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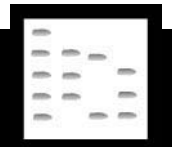
SUPERCRITICAL FLUID CHROMATOGRAPHY

See **III / OILS, FATS AND WAXES: SUPERCRITICAL FLUID CHROMATOGRAPHY**

FATTY ACIDS: GAS CHROMATOGRAPHY

See **III / LIPIDS: Gas Chromatography**

FLAME IONIZATION DETECTION: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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The Iatroscan is a British invention brought to fruition in Japan by Iatron Laboratories of Tokyo, which is basically a hospital equipment company. It has become unexpectedly popular in such diverse analytical areas as marine lipids and heavy petroleum fractions. The combination of the resolving power of thin-layer chromatography (TLC), itself only somewhat more than 40 years old, with the simplicity and sensitivity of the hydrogen flame ionization detector (FID), developed about that time as a superb detector for gas–liquid chromatography (GC), was a happy marriage, usually summarized as TLC-FID. The basic separation technology of the Chromarod-SIII is conducted on a quartz rod 0.9 mm in diameter and 152 mm in length, coated with 75 μm thickness of silica gel (10 μm particles) held in place by a soft glass frit. Ten Chromarods are conveniently held in a stainless steel rack for application of samples and subsequent development in a covered solvent tank, exactly as for planar TLC. The removal of solvent takes only a few minutes and the rack can then be dropped into a holding frame in the Iatroscan proper for scanning. This process can be controlled for maximum sensitivity but usually takes less than 10 min.

A virtue of the 10 Chromarods is that 10 different samples can be quickly compared or any combination can be replicated or compared to calibration standards run at the same time. The basic mechanism for passing the rod through the flame is fully automated. In the popular Mark III Iatroscan, the frame holding the development rack of up to 10 Chromarods was inclined. This has been replaced in the Mark V unit (**Figure 1**) with a horizontal frame. In the Mark IV Iatroscan the TLC-FID principles remained the same

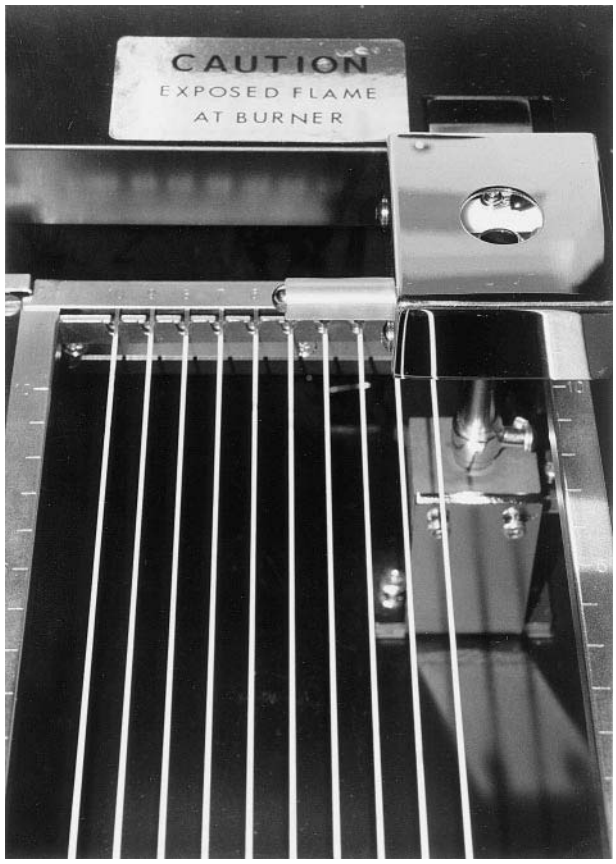

Figure 1 Top view of Mark V Iatroscan with horizontal rack holding 10 Chromarods in position for automatic scanning in the FID. The flame jet on the right is visible below the ion collector. The Chromarods pass between the two FID parts as the frame holding the development rack cycles for scanning. After each scan the frame moves sideways and returns between the Chromarods, bypassing the FID and moving sideways to start the next Chromarod. The right-hand rod is reflected in part of the ion collector.

but some improvements in quantitation of lipids were found in a new detector design, and that development led to an improved FID arrangement installed in the Mark V. It has not yet been rigorously evaluated for quantitation, for example in conjunction with hydrogenation of complex lipid extracts, but should be an improvement over the robust Mark III as regards quantitation.

General Considerations

GC and high performance liquid chromatography may frequently require an hour for each analysis. With several sets of Chromarods at hand, an analyst can conduct several types or sets of analyses per hour, since the development times (40–50 min) and scan times ($\sim$10 min) can overlap. Tanks with different solvent systems can be ready to participate in this process. Generally, the Iatroscan has not found wide application in the food industry. The response of carbohydrates in the FID is low because of the high oxygen content of the molecules.

The first problem in taking up TLC-FID is that those familiar with planar TLC often think in mg, and must adapt to μg – usually not more than 20 μg total per Chromarod. The second is that the application of a few micrograms of nonvolatile material in 1 μl of solvent can be automated or manual, but always results in some band spreading at the point of application. Solvent focusing has been found to overcome this usually minor problem and to narrow the sample band mixtures prior to actual development. Usually the choice is of a poor solvent for the materials in question, and for focusing, a development of the solvent front of less than 1 cm is adequate. An example is presented in **Figure 2**.

It is rare to find any unburnt organic material after analysis but it is good practice to clean the silica gel Chromarods regularly overnight in strong sulfuric acid, rinsing thoroughly in water, and passing through the scan cycle prior to use. If the previous samples generate any residue problems, such as from the calcium, magnesium and zinc of phytic acid, it will show up in this conditioning scan.

Early Chromarods showed variations in thickness and polarity that were mostly overcome with the introduction of the machine-produced silica gel Chromarods S-III. Alumina rods are also available but the literature does not indicate their wide use. Although there is a tendency to regard many solids and liquids as nonvolatile, this can be a tricky subject. Polar groups such as those of fatty acids and esters

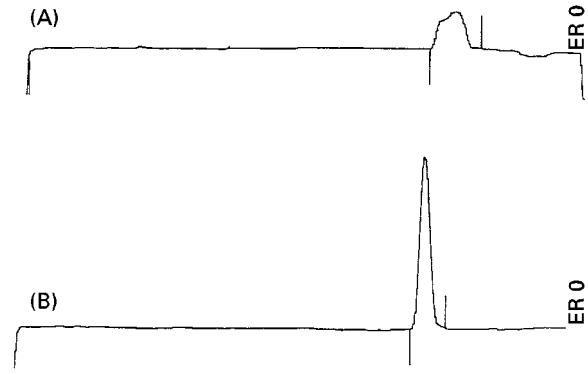

Figure 2 (A) Caffeine deposited on a Chromarod S-III from an queous solution and then developed and scanned. (B) Benefit of solvent focusing with methanol prior to development. (Reproduced with permission from Ackman RG and Heras H (1997) Recent applications of Iatroscan TLC-FID methodology. In McDonald RE and Mossoba MM (eds) *New Techniques and Applications in Lipid Analysis*, pp. 325–340. Champaign, IL: AOCS Press.

adhere to the silica gel quite well. Sterols are polar (R-OH) molecules of reasonably high molecular weight (387 for cholesterol), but the planar molecule may make hydrogen bonding difficult, and erratic calibration factors have been reported. It is assumed that the radiant heating of the approaching flame can sometimes vaporize part of the sterol band before it can be combusted to form ions. Squalene (molecular weight 411) had practically no binding capability and can lose half its apparent mass for similar reasons, but we have found that it is easily made less volatile and gives a full response if the Chromarod is exposed to iodine vapour for a few minutes prior to scanning.

The Chromarod-Iatroscan technology for analysis of nonvolatile materials is especially useful for high-molecular-weight polymeric oxygenated materials such as are found in oxidized oils. These are usually not easy to move along the Chromarod with developing solvents, whereas simple dimeric and trimeric triacylglycerols can be resolved by development. With the use of a nitrogen-specific attachment, the FID has greatly augmented sensitivity in the N-sensitive mode. This thermionic detector mode has long been available in GC, and is notoriously temperamental. It can extend TLC-FID into the selective analysis of many shellfish toxins, many of which contain a few atoms of nitrogen in very large molecules (e.g. mol. wt 301, $7 \times$ N, for saxitoxin).

For brevity this review will focus on two materials, marine lipids and heavy hydrocarbon fractions, but the possibilities for analysing reasonably large molecules are almost unlimited.

Marine Lipids

The first installation of an Iatroscan in North America was in 1976 in a marine lipids laboratory. The resulting publications on analyses of various complex materials attracted much attention among lipid chemists and biochemists, leading to a special issue of the journal *Lipids* in August 1985. Lipids of individual small marine organisms could be analysed for the first time and the sensitivity enabled extraction of water-soluble lipids to be modified to collect and extract less sample, and thus conserve on solvent use. The Iatroscan was quickly adopted in many countries with marine research programmes.

It is not often recognized that many human body lipids, especially those of muscle, liver and the blood, have fatty acid compositions spanning the same range as are found in fish oils and lipids. The latter include all varieties of lipids found in ourselves and other animals, and can be good materials to train with. Some will be featured in the few following examples of separations as demonstrations. In fish muscle lipids the fatty acid extremes in all lipid classes are the relatively short chain myristic acid (14 : 0) and palmitic acid (16 : 0) on the one hand, and the long chain, highly unsaturated eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) on the other hand. A superefficient separation is shown in **Figure 3**. In the A and B chromatograms the free fatty acids and sterol esters are split into two respective subclasses, one with 14:0 and 16:0 as the principal fatty acids and the other with 20:5 + 22:6 as the dominant fatty acids. After hydrogenation,

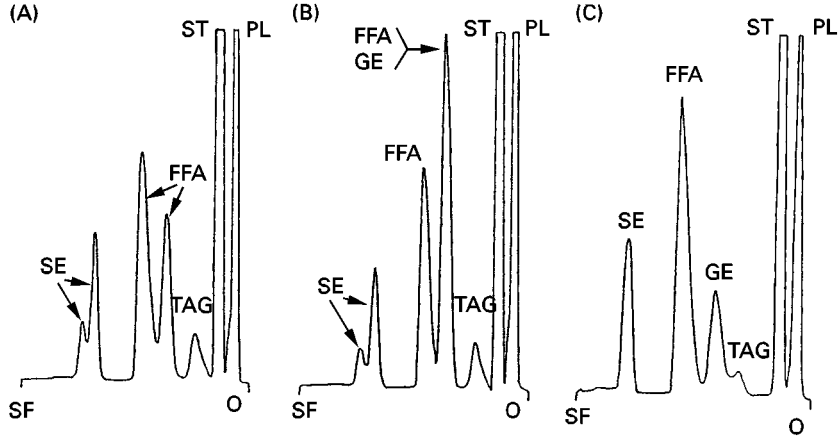

Figure 3 Iatroscan TLC-FID chromatograms of a fraction enriched with neutral lipids isolated from cod flesh lipids. (A) Neutral lipid (NL) fraction from cod flesh stored on ice for 3 days after being caught; (B) NL spiked with authentic 1-0-palmityl glyceryl ether dipalmitate (GE) coinciding with highly unsaturated free fatty acid; (C) Hydrogenated NL spiked with GE. Solvent system hexane:diethyl ether:formic acid; 97 : 3 :1. FFA, Free fatty acid; PL, phospholipids; SE, steryl ester; SF, solvent front; ST, free sterol; TAG, triacylglycerol. (Reproduced with permission from Ohshima T, Ratnayake WMN and Ackman RG (1987) Cod lipids, solvent systems and the effect of fatty acid chain length and unsaturation on lipid class analysis by Iatroscan TLC-FID. *Journal of American Oil Chemists' Society* 64: 219–223.)

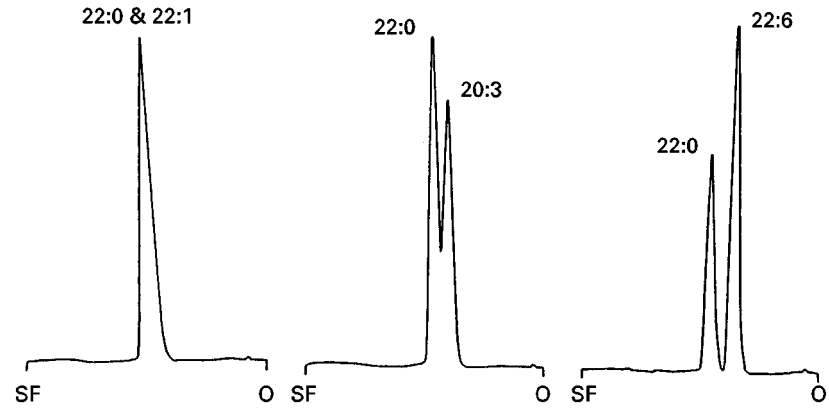

Figure 4 Iatroscan TLC-FID showing the effect of the degree of unsaturation on the separation of C_{22} free fatty acid standards on Chromarods-SII. Experimental conditions are development in hexane:diethyl ether:formic acid (97:3:1, v:v:v) for 40 min. O, Origin; SF, solvent front. Shorthand gives chain length and number of methylene interrupted ethylenic bonds.

chromatogram C shows that the pairs have collapsed into single peaks as the Chromarod behaviours of the resulting 14:0, 16:0, 20:0 and 22:0 are not very different. This is shown by the behaviours of selected sets of fatty acids and triacylglycerols (**Figures 4** and **5**).

Hydrogenation is not possible with many classes of organic compounds: it is not only feasible in analyses of lipid classes, but it has a unique advantage. The hydrogenated lipid fatty acids, unlike the natural highly unsaturated fatty acids, are stable to oxidation and can be studied and analysed at leisure, or with different solvent systems. Peaks are also sharper, improving sensitivity limits slightly.

For most simple lipid classes such as are found in vegetable oil products and mixtures, separation by lipid classes is facilitated by the fact that the common fatty acids are palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2n-6) and α-linolenic (18:3n-6). Except for palmitic acid, these are all identical in chain length (C_{18}), and the unsaturated acids differ only in having the 1, 2 or 3 ethylenic bonds. Chromarods dipped in silver nitrate can resolve such mixtures as well as handle some types of *cis-trans* separations, but these simple vegetable lipid cases are usually best handled by GC.

It is possible to develop one or more classes of lipids along the Chromarod, while 'parking' the rest at or near the point of application, scanning partway down the Chromarod to determine the most mobile class, then redeveloping the balance of the material applied to whatever extent is desired into the clean space thus presented by the first scan. This means that multiple scans are always of the same original sample and conducted on the same Chromarod.

A good example of multiple development is provided by a lipid class analysis of the total lipids of the muscle of the fish silver hake (**Figure 6**). The actual separation of the lipid classes was conducted with a development sequence of three different solvent systems. The extracted lipids were dissolved in

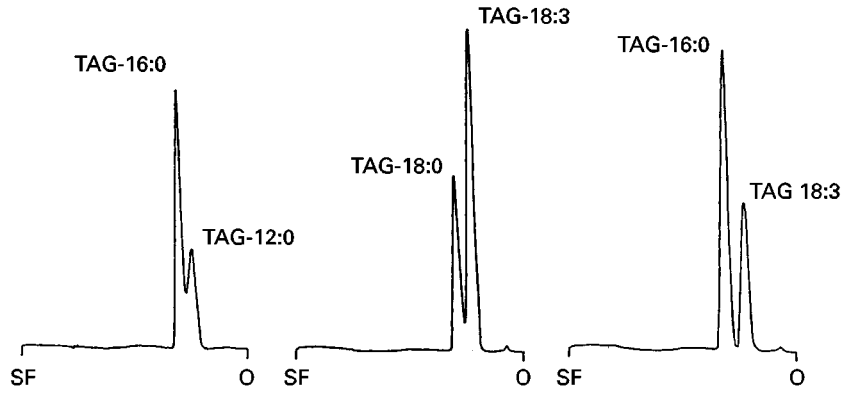

Figure 5 Iatroscan TLC-FID showing the effect of the degree of unsaturation and chain length on the separation of triacylglycerol standards on Chromarods-SII. Experimental conditions and abbreviations are the same as in Figure 4. TAG-16:0, tripalmitin; TAG-12:0, trilaurin; TAG-18:0, tristearin; TAG-18:3, trilinolenin. (Reproduced with permission from Ohshima T and Ackman RG (1991) New developments in Chromarod/Iatroscan TLC-FID: analysis of lipid class composition. *Journal of Planar Chromatography* 4: 27–34.)

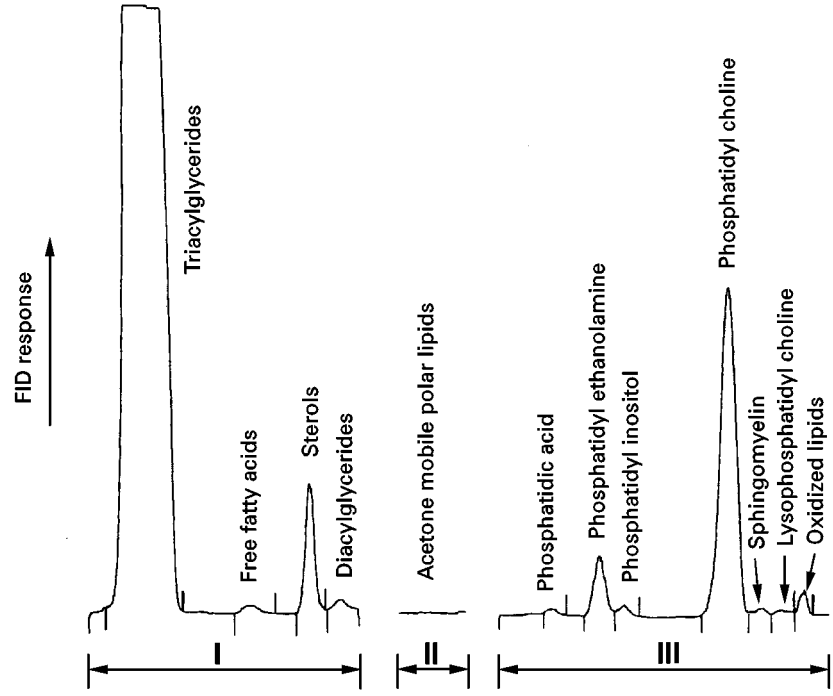

Figure 6 Sequential TLC-FID profiles of partial chromatograms of the lipid classes extracted from silver hake muscle tissue. I, II, and III represent the three-stage development sequence to separate total lipids on a silica gel Chromarod-SIII as described in the text. (Reproduced with permission from Zhou S and Ackman RG (1996) Interference of polar lipids with the alkali metric determination of free fatty acids in fish lipids. *Journal of the American Oil Chemists' Society* 73: 1019–1023.)

chloroform at an appropriate concentration, and this solution was then spotted on to Chromarods-SIII in 1 μL volumes from glass Microcap 1 μL disposable pipettes. The Chromarods were then conditioned in a constant humidity chamber for 5 min. The first development was carried out for 55 min in hexane:chloroform:propan-2-ol:formic acid; 80:14: 1:0.2, by vol. The Chromarods were then dried at 100°C for 1.5 min and partially scanned from the top to a point just below the diacylglycerol peak (**Figure 6**I). The Chromarods were then redeveloped in acetone for 15 min, dried at 100°C for 1.5 min and partially scanned to below the acetone–mobile polar lipid position (**Figure 6**II). Finally, the Chromarods were again developed in chloroform:methanol:water (70:30:3, by volume) for 60 min, dried at 100°C for 3 min and completely scanned to reveal different phospholipids (**Figure 6**III).

In this example the free fatty acids are clearly separated from triacylglycerols. This is sometimes difficult to achieve in a single development of a mixture of animal lipids with one of the common lipid class solvent systems such as hexane:diethyl ether:formic acid 85:9:1 (by volume). The problem can be clarified by considering the free fatty acids as having a key position on the silica gel of the Chromarod, and adjusting the solvent polarity to achieve relative movement of the rest of the neutral lipids, which usually develop in the order wax/sterol esters, triacylglycerols, cholesterol, and di- and monoacylglycerols, to positions where there is no conflict with the free fatty acids.

Solving such problems with TLC-FID may be compared with GC with only one column, and changes in temperature programming may be the only variable possible. With the Chromarod an unlimited choice of solvent systems is available and, when combined with scan and redevelopment, almost any lipid class separation is possible.

Figure 7 is of a commercial animal lipid mixture. The A chromatogram appears to show that the dominant triacylglycerol is accompanied by two peaks matching exactly 1,3-diacylglycerols and 1,2-diacylglycerols. This was considered to be an unusual composition. To verify it, hydrogenation of 10 mg of the sample (a simple process carried out by stirring in methanol:hexane : : 3:2 under hydrogen for 1 h, with a few mg of PtO_2), gave the materials of the B chromatogram. The triacylglycerol peak is sharper and the supposed 1,2-diacylglycerol is now added to the original 1,3-diacylglycerol peak. Clearly, the supposed 1,2-diacylglycerol component consisted of two highly unsaturated fatty acids, probably a mixture of arachidonic acid (20:4n-6) and docosahexaenoic acid

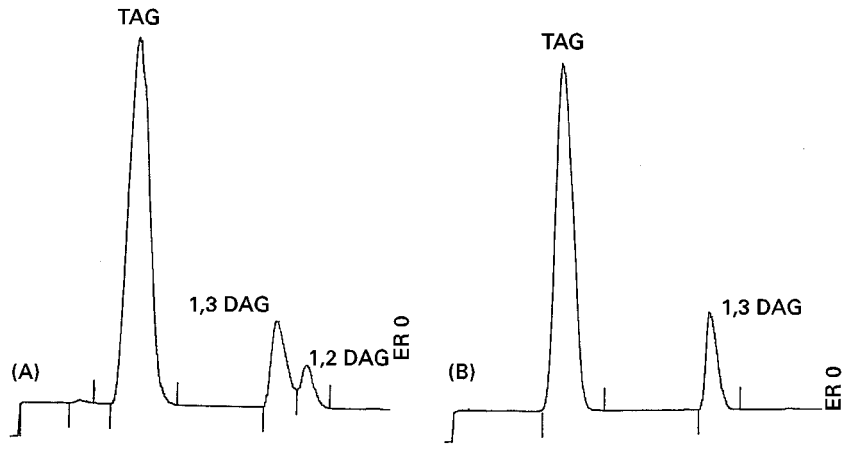

Figure 7 A commercial lipid product developed (A) in a solvent mixture of hexane:ethyl acetate:formic acid; 94:6:1 (v:v:v) hydrogenated, and (B) reanalysed. The smaller peaks, ostensibly 1,3-diacylglycerols (1,3 DAG) and 1,2-diacylglycerols (1,2 DAG), were shown to be two types of 1,3-diacylglycerol. TAG, Triacylglycerol.

(22:6n-3), materials currently of interest in infant nutrition.

Heavy Hydrocarbon Fractions

At one time coal provided a variety of liquid and semisolid materials, the latter usually referred to as pitch. The high-molecular-weight materials consisted of polycyclic aromatic hydrocarbons that could be individually defined with some difficulty, and more complex materials that were defined, mostly by solubility, as maltenes, asphaltenes and pre-asphaltenes. The use of TLC-FID in their analysis has been investigated for more than a decade and it promises to reduce solvent use and speed up analysis time enormously. The trend to coal liquification to produce fuel fractions competing with petroleum fractions makes new analytical technology even more useful to that industry, and at the same time the petroleum industry is turning to heavy crude oils and raw materials recovered from tar sands.

The products recovered from crude petroleum range all the way from hydrocarbon gases to alkanes of chain lengths up to C_{100}, polycyclic aromatics ranging from naphthalene upward, and other very complex high-molecular-weight materials, often incorporating nitrogen or sulfur. In the petroleum industry, standard methods tend to be time-consuming and complex. To make the life of the petroleum analyst even more difficult, 'cracking' to produce more valuable volatile fractions leaves residues of heavy materials such as asphaltenes. The application of TLC-FID to the latter has shown superiority to conventional methods, and has gradually been accepted, as shown by numerous publications.

The problem in crude petroleum analyses was basically the lack of natural standards, so that the quantitation of the FID response would reflect the mass of the particular complex fraction and pure chemicals representative of a fraction were unsatisfactory reference materials. For North Sea crude oils this difficulty has been overcome by preparation of appropriate standard fractions from typical crude oils, so that TLC-FID can provide data reliable for interlaboratory comparisons. In the petroleum laboratory particular difficulty is found with methods for heavy aromatic fractions and the more polar classes of materials. The latter often contain sulfur and nitrogen molecules and this makes some reporting technologies of little use, but the impact on TLC-FID response is not very significant.

One reason for industry laboratory problems is the obsolescence of standard methods, a problem not limited to the petroleum industry alone. When very large volumes of commodities are bought and sold there must be standards (and applicable methods) agreed on by all parties. Many have been around for decades with no changes. Meanwhile the internal combustion engine has been progressively fine-tuned to conserve energy, and even the robust diesel engine needs higher standards for volatile distillates. Complex refining and cracking steps produce even more heavy residues which must be investigated and utilized. The TLC-FID, introduced in about 1976, was immediately seized on by the petroleum industry and offers distinct advantages. The example given in **Figure 8** is taken from a recent paper on the subject. The Chromarod scans illustrate the weaknesses of the ASTM method D2007-91, based on rather lengthy and cumbersome open column chromatography on clay and silica gel columns in series.

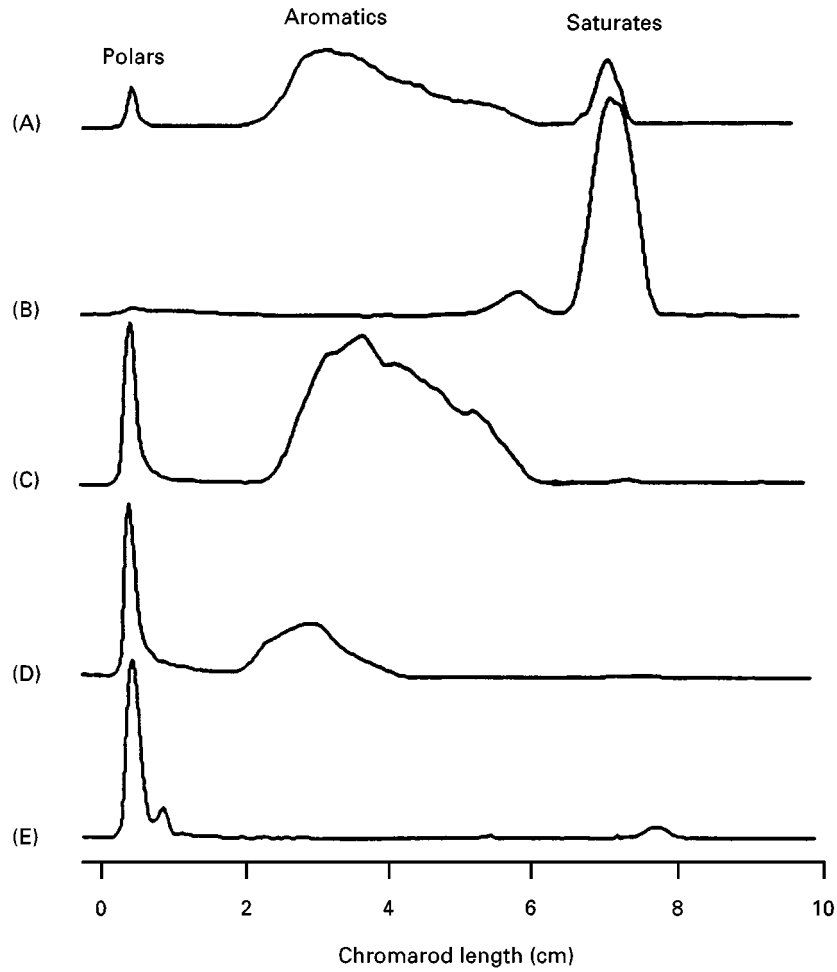

Figure 8 Superiority of Iatroscan TLC-FID over ASTM D2007 method in hydrocarbon analyses as exemplified with an aromatic petroleum extract and its fractions from the ASTM method. Chromatograms are for (A) TLC-FID of aromatic extract; (B) saturates by ASTM D2007, (C) aromatics by ASTM D2007, (D) polars by ASTM D2007; (E) residual polars from clay. Two-step Chromarod development of *n*-heptane for 30 min, followed by development with toluene for 5 min. (Reproduced with permission from Barman BN (1996) Hydrocarbon-type analysis of base oils and other heavy distillates by thin-layer chromatography with flame ionization detection and by the clay-gel method. *Journal of Chromatography Science* 34: 219–225.)

Conclusion

A recent paper on supercritical fluid chromatography suggested that often attempts to replace older and proven GC and HPLC methods with novel technology can be disappointing. That the TLC-FID system has been popular in only a few analytical fields may be due to the need for close interaction between the analyst and the method – almost a lost art in the face of contemporary automated equipment.

One exception to this is the TLC-FID of the Chromarod-Iatroscan combination, mostly used in research laboratories. As long as researchers have relatively nonvolatile organic materials to analyse, their resolution and determination by TLC is often a challenge for which the flexibility of the Iatroscan is ideally suited. Their chemical nature may have been defined by decades of patient work by others, but the adaptation to rapid analysis by thin-layer silica gel chromatography on a microgram scale may require a combination of imagination, knowledge and perseverence. The TLC-FID is a system that offers the challenge that makes science enjoyable!

See also: **III/Geochemical Analysis: Gas Chromatography. Lipids:** Gas Chromatography; Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Oils, Fats and Waxes: Supercritical Fluid Chromatography. Petroleum Products:** Thin-Layer (Planar) Chromatography.

Further Reading

Lipids

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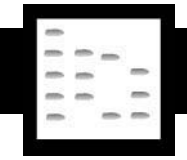
FLASH CHROMATOGRAPHY



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Flash chromatography and related techniques are widely used for laboratory-scale fractionation of mixtures from organic synthesis or for analysis when only a modest increase in resolution over conventional column liquid chromatography is required. These techniques employ short columns packed with particles of an intermediate size (typically 40–60 μm) combined with accelerated solvent flow achieved through modest pressure or suction. Compared to conventional column liquid chromatography, separations are obtained in less time; isolated compounds are often purer because resolution between bands is increased and band tailing is reduced; and compounds that are degraded or altered during chromatography are recovered in higher purity because of the shorter contact time with the chromatographic system.

The main applications of flash chromatography are purification of synthetic products, isolation of target compounds from natural products, the simplification of mixtures prior to high resolution preparative (usually) liquid chromatography and the fractionation of complex mixtures into simpler groups for analysis. Its primary virtue is low cost, since virtually no special equipment is required, and the stationary and mobile phases are inexpensive enough to be discarded after a single use, or can be recycled. Resolution is less than that obtained by medium and high pressure liquid chromatography but the operational costs and equipment needs are greater for these techniques. Flash chromatography is often employed as a pre-separation technique to remove particulate matter and sample components that are either weakly or strongly retained on the separation column in medium and high pressure liquid chromatography. This allows higher sample loads to be separated under more selective separation conditions and avoids column contamination and regeneration problems. The production costs of the isolated products are thus rendered more favourable.

Dry-column Chromatography

Dry-column chromatography (**Figure 1**) is a variant of preparative thin-layer chromatography with similar resolution but a higher sample loading capacity. A glass column or nylon tube is packed with thin-layer chromatographic grade sorbent, usually silica gel, to a height of 10–15 cm. Sample is added as