerical values (codes) for colour reactions. The 99 rows represent the number of drugs used for experimental work. In order to compare the suitability of techniques and/or systems, the mean list length (MLL) concept was developed /7,8/. The MLL represents the mean number of candidates that would qualify for the identification of an unknown compound when a given technique/system is used, alone or in combination. A compound is precisely identified when the MLL value is 1.00. Thus, the MLL represents an objective criterion to establish the identification capacity of a single system, combination of systems or even combinations of different analytical techniques such as gas-chromatography (GC), TLC and UV-spectrometry /5,9/. The MLL calculation for the matrix of R_f^c values, colour codes and the 99 drugs was performed using the Color Tox program, in Turbo Pascal under MS-DOS /5/. Even when the best combination of the three independent eluent systems plus colour codes was used, the obtained MLL value of 1.22 is still slightly higher than the optimal value 1.00, and thus the 99 compounds in the drug set cannot be unequivocally identified /5/. Finally, it should be outlined that a list of the number of coincidences that come into consideration can be listed by computer, in ranking order, on the basis of probability and similarity index. The index shows concordance between observed and listed data in the database /10/. The rank order is of supplementary help in the final identification process /5/.

3. THIN LAYER CHROMATOGRAPHY — UV/Vis SPECTROMETRY AND FLUORIMETRY

Though UV/Vis spectrometry and fluorimetry provide less structural information compared to IR and Raman spectrometry, the first techniques

are frequently used in analytical chemistry. In the last decade, modern instruments for qualitative evaluation of thin-layer plates were developed, increasing performance and optimising computer data-handling.

UV/Vis and fluorescence spectra are usually obtained in scattered reflection mode from thin-layer plates, and are specific for each substance. The compound identification is, in fact, a simple comparison of the *in situ* spectra obtained from the thin layer plate, in the same condition for the unknown substance and for a standard one. When standard substances are not available, the comparison can be performed using spectra libraries, taking into account that, usually, these spectra are obtained from solutions. Both R_f values and spectra for unknown and standard substances must be the same for a proper identification.

The new generation of densitometers are equipped with photodiode array detection and with a minimum number of mobile parts. In these conditions, UV/Vis *in situ* spectra can be obtained in less than 0.1 s.

Ebel and Wuthe /11/ have adapted a Schoeffel TLC Scanner SD 3000 for photodiode array detection and several other researches concerning the improvement of optical detection in TLC are reported in the literature. Early techniques involving TLC coupled with UV spectrometry using an optical fibre bunch were reporting during the sixties /12,13/. In 1989, Gaulitz *et al.* /14/ described the introduction of photodiode array detection in densitometry. Two years later, Ebel and Windmann /15/ reported the photodiode array scanning coupled with the transmission of the optical signal through "Y" shaped optical fibres. One lane of the "Y" shape was used to transmit the light from the source to the spot on the plate, and the other lane to collect and lead the reflected light towards the detection system. Computer data-handling offers the possibilities to store the recorded spectra in the memory. The *in situ* spectra can be obtained within the range 200-620 nm with a precision of 0.1 nm and a resolution of 2.4 nm. The spectra are corrected by measuring the layer reflectance, usually near the spot of interest. It was experimentally observed, on caffeine and Sudan Red, that the baseline noise is not improved further by using the mean of more than 100 spectra, and this operation can be performed in less than 1.5 s.

The *in situ* spectra are significantly influenced by the layer structure /16,17/. The spectra of aromatic compounds are the same on silica layers and on ion-exchange resins with 20% silica, but are significantly different on alumina layers.

The UV spectrometry was applied for herbicides /18/ and phytohormones /19/ identification in TLC. The method was also used for drug identification. Several barbiturates and butyrophenones were identified by comparing their *in situ* spectra with a spectra library /20/. The principal polar aromatic flavour compounds of commercial cinnamon /21/ and traces of trinitrotoluene and its metabolites in human urine /22/ were identified in the same way.

Advances in obtaining structural information for sample identification on separated compounds by TLC-UV spectrometry are shown in a detailed review /23/. In another recent paper, Butz and Stan /24/ have analysed a number of 283 pesticides by TLC with automated multiple development (AMD). The compound identification was performed taking into account the migration distance, the UV spectra and the detection limits.

In situ UV/Vis spectra are often significantly different from the spectra obtained for the same compound in solution. Therefore the spectra libraries obtained for the same compound in solution cannot be used for direct component identification in TLC /25/.

TLC-fluorimetry is another method for compound identification by comparison the *in situ* spectra with a library of spectra obtained in the same conditions and was successfully applied for polycyclic aromatic hydrocarbons in different matrices /26-31/.

4. THIN LAYER CHROMATOGRAPHY – IR SPECTROMETRY

TLC can be coupled with IR-spectrometry in different ways and the methods were improved according to the development of spectrometric and chromatographic techniques /32/. The methods using IR-spectrometry for component identification in TLC can be divided into two groups. The first group involves the removing of the substance from the spot, the depositing on an IR-transparent material (KBr or KCl) and the analysis. The second group contains the *in situ* methods, spectra recording directly from the plate.

Fourier transform infrared spectrometry (FTIR) offers the advantages of good sensitivity, high resolution and high speed for data collecting, thus a spectrum can be obtained in less than 1s. On the other hand, the FTIR instruments are very complex and expensive. These instruments do not contain prisms or diffraction grids and all wave-lengths are simultaneously

detected and measured. Therefore, the signal must be modulated so that all the wave-lengths can be separated using a high-speed computer.

4.1. TLC-IR Transfer Methods

The methods which involve the component transfer from the spot to a "transparent" substance for IR analysis are laborious and consist of three steps. First of all, the spot of interest must be precisely located using a non-destructive method, and then the substance with the layer is removed from the plate. The second step is the extraction of the substance with an appropriate solvent, followed by filtration or centrifugation in order to separate it from the layer particles. The solution of analysed substance is concentrated, usually by evaporating the solvent to dryness and dissolving the residuum in a smaller amount of solvent. The third and last step is the impregnation of a small quantity of KBr or KCl with the concentrated solution, the evaporation of solvent and then the preparation of a pellet suitable for IR analysis. The major disadvantage of this method is that the sample can be contaminated with small particles from the layer, of which the signals can overlap the sample signals or can modify the spectra. For example, silica has a strong signal at $9\text{ }\mu\text{m}$ /33/.

Different transfer methods have been reported. Issaq /34/ used an Eluchrom (Camag) device for the *in situ* elution of a substance from the spot to a small quantity of KBr, followed by solvent evaporation and compressing the powder as a micropellet. About $5\text{ }\mu\text{g}$ of substance are necessary for a spectrum. A similar method was reported by Rice /35/ and de Klein /36/, who used the direct transfer of the substance from the spot to KBr powder.

Goodman /37/ developed another transfer method using a plate covered with a KBr layer. The spot is identified on the thin-layer chromatographic plate and then the plate is rotated by 90^0 as in bidimensional development and eluted again. The KBr plate is placed layer over layer, in the direction where the spot is moving. The detection limit is $50\text{ }\mu\text{g/spot}$.

The spot and the layer can be placed in a special device on the top of a hypodermic needle. The needle contains a small quantity of KBr as a filter. Using a syringe, an adequate solvent will elute the substance, drop by drop, from the layer to 10 mg KBr. Each drop is followed by evaporation and 20 drops are usually sufficient. The KBr powder from the needle is homogenised with the substance and is compressed as a 1.5 mm diameter pellet. Excellent spectra are obtained for $5\text{-}30\text{ }\mu\text{g}$ of substance /38/.

Garner and Packer /39/ have described an absorption method using a KBr pyramid. The spot and the layer are removed from the chromatographic plate and introduced in a glass cylinder. The solvent will dissolve the substance from the layer and will migrate through the KBr pyramid towards its top where the substance will be concentrated by solvent evaporation. The pyramid top, containing the analysed substance will be cut, dried and used to make a micropellet. Good spectra were reported for 10 μg of substance and a recovery of 50 - 80 %. The technique can be applied using the commercial "Wick-Stick".

Using this procedure, Alto and Szekely /40/ successfully identified the components of an indigo dye by TLC-IR spectroscopy.

Chalmers *et al.* /41/ have reported a method for substance transfer from the spot to a KCl pellet followed by diffuse reflectance – Fourier transform infrared spectrometry (DR/FTIR). Spectra obtained from standard substances and some additives extracted from polypropylene, which were previously separated by thin-layer chromatography, show excellent similarities for 40 μg of substance and probably for 10-20 μg in favourable conditions. This method has the same sensitivity as the "Wick-Stick" method, but the probability of sample contamination is lower in the first one /42/.

Other authors have also applied the KBr micropyramid technique for substance transfer from a thin-layer plate followed by FTIR. Iwaoka *et al.* /43/ have performed a complex study regarding the substance transfer via silica layer - organic solvent - KBr support, including the estimation of silica solubility in different organic solvents by measuring the absorbance at 1095 cm^{-1} . Chloroform, methylene chloride and acetonitrile are strongly recommended solvents, but ethyl ether, n-hexane, benzene, ethyl acetate, ethanol or acetone can also be used. For aqueous solutions, the best results are obtained with water-acetic acid and the worst results with aqueous ammonia solutions. The recovery rates for the transferred substance were 85 - 100 %. The KBr pellet diameter, r , should be as low as possible for a FTIR measurement, in order to increase the detection limit which is proportional to $1/r^2$. A 4-5 mm diameter is recommended for the pellet. The study includes data on the value of necessary pressure and the pressing time for KBr pellets. A force of 1,5 tons and 5 s pressing time are recommended for KBr pellets of 4-5 mm diameter. The first step after chromatographic separation on thin layer plates is the determination of the exact position of each spot using a non-destructive method. The spots are bordered by two

lines traced in the layer to prevent transversal diffusion of solvent during the second elution. The direction of the second elution is perpendicular to the first elution direction, as in bidimensional TLC. The KBr pyramids are placed on the side of the plate which will be reached by the eluent front after the second elution, their axes perpendicular to the migration direction. Excellent FTIR spectra were obtained for several aminopyrines using this technique.

Shafer and Griffiths /44/ have described a substance transfer technique from the thin-layer plate to an IR transparent material. They used 2 x 8 cm thin-layer plates for the separation. The plates are rotated by 90° so that the longer side is in the horizontal position, and an aluminium sheet is attached to the top side. The aluminium sheet has small holes filled with an IR-transparent glass powder. The thin-layer plate is developed with a suitable eluent which enables the transfer process in about 2 minutes. The metallic band is detached from the thin-layer plate and dried at 40°C. DR/FTIR spectra were obtained for concentrations of 40 µg/µl.

4.2. TLC - IR *in situ* methods

The first attempts at *in situ* determinations were of Percival and Griffiths /45/ who prepared chromatographic plates with the layer deposited directly on an IR transparent material. Spectra were obtained for the separated compounds in transmittance mode.

Diffuse reflectance - Fourier transform infrared spectrometry (DR/FTIR) can also be applied for *in situ* compound identification /46,47/. The optic system was described and the detection limits were of 1 µg dispersed substance in KCl or of 1 µg substance/ spot.

The theory of diffuse reflectance was developed by Kubelka and Munk /48,49/, and allows the correlation between sample concentration and the intensity of scattered light in a similar way as Bouguer-Beer law can predict for spectrometry by transmittance.

Kortum *et al.* /50/ have demonstrated that for diluted samples in a non- or slightly-absorptive matrix the following equation is valid:

$$k = 2.303 \epsilon c$$

where k = molar absorption coefficient

ϵ = molar absorptivity

c = molar concentration

Zuber *et al.* /51/ have proved that a single spot on a thin-layer plate can be analysed *in situ* in 30 minutes or less by DR/FTIR, the detection limit being 1 µg. 2-8 mm diameter spots can be placed manually under the IR beam (1 mm diameter). The precise position of spots on the thin-layer plate can be obtained with an UV lamp or using a second plate as reference, eluted in the same conditions and sprayed with a colour reagent. All solvent must be carefully removed from the plate prior to spectra recording. The spectra can be corrected by subtracting the layer absorption, measured on a reference plate which was eluted in the same conditions as the plate used for sample separation. The major disadvantage of this technique is that even with layer absorption correction, it is very difficult to obtain reliable spectra in the regions where the layer shows strong IR absorption. For example, silica shows storage absorption in the 3700 - 3100 cm^{-1} and 1650 - 800 cm^{-1} (especially 1300 - 1100 cm^{-1}) regions, so that information about the sample cannot be obtained. Avicel thin-layers have a lower IR absorption in the region 1800 - 1600 cm^{-1} compared to silica layers, and substances which contain carbonyl groups can be detected. It is important to compare the IR spectra of a substance only if they were recorded from the same layer, otherwise the band intensities dramatically change. The thin-layer width and the granule dimension do not significantly influence the FTIR spectra. The advantage of *in situ* DR/FTIR spectra is the elimination of the transfer procedure on KCl or KBr, which could induce substance hydrolysis.

Beauchemin and Brown /52/ used the same plate for sample separation and for background correction. The layer spectrum was recorded at 3 mm distance from the spot. The substance spectrum was recorded in the middle of 2-8 mm diameter spots on an area of 1-2 mm diameter, for a period of 2 minutes. The method was applied for quantitative analysis of photosensitive compounds (e.g. diazonaphthochinones), using reverse-phase high performance thin layer chromatography (RP-HPTLC), on RP-18 F₂₅₄ plates and visualisation in UV light. Qualitative analysis was performed between 2800-1300 cm^{-1} , where the layer spectrum has a region of the greatest reflectivity, since the strongest infrared absorbance of the diazonaphthoquinone is that of the diazo group ($\text{C}=\text{N}=\text{N}$), which occurs at 2162 cm^{-1} near the maximum diffuse reflectances of the reversed phase silica-C18. In the case of *in situ* measurements, the interaction between the adsorbed substance and the layer will lead to significant changes of the maximum absorbance position for several functional groups. An example is diazo-naphthochinonsulphonic ester which shows a doublet at 2164 cm^{-1} and 2124 cm^{-1} for diazo group on

RP-18 layer. The *in situ* degradation of diazochinonic ester on RP-HPTLC plates can be observed due to the decreasing of "azo" absorption bands at 2132-2180 cm^{-1} and the appearance of a maximum absorption at 1710 cm^{-1} corresponding to the new-formed carbonyl group.

A computer controlled IR densitometer for obtaining DR/FTIR spectra has been described /53/ and the combination automated multiple development (AMD) and DR/FTIR was shown to increase identification limit by one third /53-55/.

The various effects of different matrix on diffuse-reflectance IR spectra are the cause of difficult quantitative analysis and the sample must be prepared, as far as possible, in a reproducible way /56/. Qualitative analysis can be performed but the changes in spectra due to the interaction between the adsorbed substances and the layer must be taken into account. Otto *et al.* /57/ have studied the *in situ* spectra of different substances on KBr powder and on silica layer and they observed that the detection limit is significantly influenced in the regions where the layer shows a strong absorbance. For a proper identification, both spectra, for analysed and for reference substance, must be recorded *in situ*, on the plate layer.

Compounds of environmental interest, including surfactants /58/, phthalates /59/, dyes /60/ and pesticides /57, 61/ were analysed *in situ* by TLC - FTIR on silica, alumina, zirconium oxide and reverse phase layers.

A new TLC technique, microchannel TLC, has been reported /62/. Channels having the dimensions 0.4 x 0.2 x 50 mm were packed with a zirconia stationary phase and used for TLC. Infrared microspectroscopic detection of organic compounds separated in these channels provided excellent DR-IR spectra and an improvement in the minimum identifiable quantity is increased by a factor of 500 /59/.

Although TLC-DR/FTIR has some limitations, it is a rapid and modern method for qualitative analysis. This technique can be combined with the transfer methods of the separated substance from the layer to KBr or KCl powder and very good detection limits are obtained.

5. THIN LAYER CHROMATOGRAPHY – PHOTOACOUSTIC SPECTROMETRY

Photoacoustic spectrometry (PAS) is a technique for obtaining spectral information from strong dispersive, opaque or weak absorptive samples /63-

65/. Several theoretic studies regarding PAS /66-68/ and also a digital model /69/ are available. Photoacoustic absorbance spectra are generated by an amplifier with synchron signal detection as a function of incident radiation wavelength. IR spectra can be measured using photoacoustic spectroscopy. Usually, the sample is placed in a constant volume cell filled with an inert gas, and the incident IR monochromatic radiation reaches the sample through a NaCl window. The sample molecules are excited to superior levels of vibrational and rotational energy by absorbing radiant energy. During relaxation to the fundamental state, the absorbed energy is transformed in heat so that the temperature and the pressure of the gas in the cell increase. The change in gas pressure determines a corresponding change of a microphone capacity. The microphone output signal is amplified as a function of incident radiation wavelength, and as a consequence, the PAS spectrum is a function of incident radiation wavelength.

The first experimental absorption photoacoustic spectra were obtained from the substances which were removed with the layer from the chromatographic plate and introduced in the measuring cell /70/. The reproducibility of this method was very poor, because the sample signals were strongly influenced by the size and quantity of the layer particles introduced with the sample in the cell. In order to avoid this problem, the samples were applied on special silica discs, or chromatographic plates with flexible supports, as aluminium or polymers, were chosen, so that the spots could be cut and introduced in the measuring cell of a photoacoustic spectrophotometer /70, 71/. Discs of 1 cm diameter with silica F₂₅₄ layer (0.25 mm width) on aluminium support or with neutral alumina layer (0.20 mm width) on polystyrene terephthalate support, were used for PAS measurements. Two systems, a microphone and a piezoelectric transducer, were available for detection. Similar photoacoustic spectra were recorded for tetraphenyl-cyclopentadienone using both detection systems /71/. An area of 7 mm² around the spot of interest can be cut from the plate and introduced in the measuring PAS cell /72/. Several metals were analysed as chelates of 1-(2-pyridyl-azo)-2-naphthol obtaining the following detection limits: cobalt (0.1 ng), nickel (0.1 ng), copper (0.4 ng); an amount of 10 ng chelate is sufficient for a PAS measurement.

PAS spectrometry has a common problem with DR/FTIR: in the region where silica shows strong absorption, it is difficult to obtain significant information about the sample. An other problem is that the cell must be carefully purged with helium for at least 15 minutes, in order to remove

from the silica layer all traces of water, CO₂ or other substances, which can easily overlap the sample signal /71/. A detection limit of 1 µg was obtained for caffeine, using the absorption band of the carbonyl group and subtracting the background spectrum of silica absorption /70/.

A special method was reported, using an open photoacoustic cell which can be directly applied on the plate layer /73/. Photoacoustic spectra were obtained from thin-layer plates for 0.4 µg of Bengal Pink colorant with a signal-to-noise ratio of 3.3. The photoacoustic cell is made of aluminium and the bottom part is carefully polished for a good tightening with the layer. The light enters the cell through a flexible fibre optic probe as previously used by Cahen *et al.* /74/ in PAS; the other end of the 51 cm long probe is mounted at the exit slit of the monochromator. The optical fibre bunch is tightened with the cell by silicone rubber. The microphone is completely closed in the cell by ring shape fittings and it is electrically isolated from the cell. The entire volume of the photoacoustic cell is 1.13 cm³, and the area exposed to light is 0.35 cm². The analysis of a certain spot is made by positioning the lightened area of the cell directly on the spot. PAS is a non-destructive method and both qualitative and quantitative analysis can be performed directly on the layer. Several cosmetic colorants and Ru(bi-pyridine)₃²⁺ complex were analysed from an ion-exchange membrane using this technique.

Fourier transform near-infrared (FT-NIR) spectrometry can now be used in combination with HPTLC with a microscope equipped spectrometer /75/. Phospholipids samples were used for studies, because these substances have essentially no absorption bands in the NIR region. The detection limit of phosphatidyl-choline was under 1 µg.

6. THIN LAYER CHROMATOGRAPHY – RAMAN SPECTROSCOPY

Raman spectra, as well as IR spectra, are determined by molecular vibrations, but essentially different in the excitation procedure of molecules. Raman spectroscopy is a useful technique, especially for very symmetric molecules which are inactive in the IR region because the absorbed energy is not sufficient to determine a dipole-moment formation in the molecule. To obtain a Raman spectrum, a change in the polarizability of electrons, at the

same time as the vibrational excitation, is sufficient. When a molecule has a high IR absorption, then it will show a weak Raman effect, and vice versa.

A Raman spectrometer usually has a monochromatic excitation system and a system to analyse the diffuse light positioned perpendicularly to the incident light direction. Soper *et al.* /76/ have described an instrument which has a monochromatic source (514.5 nm). The incident light reaches the thin-layer plate under an angle of 45° , led by an optical fibre bunch which is fixed with a tridimensional system. The diffuse light is focused on the entrance slit of a double-grid monochromator and analysed by a complex optic system. The monochromator is computer controlled. The detection system is a photomultiplier and an amplifier/discriminator photon counter. Para-rosaniline was separated by HPLC and the effluent was deposited spot by spot on a silver-activated thin-layer plate. The detector and spectra of *p*-rosaniline were obtained from the plate by using the previously described Raman spectroscopy technique. The detection limit was 750 femtomols and a linear response was obtained for concentrations of 10^{-5} – 10^{-7} M.

Surface-enhanced Raman scattering (SERS) spectrometry was applied for direct analysis of nucleic purine derivatives on HPTLC plates /77/. The instrument is designed to capture the diffuse light. The diameter of the analysed spot was 2-4 mm the light beam dimensions were 1.5×0.1 mm. Spectra were obtained with the scanning speed (accumulation) of $2 \text{ cm}^{-1}/1.5 \text{ s}$ for a substance quantity per spot from 10 to 60 ng. The identification of nucleic purine derivatives was based on the R_f values and the specific Raman frequency measured with a precision of $\pm 4 \text{ cm}^{-1}$. In the case of partially overlapping spots, with very close R_f values, a second specific frequency can be considered. Guanine and adenine provide characteristic information about the methylated centres of nucleic purine derivatives, in the region $600 - 700 \text{ cm}^{-1}$.

Micro-SERS has been used to analyse amounts of pg of compounds on HPTLC plates with silver colloidal spheres deposited on the layer /78-80/. The system allows data acquisition from Raman spectra from small spots, with less than $1 \mu\text{m}$ diameter. Several basic compounds, aromatic acids, polychlorinated dibenzo-dioxines and dibenzofurans were identified in this technique. Silver activated layers have been used for identification of high fluorescent compounds as orange-acridine and 2-aminofluorene. Micro-SERS/HPTLC can be a specific method for environmental analysis, especially for isomers of pollutant compounds.

Compared with *in situ* IR spectrometry, Raman spectrometry is not influenced by humidity or background absorption of the layer /77, 81/. The detection limit is usually of 1 ng substance/spot. Resonance Raman spectrometry coupled with TLC has been applied for the detection and identification of several metal-porphyrines. Nickel and copper derivatives of uroporphyrine can be identified by changes in the intensity of some absorption bands of the spectra. The quality of the resonance Raman spectra obtained from a substance deposited on silica layer is superior to that of spectra of the same substance in solution, and some changes in the relative intensity of peaks can be observed /81/. However, an increase in laser power and a supplementary focusing of laser beam on the spot, also increase the local heating and can produce the photodecomposition of the analysed substance /82/.

Near-IR-FT Raman spectrometry is another method which has been applied for *in situ* analysis of compounds on TLC plates, especially for the identification of polypropylene additives /83/. The concentration of the substance in the spot is the limiting factor in this type of measurements because the necessary spot area for a Raman spectra is only 1 mm² from the total spot area. Reasonable spectra were obtained from sample loading equivalent to about 3 µg/mm² in the most favourable case. Good spectra were obtained also from silica gel plates.

7. THIN LAYER CHROMATOGRAPHY - MASS SPECTROMETRY

The first attempts to couple TLC with mass spectrometry (TLC/MS) were performed over 25 years ago and were presented by Kaiser in a review /84/. The new development of coupling these two techniques has begun after 1980 and was based on a new generation of mass-spectrometers with reduced dimensions and reasonable prices, and on the improvement of the ionisation sources and interfaces.

7.1. TLC/MS transfer methods by elution

Preparative thin-layer chromatography was a first solution to extract a substance from a separated spot. However, this procedure is time consuming, especially when more compounds have to be extracted, and the compounds are exposed to contamination during the extraction /85, 86/.

A similar technique to "Wick-Stick" /39/ for IR spectrometry has been used for mass spectrometry, but the prism was made of NH_4Cl powder /87/. Brown *et al.* /88/ have described a device with two possibilities of continuous extraction of substance from a spot which was completely removed from the plate. The extracted sample is continuously introduced in the ionisation source of a mass spectrometer. A small quantity of 5-10 % matrix liquid was introduced in the extraction solvent in order to increase the sputtering efficiency into the source.

7.2. TLC/MS transfer methods by probe tip

MS spectra were obtained by transferring an amount of a few μg of substance from separated spots on a TLC plate to a strip attached to the probe of a fast atomic bombardment (FAB) mass spectrometer. The spots are visualised on the plate, usually by UV/fluorescence and the probe presses the strip on the layer surface. For a successful transfer operation, a few μl of an adequate solvent (dichloromethane or methanol) and of the matrix liquid (e.g. glycerine or thioglycerine) are added to the TLC adsorbent adhering to the probe tip. Then the probe is introduced in the FAB-mass spectrometer for a normal spectrum recording. High-quality mass spectra were obtained using this technique for three coccidiostats (lasalocid, septamycin and monensin), previously separated by TLC /89,90/.

7.3. *in situ* TLC/MS systems

Another important research direction was the improvement and development of instruments able to record mass spectra directly from a chromatographic plate. Ramaley *et al.* /91,92/ have developed a complex system for detection. The chromatographic plate (1 x 10 cm) can be moved until the analysed spot is positioned under the slit of the energy source. The desorption is made by a series of CO_2 laser pulses. Chemical ionisation (CI) reagent gas sweeps desorbed materials through a heated inlet system into the ion source of a quadrupole mass spectrometer. Good peak shape and high resolution were obtained for silica layers by using this system. The detection of polar compounds is more difficult. Alcohols and carboxylic acids are less sensitive. Aminoacids cannot be detected. When the molecular mass of compounds is higher, their volatility is lower and the detection becomes more difficult. Substances with molecular mass over 300 cannot be

detected. Furthermore, the chromatographic plate can be analysed by other methods because the mass spectrometry is non-destructive.

An ion trap detector (ITD) has been coupled to HPTLC by a laboratory-made interface /93/. The ITD was interfaced with an open air cell. When the cell is positioned over a spot, a closed chamber is obtained, which communicates with a helium source and with a capillary connected with ITD. The chromatographic plate is placed on a plate holder, with the layer towards the cell. The plate holder has the possibility of warming the spot, evaporating the substance which will reach the ITD with a helium flow. By programming the heating procedure and using a small column (1.5 m) in the long transfer line of the ITD, a separation of different components present in the same spot become possible. This system was tested for 10 ng naphthalene deposited on a HPTLC plate, when a high response on the ITD and a full spectrum could be recorded.

Stanley and Busch /94/ have described a more perforated interface than the interface reported in detail in the literature /95.96/ for the analysis of chromatographic plates by secondary ion mass spectrometry (SIMS). The SIMS system adapted for TLC has a vacuum chamber for chromatographic plates which can be mounted on a 25 cm² heatable computer-controlled sample platform. The vacuum chamber has Plexiglas windows to give visual access to the plate. A quadrupole mass analyser, with a mass range of 4700 Daltons, is located at a 90° angle relative to the TLC plate. An electrostatic energy analyser with associated focusing lenses precedes the quadrupole. The primary particle beam is positioned at 45° relative to the sample plate, having the possibility to adjust the angle to the target surface, so that the point where the beam strikes the surface must be in alignment with the secondary ion optics. This instrument has a flexible design and several sources for primary beam can be used, including a Cs⁺ thermoionic gun or a saddlefield FAB (Ar⁰) gun, which produce quality mass spectra for discrete samples, while a focused liquid metal Ga ion gun provides a small diameter primary beam, needed for high-resolution imaging analysis.

A new system for TLC/MS, with a surface tracking capillary within the vacuum chamber of the mass spectrometer, was reported /88/. This technique enhances the number of suitable solvents, and eliminates the procedure of preparing the layer surface previous to mass-spectrometry analysis. The solvent reaches the spot on the chromatographic plate through a capillary, extracts the substance, and introduces it in the vacuum chamber of the spectrometer. The solvent flow is maintained by a syringe pump, and

the flow rate is 2-5 $\mu\text{l}/\text{min}$. The solvents are deposited on the layer in the same place where the primary beam reaches the layer. The advantage of this instrument is that the deterioration of analyte spatial resolution via diffusion of a sample compound in the liquid matrix is minimum. The solvent flow rate through the capillary is constant, continuously filling the matrix of the bombardment centre of primary beam. The high-quality spectrum of a phosphonium salt, obtained with positive-ion FAB from the surface of a TLC plate using surface-tracking capillary, proves the usefulness of this system which is able to record spectra in about 10 s.

The time-of-flight secondary ion mass spectrometer (TOF SIMS) is a technique which applies a simple principle. When several ions are accelerated with the same energy, those which have a smaller molecular mass will "fly" at a greater speed. For a fixed distance, the ions time-of-flight is a function of their masses: the lighter ions will go through this distance earlier. The correlation between the time-of-flight, t_f , and the ratio m/e is $t_f \sim (m/e)^{1/2}$. The electrons bombard the gaseous sample in frequent short period of time (0.25 μs), when ions with the same energy are obtained. The ions are accelerated by an electric field and will "fly" through a 1 m vacuum tube, towards the detector. The first ion will be that with the smallest mass, and the others will follow in increasing mass order. The frequency of ions is set so that the heaviest ion from a pulse will not overlap the lightest ion from the next pulse. A detailed description can be found in the literature /97/.

In the TLC/TOF SIMS coupling, the intact chromatogram plate is placed within a source chamber and a spatially resolved map of organic or bio-organic compounds distributed across the surface is generated. Successful imaging of samples from the TLC plates with high spatial resolution, high mass resolution, and high sensitivity has been demonstrated /98/. The data were measured in the ion microscope mode with a total image field of 200 μm . Sensitivity is increased by using a high transmission instrument designed to collect the majority of the secondary ions from the sample surface (TLC plate).

7.4. TLC/MS component desorption and ionisation

The desorption of organic substances from the layer (silica) by heating is achieved with some difficulty /99,100/. Several studies have proved that even at high temperatures (over 600°C) the substances are not completely

desorbed from the layer /101/. In some cases, the substances were pyrolysed or burned before desorption could occur. For this reason, the composition of the layer is very important, and the best results were obtained on silanised silica and polyamide /91/. Some lipids, steroids and terpenoids cannot be completely pyrolysed on silica layers even at 800-900°C /102/.

In the case of thermal stable compounds with low volatility, the use of a low power CO₂ laser pulse instead of an incandescent filament is recommended. In this way, the sample is heated more quickly and the desorbed molecules show less decomposition /103,104,/. When laser desorption is coupled with mass-spectrometry, both desorption and ionisation of compounds can be obtained. However, using a laser, the quantity of neutral molecules is higher, especially at a power density of about 10⁷ W/cm²; but laser mass spectrometry (LMS) is a suitable technique for TLC /104-107/. The laser beam can be precisely positioned either on small spots (5 µm diameter) or on larger spots /108/, and the technique can be applied for direct analysis of chromatographic plates with a good spatial resolution /109/. It is also possible to map samples for organic fragments, a capability ideally suited to characterisation of TLC plates by LMS /110/. The combination of TLC/LMS was established as a potentially powerful analytical technique. TLC/LMS has been applied for the detection and identification of aromatic hydrocarbons and purinic bases, on polyamide layers. The R_f values can be accurately measured with TLC/LMS. The technique can determine if the broad TLC spots are due simply to tailing or are overlapping spots of two components /111/.

Resonant two-photon ionisation spectroscopy with pulsed laser desorption/volatilisation into supersonic jet expansion was also applied for the analysis of chromatographic plates /112/. Pulsed laser desorption has been used for direct evaporation of substances from TLC spots. The resulting neutral plume of molecules is then entrained into a supersonic jet expansion of CO₂ and transported towards TOF MS where resonant two-photon ionisation is performed. Various thermally labile biological molecules, including indoleamines, catecholamines, peptides, and drugs have been detected from silica gel layers with production of molecular ion in a TOF MS instrument. The technique combines the advantages of optic spectrometry and mass spectrometry. In favourable cases, detection limits in the low nanogram range can be obtained.

The ionisation methods of conventional mass-spectrometry, as electronic impact (EI) or chemical ionisation (CI), are limited to volatile compounds.

FAB-MS enables the spectra recording of compounds thermally unstable or with low volatility, direct from the chromatographic plate /89,90,113-123/.

Secondary-ion mass-spectrometry (SIMS) or liquid secondary-ion mass-spectrometry (LSIMS) is a suitable method for *in situ* spectra recording on thin-layer plates /96,124-144/. SIMS and LSIMS spectra have, in many cases, similar characteristics with Chemical Ionisation Mass-Spectrometry (CIMS).

7.5. TLC/ Tandem Mass-Spectrometry

Tandem mass-spectrometry (MS-MS) coupled with planar chromatography (TLC or HPTLC) has already been reviewed /145/. A tandem MS-MS system is formed by two mass-spectrometers, MS1 and MS2, connected on-line. The first spectrometer, MS1, has the role to select the molecular ion, and to separate it from the background ions formed by the glycerol matrix, while the second spectrometer, MS2, induces fragmentation in a collision cell containing helium, and it is able to separate and detect the fragments which provide analyte characterisation. Although the quality of the resulting spectra by TLC/MS-MS is much superior to the quality of the spectra obtained by TLC/MS, the number of practical applications is still a small part in the TLC/MS experimental work.

Sample introduction in TLC/MS-MS or TLC/MS systems can be performed by using either manual or instrumental approaches, depending on the available equipment. In both cases, the area of interest on the TLC plate must be impregnated with an appropriate matrix (e.g., glycerol) if FAB or LSIMS systems are used for sample ionisation.

Although a silica layer was used in most TLC/MS-MS studies, diols, amino- and cyano-bonded phases have also been applied /146,147/. In the instrumental technique, coated aluminium plates are generally used because good contact with the probe can be ensured. TLC/MS-MS can be applied for different classes of compounds such as: pharmaceuticals, including salicylic acid and its glycine conjugate salicylhippuric acid, phenacetin, ibuprofen, diclofenac, indometacin, flurbiprofen, and apoxen /145/; antipyrine and its metabolites in extracts of human urine /148/; rhamnolipids /149/; ecdysteroids (polar polyhydroxylated steroids) /120,150/; and polymer additives /151/.

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