gram can be obtained by linking the GC to a simple behavioural assay, for example the wing-fanning response elicited from male aphid parasitoids by the female sex pheromone (**Figure 4**). By monitoring the responses of individuals or groups of insects in the bioassay chamber as they are exposed to the effluent from the GC, it is possible to locate, quite accurately, the elution time of the semiochemical. This technique was widely used in the 1970s, particularly in studies on lepidopterous sex pheromones. More recently, this approach has been used to investigate the role of learning in mixture recognition by foraging honeybees.

Reaction Gas Chromatography

GC–MS is the main identification tool for chemical ecologists, but frequently does not provide sufficient information for a full characterization of the compounds of interest. Thus, in terms of lepidopterous sex pheromones, which usually comprise long chain fatty acid derivatives with varying degrees of unsaturation, MS frequently cannot locate the positions of double bonds, nor distinguish between (*Z*)- and (*E*)-isomers. Microscale reactions conducted before chromatography, or even on-column, can be used to provide information about the class of compound and its functional group, or even to convert them into more stable derivatives. It is possible to carry out a surprising number of reactions on nanogram quantities of material where the reaction is reproducible, quantitative and gives simple products. Various methods have been described for hydrogenation, ozonolysis, epoxidation, reduction, hydrolysis and esterification on nanogram sample levels, or even the use of subtraction loops or specific reactions to remove particular classes of compounds from the mixture (**Figure 5**).

Conclusions

For the chemical ecologist, GC is not just a technique that enables high resolution and separation of complex natural product extracts. It can also provide considerable structural information. At the simplest level, noting the retention times of compounds of interest on polar and nonpolar stationary phases can give information on the molecular mass and polarity of a compound. However, when appropriate microscale reactions are included in the repertoire, the GC can prove to be a considerable aid in the identification of semiochemicals. When combined with MS, the availability of structural information is increased considerably.

See also: **II/Chromatography: Gas:** Column Technology; Detectors: Mass Spectrometry; Headspace Gas Chromatography. **III/Chiral Separations:** Gas Chromatography.

Further Reading

Allenmark S (ed.) (1991) *Chromatographic Enantioseparation Methods and Application*. England: Ellis Horwood.

Attygalle AB and Morgan ED (1988) Pheromones in nanogram quantities: structure determination by combined microchemical and gas chromatographic methods. *Angewandte Chemie* 27: 460.

Baugh PJ (ed.) (1993) *Gas Chromatography: A Practical Approach*. New York: Oxford University Press.

Cardé RT and Bell WJ (eds) (1995) *Chemical Ecology of Insects* 2. New York: Chapman & Hall.

Hummel HE and Miller TA (eds) (1984) *Techniques in Pheromone Research*. New York: Springer-Verlag Inc.

McCaffery AR and Wilson ID (eds) (1990) *Chromatography and Isolation of Insect Hormones and Pheromones*. New York: Plenum Press.

Millar JG and Haynes KF (eds) (1998) *Methods in Chemical Ecology: Chemical Methods*. New York: Chapman & Hall.

Nordlund DA and Lewis W (1976) Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *Journal of Chemical Ecology* 2: 221.

Pickett JA, Wadhams LJ, Woodcock CM and Hardie J (1992) The chemical ecology of aphids. *Annual Review of Entomology* 37: 67.

Sandra P (ed.) (1985) *Sample Introduction in Capillary Gas Chromatography*, vol. 1. Heidelberg: Dr Alferd Huethig Verlag.

Thin-Layer (Planar) Chromatography

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Thin-layer chromatography (TLC) in the study of pheromones is more a subject of potentials than of wide application. Its use, some examples of its application in general and specific problems, and some sources where the reader can find procedures to follow are described here.

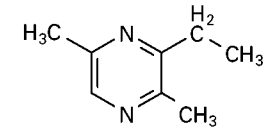
There is a constant argument between those who advocate simpler methods and techniques (often the

older practitioners) and those (sometimes the younger scientists) who believe that the latest and most elaborate and expensive equipment gives the best result in the shortest time. TLC lies in the thick of this argument. Its inherent simplicity, low cost, rapidity and ready interpretation are among its great advantages. Its detractors draw attention to its limited resolution and relatively poor limit of detection. Nevertheless, it is a useful and time-saving technique, worthy of consideration, and there are several recent books devoted entirely to the subject. The technique can be very simple, with hand-applied mixtures and simple spectroscopic, chemical or just bioassay detection methods, or it can be as instrumentally complex as one wishes, with automatic application, scanning densitometers, linking to nuclear magnetic resonance (NMR) and mass spectrometers. A new automatic device is available for collecting bands of silica from a TLC plate and transferring them by suction to solid-phase extraction (SPE) cartridges, which are then extracted with solvent.

Wilson has done much to show the capabilities of TLC allied to other techniques. Modern NMR spectroscopic methods make it possible to place the silica band containing the compound of interest directly into the NMR tube and obtain high resolution spectra. Mass spectroscopists were reluctant at first to put fine silica powder from TLC plates directly into their instruments but now commercial devices are available for doing just this. Highly polar compounds are not suitable for electron ionization–mass spectrometry (EI-MS) by direct insertion of the silica band from TLC, but several groups have shown that fast atom bombardment–mass spectrometry (FAB-MS) can be performed directly on this material, and that with suitable instruments, fragmentation information can be obtained by thin-layer chromatography–tandem mass spectrometry (TLC-MS-MS). Fell has written a comprehensive review on TLC in the study of insects (divided into carbohydrates, lipids, ecdysteroids and terpenoids, amines and pigments). Advocates of TLC, however, have not had a great influence on the field of pheromone studies, but TLC can have great advantages in preliminary studies of new pheromones.

Whatever one places on a TLC plate can be recovered (provided it does not decompose in the meantime), but with both gas chromatography (GC) and high performance liquid chromatography (HPLC) compounds can be either lost in the solvent front or never eluted from the column. Without a good quantitative bioassay or the use of radio-labelled samples the loss would not be detected and the research would be defective. With a simple bioassay, TLC can quickly yield information. An example from personal experience is illustrative. At the beginning of a study of the trail pheromones of *Myrmica* ants, no candidate peaks could be seen in a GC of an extract of the glands. To make sure the material was stable enough to purify, several poison glands known from bioassays to be the source of the pheromone were placed at the origin of a TLC plate, and chromatographed with hexane–acetone (7 : 3). Then the silica from the origin to the solvent front was divided into several bands, which were scraped from the plate, and eluted with acetone or methanol and the extracts submitted to a simple bioassay. There was far too little material there to be visualized by a chemical test. The active band containing the pheromone was readily recognized by a positive bioassay; the other bands were inactive (**Figure 1**). There was no correlation evident between activity and visualized bands because there was so little of the pheromone present. By trying a few other solvent systems, a good idea of the polarity of the pheromone was quickly obtained. By a few further TLC experiments (adding $NaBH_4$, NaOH or HCl) it was quickly established that the substance did not contain a reducible ketone group, was not acidic, but was basic. This knowledge greatly encouraged and simplified the subsequent isolation and identification of the pheromone (3-ethyl-2,5-dimethylpyrazine; structure I) with GC-MS. A little chemical imagination can devise other simple reactions that can be used with TLC to learn about functional groups. Once the simple TLC with bioassay has been mastered, more advanced techniques suggest themselves.

Structure I



3-Ethyl-2,5-dimethylpyrazine

Insects

Since many pheromones are readily volatile and transmitted through the air, gas chromatography has been the technique of first resort. It is too often forgotten that some pheromones are not volatile. A good example of a totally involatile pheromone is the oviposition-deterrent pheromone (structure II) of the cherry fruit fly *Rhagoletis cerasi*, identified by a large group led by Hurter, and for which a great deal of TLC was used.

When the female fly lays her egg inside the cherry, she places the pheromone on the cherry surface to warn other flies that this cherry is already occupied. It must remain there for the life of the larva, and withstand sunlight and rain. Isolation began with 7.3 g of female fly faeces, extracted with methanol and

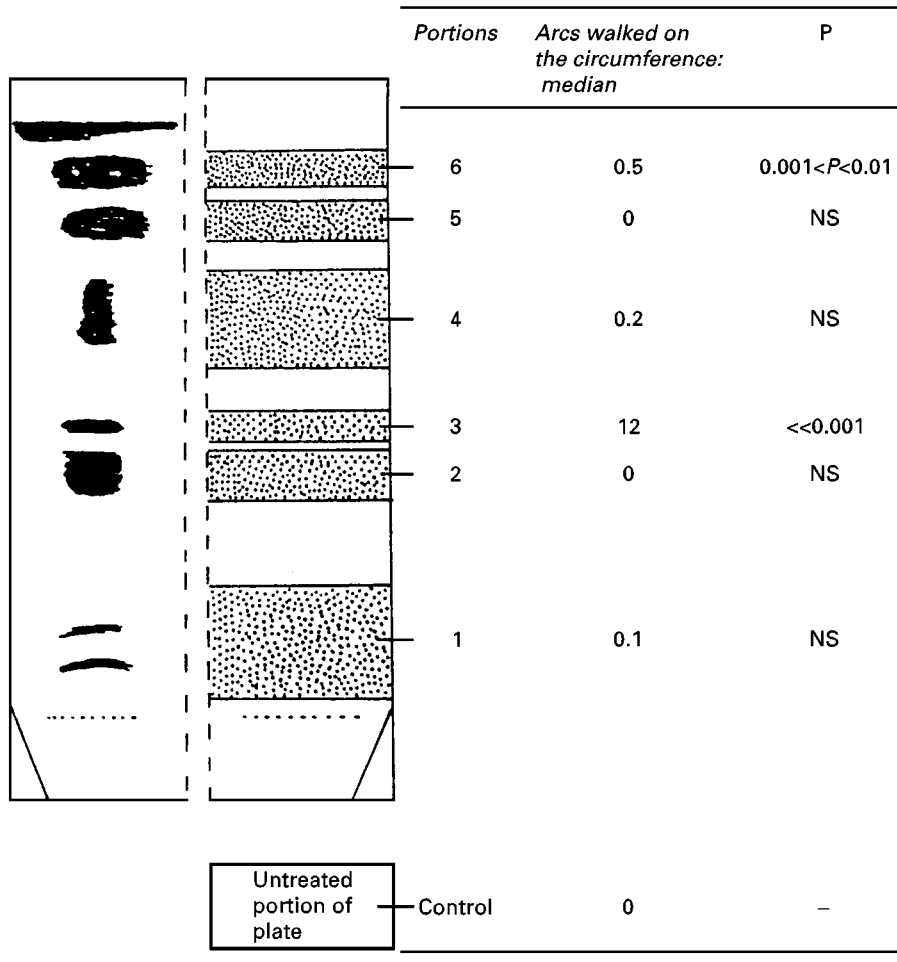
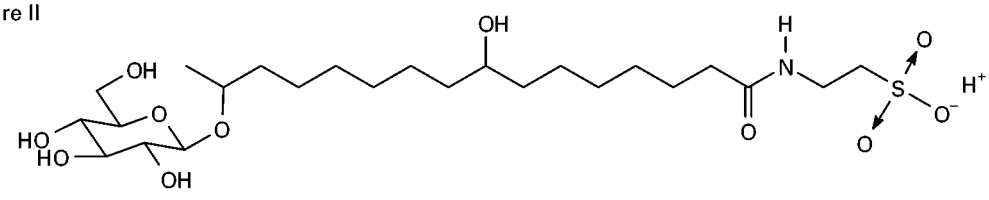


Figure 1 Thin-layer chromatogram of poison glands of *Myrmica rubra* ants applied to the origin of a silica plate and eluted three times with hexane–acetone (85 : 15). The plate was partly sprayed with phosphomolybdic acid for visualizing, and partly cut and the silica removed for elution of activity as shown. The activity of the fractions as indicated by worker ants following a circular trail is also shown. *P*, Level of probability; NS, nonsignificant difference for $P = 0.01$. (Reproduced with permission from Cammaerts-Tricot *et al.* (1997) Isolation of the trail pheromone of the ant *Myrmica rubra. Journal of Insect Physiology* 23: 421.)

cleaned up on a cellulose powder column. Preparative TLC on PSC cellulose, developed with ethanol–water (1 : 1), and cutting 0.1 R_F bands located all the activity in the 0.9–1.0 R_F band. This was subjected to HPLC and the active band was further separated on Antec OPTI-UP C-12 plates, developed with methanol, and the activity was found this time in the 0.5–0.7 R_F band. Later, when the pure pheromone was obtained, the taurine fragment was identified by TLC on Merck Silicagel $60F_{254}$, using *n*-butanol–99% acetic acid–water (8 : 3 : 1), and *n*-propanol–25% aqueous ammonia (8 : 2) and visualizing with Cl_2, I_2 and N,N,N′,N′-tetramethyl-4,4′-diaminodiphenylmethane.

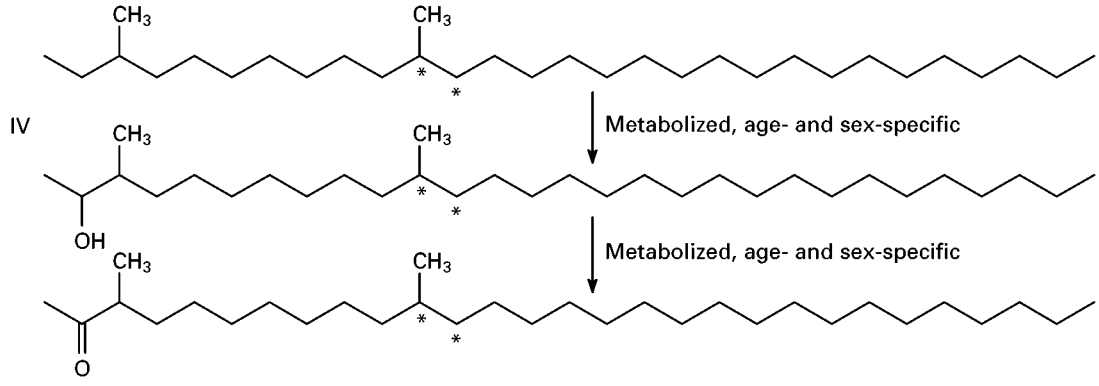
The sex attractant pheromones of Lepidoptera are not stored in the pheromone-producing gland but are synthesized and released as required. Aldehydes are the immediate precursors in *Manduca sexta*, but

Structure II



N-[15-(β-glucopyranylosyl)oxy-8-hydroxypalmitoyl]-taurine

Structures III and IV



[11,12-$^3H_2$]-3,11-dimethyl-2-nonacosanone
*Indicates position of tritium label.

to find which of the lipid classes was the source of the aldehydes, conventional lipid TLC was used effectively. Reversed-phase TLC has been used in the actual identification of lepidopteran sex pheromones too. Merck RP-8F_{254S} plates with acetonitrile–water (9 : 1) were used by Ando and others to separate C_{12} to C_{16} alcohols, aldehydes and acetates. Chromatograms were presented; this demonstrates how it is a useful tool in determining the chain length and number of double bonds. Separation of the *cis-trans* isomers so common in lepidopteran pheromones is very simple by argentation TLC.

Sex pheromone activity of the brown-legged grain mite *Aleuroglyphus ovatus* (strictly, mites are not insects) was not observed by Kuwahara in either a crude hexane extract or in column chromatography fractions. But when carefully separated from masking alarm pheromone activity of citral by TLC, the activity was readily recognized and the pheromone identified as 2-hydroxy-6-methylbenzaldehyde. The different pheromones of the camel tick and the dog tick were first studied by TLC, which showed them to be in the cholesteryl ester fraction of the surface lipids of the ticks, then HPLC was used to identify the exact compounds. Two-dimensional reversed-phase high performance thin-layer chromatography (RP-HPTLC) was used in the final stage of purification of a group of long chain branched and unbranched alcohols and acetates in looking for the female-produced sex pheromone of the screwworm fly, a serious pest of cattle. Plates were eluted once with hexane–ether (94 : 6) in one direction, then five times with hexane–benzene (96 : 4) in the second direction.

TLC is an excellent way to study the biosynthesis and metabolism of pheromones and precursors using autoradiography. The biosynthesis of the sex pheromone (3,11-dimethylnonacosan-2-one; structure III) of the German cockroach was studied through radio-TLC and radio-GC of a tritiated hydrocarbon precursor, 3,11-dimethylnonacosane (structure IV). When the hydrocarbon was applied to the surface of the insects, it was poorly converted to the ketone (structure III), but also to the intermediate alcohol. The alcohol, when applied, was efficiently converted to the ketone (**Figure 2**).

The effect of age and day length on the production of the main component ((*Z*)-11-hexadecenal) of the sex pheromone of the important grain pest *Helicoverpa zea* was quantified by TLC and GC. Radioactivity in the TLC fractions was monitored by scraping off bands of the silica for scintillation counting. Later the direct nervous control of the synthesis of this compound was demonstrated with radio-TLC, radio-HPLC and column chromatography. Further studies of the stimulation of pheromone production in glands of *H. armigera* and production of its fatty acid precursors were both measured by TLC. Similar methods were used to correlate the amounts of pheromone aldehydes and their triacyl glycerol precursors in the glands of the tobacco hornworm *M. sexta*.

Other Animals

TLC can be still more useful in the separation of the complex mixtures often encountered in mammalian pheromones. Female mole rats (blind, solitary rodents), when they are on heat, are attracted to substances in the urine of adult males which they would otherwise avoid. The urine was extracted with dichloromethane and the activity was shown by TLC to be correlated with the sterol and fatty acid ethyl ester fraction, but TLC also showed that this was only part of the activity found in the urine.

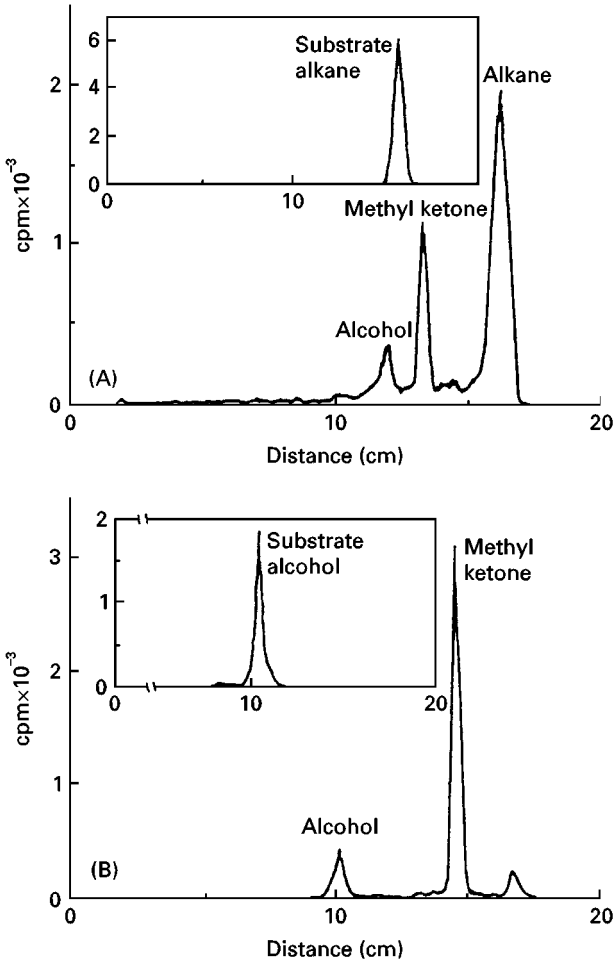


Figure 2 (A) Radio-TLC of cuticular extracts from female German cockroaches treated with [11,12-$^3H_2$]-3,11-dimethylnonacosane (inset: TLC of the pure alkane used). (B) Similar TLC of products when female insects were treated with [11,12-$^3H_2$]-3,11-dimethyl-2-nonacosanol (inset: TLC of pure alcohol used). (Reproduced with permission from Chase *et al.* (1992) Biosynthesis and endocrine control of the production of the German-cockroach sex-pheromone 3,11-dimethylnonacosan-2-one. *Proceedings of the National Academy of Sciences of the USA* 89: 6050.)

Tigers, like domestic cats, mark territory by spraying a marking pheromone upwards and backwards with their urine. The more volatile components are held from too rapid evaporation by lipid fixatives. The composition of the fixative has been studied by TLC and separated into cholesteryl esters, wax esters, tri-, di- and mono-glycerides, free fatty acids, sterols and phospholipids. The nature of the components could then be determined by transesterification and GC.

Marine Animals

Even fish pheromones have yielded to a study by TLC. The male yellowfin sculpin from Lake Baikal releases a sexual pheromone with its milt to attract females. An ether extract of male urine was separated into fractions, much as described above for *Myrmica* ants, and the active fractions from TLC were further purified by HPLC, and mass spectrometry identified testosterone and 11β-hydroxytestosterone. A third component was probably farnesol. TLC was used similarly to identify androgens in the serum of male Australian lungfish, and showed that testosterone was the main androgen. The exploration of maturation pheromones in goldfish has been studied through metabolism of [^{3}H]-17α-hydroxyprogesterone by ovarian follicles and following the products with autoradiography of TLC plates. Even corals produce pheromones at reproduction time. The identification in, and metabolism of, [^{3}H]progesterone and [^{3}H]androstenedione by Antarctic soft corals was studied by TLC with multiple elution. The metabolites are thought to be part of their chemical signalling or defensive metabolites.

Enantiomers

Enantiomer identification and separation are very important in pheromone isolation and synthesis, although no example of TLC used for this purpose can be found. Lepri's review of enantiomer separation by TLC gives many examples of its use that could be applied in pheromone studies.

Concluding Comments

Everyone knows about TLC, and everyone will have used it in their undergraduate days. All the techniques are in place for the greater use of TLC in pheromone research. All that is needed is for investigators to bear it in mind. It can save a great deal of time and effort in the preliminary stages of an investigation, give some indication of the characteristics of the compound being investigated, and provide a guide to the best instrumental method for further separation or purification. Mass spectrometry has long been able to handle the material from a single TLC band; with modern NMR instruments, there can be enough material on a single plate to obtain a good spectrum. The future of TLC in pheromone work could be bright: its greatest enemy is inertia.

See also: **II/Chromatography: Thin-Layer (Planar):** Mass Spectrometry. **III/Chiral Separations:** Thin-Layer (Planar) Chromatography. **Pheromones:** Gas Chromatography.

Further Reading

Ando T, Hasegawa Y and Uchiyama M (1986) Separation of lepidopterous sex-pheromones by reversed-phase thin-layer chromatography and high-performance liquid-chromatography. *Agricultural and Biological Chemistry* 50: 2935.

Bertsch WS, Hara S, Kaiser RE and Zlatkis A (eds) (1987) *Instrumental HPTLC*. Heidelberg: Hütig.

Cammaerts-Tricot M-C, Morgan ED and Tyler RC (1977) Isolation of the trail pheromone of the ant *Myrmica rubra*. *Journal of Insect Physiology* 23: 421.

Chase J, Touhara K, Prestwich GD *et al*. (1992) Biosynthesis and endocrine control of the production of the German-cockroach sex-pheromone 3,11-dimethylnonacosan-2-one. *Proceedings of the National Academy of Sciences of the USA* 89: 6050.

Fell RD (1996) Thin layer chromatography in the study of entomology. In: Fried B and Sherma J (eds) *Practical Thin-layer Chromatography*, ch. 5, p. 71. Boca Raton: CRC Press.

Hurter J, Boller EF, Stadler E *et al*. (1987) Oviposition-deterrent pheromone in *Rhagoletis cerasi* L. – purification and determination of the chemical constitution. *Experientia* 43: 157.

Katsel PL, Dmitrieva TM, Valeyev RB and Koslov YP (1992) Sex pheromones of male yellowfin Baikal sculpin (*Cottocomephorus grewingki*) – isolation and chemical studies. *Journal of Chemical Ecology* 18: 2003.

Kuwahara Y, Sato M, Koshii T and Suzuki T (1992) Chemical ecology of astigmatid mites. 32. 2-Hydroxy-6-methyl-benzaldehyde, the sex-pheromone of the brown-legged grain mite *Aleuroglyphus ovatus* (Troupeau) (Acarina: Acaridae). *Applied Entomology and Zoology* 27: 253.

Lepri L (1997) Enantiomer separation by thin layer chromatography. *Journal of Planar Chromatography* 10: 320.

Lessman CA (1991) Metabolism of progesterones during *in vitro* meiotic maturation of follicle-enclosed oocytes of the goldfish (*Carassius auratus*). *Journal of Experimental Zoology* 259: 59.

Poddar-Sarkar M (1996) The fixative lipid of tiger pheromone. *Journal of Lipid Mediators and Cell Signalling* 15: 89.

Sherma J and Freid B (eds) (1991) *Handbook of Thin-layer Chromatography*. New York: Marcel Dekker.

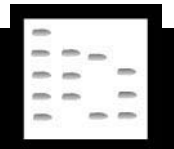
Somsen GW, Morden W and Wilson ID (1995) Planar chromatography coupled with spectroscopic techniques. *Journal of Chromatography A* 703: 613.

Wall PE (1997) Argentation thin layer chromatography. *Journal of Planar Chromatography* 10: 4.

Wilson ID (1996) Thin-layer chromatography: a neglected technique. *Therapeutic Drug Monitoring* 18: 484.

Wilson ID and Morden W (1996) Advances and applications in the use of HPTLC-MS-MS. *Journal of Planar Chromatography* 9: 84.

PHYSICOCHEMICAL MEASUREMENTS: GAS CHROMATOGRAPHY



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Introduction

Chromatography has been used for making physiochemical measurements for as long as it has been used for chemical analysis. When Martin and Synge introduced liquid–liquid chromatography in 1941 they also described how they used it to determine the distribution coefficient of the solute between the two (mobile and stationary) liquid phases.

In the early publications on gas–solid chromatography (GSC), by Wicke in 1940 and 1947 and Cremer and Prior in 1947 and 1951, the use for chemical separation is described simultaneously with physicochemical applications to the measurement of adsorption isotherms and free energies of adsorption. The earliest physicochemical measurements by gas–liquid chromatography (GLC) date from 1955 and 1956, when several authors described how to measure boiling points, partition coefficients and heats and entropies of solution for a volatile solute dissolved in a nonvolatile solvent. Since then, the scope of gas chromatographic (GC) techniques of physicochemical measurement has branched out in many different directions and now extends into a great variety of fields. These techniques are used by chemists and chemical engineers in many different specialist areas.

It is estimated that several thousand research papers have been published to date on the physicochemical applications of GC. Some of these applications have analogues in liquid chromatography, which is the subject of a separate article (Physico-Chemical Measurements).

Types of Measurement

The different types of physicochemical properties which can be measured by GC are classified in