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QUANTITATIVE ANALYSIS OF LIPIDS ON COPPER(II) SULPHATE-IMPREGNATED CHROMARODS

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SUMMARY

Impregnation of Chromarods with copper(II) sulphate was found to remove the irregularities observed with the Chromarod/flame-ionization detection system (Iatroscan) in quantitative analysis. Of the operating variables, the scan speed affected the response most. At a scan speed of 4.17 mm/sec, quantitative analysis of eleven classes of lipids was achieved with Chromarods impregnated with 5% copper(II) sulphate solution. Under the conditions described, various compounds gave uniform responses so that response factors were not necessary except for phospholipids and cholesterol. It appears that with copper(II) sulphate-impregnated silica gel, the detector behaves similarly to a hydrogen atmosphere flame-ionization detector, with the result that the response is a function of mass and not of the structure of the compounds being analysed. Impregnation with copper(II) sulphate also improves baseline stability, minimizes rod to rod variations and makes the solvent front clearly visible.

INTRODUCTION

Thin-layer chromatography (TLC) is an indispensible tool in the separation and analysis of complex lipids of plant and animal tissues. Attempts have been made from time to time to quantitate the technique[1,2]. An often used method involves the separation of various components by TLC followed by transesterification and analysis by gas chromatography (GC) using an internal standard. This method has the inherent drawback, in addition to the inaccuracies in marking, scraping off and recovery, that components that are not fatty acid esters (for example, sterols) cannot be determined simultaneously.

Iatroscan (Iatron Laboratories, Tokyo, Japan) is an instrument that combines the resolution capabilities of TLC with the possibility of quantitation by employing flame-ionization detection (FID), and has been adopted by a number of laboratories for lipid analysis[3–11]. Developments in the field have been reviewed by Ackman[12]. Unfortunately, this method also has disadvantages. Recently, Crane *et al.*[13] concluded that this method is not suitable for quantitative lipid analysis. While some of their conclusions appear to be unwarranted, a perusal of the literature[3–12] shows that

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the responses of various lipids vary not only from rod to rod, run to run and compound to compound, but also from laboratory to laboratory. All operating variables appear to have a bearing on the results obtained.

It appeared that the non-uniformity in the chromatographic properties of the Chromarods was largely responsible for the inconsistent results and the method has little scope as a quantitative technique. In this investigation, the inhomogeneities were corrected by impregnating the rods with a dilute solution of copper(II) sulphate. By this treatment, all lipids except cholesterol and phospholipids gave uniform responses so that correction factors were not necessary except for the two species mentioned.

EXPERIMENTAL

Materials

Eicosane was purchased from Larodan (Sweden), triolein and methyl oleate from Acme Synthetic Chemicals (Bombay, India), "Triglyceride Mix C" (a mixture of equal proportions of trimyristin, tripalmitin, tristearin and triarachidin) from Applied Science Labs. (State College, PA, U.S.A.) and cholesterol from Loba-Chemie (Bombay, India).

Sitosterol palmitate was prepared[14] from sitosterol (Fluka, Buchs, Switzerland) and palmitic acid and purified by TLC. Mono- and diacylglycerols were prepared by pancreatic lipase hydrolysis of triolein followed by TLC of the reaction mixture. Phosphatidylcholine was isolated from egg lecithin. Other lipid standards available in the laboratory were lauroyl palmitate (wax ester) and behenyl alcohol (fatty alcohol). Each sample was checked for homogeneity by TLC, and GC wherever possible. Individual lipids were dissolved in chloroform to give concentrations of 2–5 mg/ml. Typically, 1 μl of the solution was spotted on the Chromarod and developed in a filter-paper-lined chamber with *n*-hexane–chloroform (89:11), the latter component containing 5% of isopropanol and 0.5% of formic acid.

Treatment of Chromarods

A set of ten Chromarod SII (Iatron, Tokyo, Japan) were left in concentrated sulphuric acid overnight, washed free of acid with distilled water and dried at 100°C for 30 min. After cooling to ambient temperature, five of these rods were transferred into another frame and immersed in 5% copper(II) sulphate solution ($CuSO_4 \cdot 5H_2O$; analytical-reagent grade) for 30 min. The frame containing the rods was removed and shaken to dislodge any droplets sticking to the rods and dried at 110°C for 30 min. The five untreated and the five treated rods were placed side-by-side on the scanning frame of the Iatroscan and scanned twice at a speed of 3.14 mm/sec (using speed gear No. 30), when the treated rods turned brown and were ready for use. At a higher scan speed (4.17 mm/sec., using gear No. 40), the top portion of the rods (in relation to the flame) was not charred uniformly.

The Iatroscan was run under the following conditions: hydrogen flow-rate, 160 ml/min; air flow-rate, 2000 ml/min; recorder sensitivity, 100 mV; chart speed, 24 cm/min; scan speed, 2.13–5.45 mm/sec.

For calculation of the response factors of individual lipid standards, the rods after spotting were developed only to a distance of 5 cm from the origin in the solvent

system indicated. Development in the solvent was necessary to prevent splitting of the peaks. The developed rods were dried briefly in an air oven at 110°C and scanned. Peak area was measured manually by multiplying the peak height by its width at half-height.

RESULTS AND DISCUSSION

Quantitation has always been the most unsatisfactory aspect of TLC. Among the earlier methods of quantitation[15], densitometry of the charred spots has been the most popular. Precision was lacking because the spot intensities are not necessarily proportional to the carbon contents of the compounds analysed. Further, the method was applicable to only a limited range of sample concentrations. Impregnation of the plates with ammonium sulphate[16,17], spraying the developed plate with copper(II) acetate solution[18] and impregnation of the plate with copper(II) sulphate[19] were used to overcome the problem of non-uniform charring of the spots.

The data available on the quantitative aspects of the Chromarod–FID system presents a confusing picture. Different classes of lipids have different responses and all operating conditions affect the response, with the result that different workers have reported different response factors for the same compound. Crane *et al.*[13] reported that the response is also affected by the amount of the lipid analysed. They observed that the response factor for tripalmitin increased three-fold on going from 1 to 10 μg. The response was also found to vary with scan speed and the relative position of the spots on the rod. These observations led them to conclude that "we need a much better understanding of the manner in which all tested compounds behave under analytical conditions before quantitation can become feasible".

Hence it is apparent that the Chromarod–FID system does not generate reliable quantitative data. The Chromarod being the heart of the system, it was felt that any improvement of the method should begin with the rod. It was hoped that treatment of the rods with ammonium sulphate or copper(II) sulphate, which could improve the charring of lipids on the TLC plate, would improve the burning and hence ionization of organic compounds in the flame of the detector. Hence copper(II) sulphate- and ammonium sulphate-impregnated rods were tested to see whether the response could be improved and made at least reproducible. Ammonium sulphate-impregnated rods gave a lower response than untreated rods and were not pursued further. However, the copper(II) sulphate treatment was found to improve the response of various lipid species considerably.

Table I compares the results of the analysis of eleven classes of lipids on both untreated Chromarod SII and rods treated with copper(II) sulphate. Analyses on both types of Chromarods were run simultaneously for each compound with identical sample loadings. Results are given at three scan speeds as it was found that among the different operating variables, the scan speed affected the response to the maximum extent. For purposes of easy comparison the areas calculated for 10 μg of each lipid are given in Table I.

Untreated Chromarods

The large increase in peak area for the various compounds with increasing scan speed is obvious from Table I. At a scan speed of 2.13 mm/sec the highest

TABLE I

AREA CALCULATED FOR 10 μg OF LIPIDS ON UNTREATED AND COPPER(II) SULPHATE-TREATED CHROMARODS AT DIFFERENT SCAN SPEEDS

Samples spotted varied from 2 to 5 μg of each lipid. Five untreated and five treated Chromarods in a frame were spotted and developed for 5 cm. HC = Hydrocarbon (eicosane); SE = Sterol ester (sitosteryl palmitate); WE = wax ester (lauroyl palmitate); ME = methyl ester (methyl palmitate); TAG = triacylglycerol (a mixture of trimyristin, tripalmitin, tristearin and triarachidin); FA = fatty acid (stearic acid); F Alc = fatty alcohol (behenyl alcohol); DAG = diacyl glycerol (diolein); CHOL = sterol (cholesterol); MAG = monoacyl glycerol (monoolein); PC = phospholipid (phosphatidylcholine). Instrument conditions: hydrogen flow-rate, 160 ml/min; air flow-rate, 200 ml/min; recorder sensitivity, 100 mV; chart speed, 24 cm/min. Scan speed varied as shown; speeds of 2.13, 3.14 and 4.17 mm/sec correspond to scans with speed gears Nos. 20, 30 and 40, respectively. Values are reported as means ± standard deviations with the number of determinations in parentheses.

Lipid	*Area (mm^2)*					
	Untreated Chromarod			*$CuSO_4$-treated Chromarod*		
	2.13 mm/sec	*3.14 mm/sec*	*4.17 mm/sec*	*2.13 mm/sec*	*3.14 mm/sec*	*4.17 mm/sec*
HC	529 ± 23(8)	841 ± 31(5)	769 ± 47(13)	594 ± 22(6)	1177 ± 44(5)	1125 ± 58(10)
SE	506 ± 28(5)	652 ± 35(5)	1162 ± 43(5)	636 ± 40(5)	977 ± 30(4)	1235 ± 96(10)
WE	461 ± 20(5)	650 ± 32(6)	581 ± 57(5)	767 ± 33(8)	1353 ± 57(7)	1261 ± 35(10)
ME	343 ± 27(10)	635 ± 35(8)	749 ± 21(6)	462 ± 21(8)	1063 ± 63(9)	1111 ± 14(8)
TAG	351 ± 10(5)	535 ± 24(7)	758 ± 26(5)	795 ± 13(5)	1298 ± 24(7)	1275 ± 40(8)
FA	274 ± 21(10)	448 ± 42(8)	703 ± 21(5)	791 ± 72(14)	1311 ± 44(6)	1243 ± 50(10)
F Alc	460 ± 18(5)	560 ± 56(5)	984 ± 40(6)	726 ± 44(5)	986 ± 40(5)	1210 ± 21(8)
DAG	–	517 ± 20(5)	802 ± 103(5)	–	1168 ± 53(5)	1243 ± 72(10)
CHOL	398 ± 10(5)	819 ± 37(5)	713 ± 61(5)	393 ± 29(5)	723 ± 18(5)	651 ± 48(10)
MAG	–	451 ± 42(5)	667 ± 17(5)	–	–	1282 ± 76(10)
PC	1134 ± 23(5)	1504 ± 40(5)	1095 ± 20(5)	1068 ± 48(5)	1477 ± 37(5)	1025 ± 20(10)

response was given by phosphatidylcholine (PC), followed by hydrocarbon (HC) and steryl ester (SE). At 2.13 mm/sec the different lipids could be arranged in the order PC > HC > SE > wax ester (WE) = fatty alcohol (F Alc) > cholesterol (CHOL) > triacylglycerol (TAG) > methyl ester (ME) > fatty acid (FA), based on their relative response. At 3.14 mm/sec, PC still gave the highest response, but there was a considerable improvement in the response of CHOL and the order changed to PC > HC > CHOL > SE = WE = ME > F Alc > TAG > DAG > MAG = FA. Increasing the scan speed from 2.13 to 3.14 mm/sec generally increased the response of all the compounds tested. Increasing the scan speed further to 4.17 mm/sec decreased the response of PC, CHOL, HC and WE. At this speed SE gave the highest response. The response also improved for F Alc, ME, TAG, FA, DAG and MAG, so that the order now was SE > PC > F Alc > DAG > HC > TAG > ME > CHOL > FA > MAG > WE.

Hence it is clear that scan speed affects different compounds to different extents, which might explain the different response factors reported by different workers for the same compound.

The hydrocarbon eicosane might be expected to give the highest response owing to the absence of oxygenated carbon atoms in the molecule. This was not observed, however. The difference in response between various classes of compounds was attributed[4,12] to the difference in the ionizable carbon contents, which vary from

compound to compound. While this is true with the FID system of a gas chromatograph, that of the Iatroscan does not appear to respond to different compounds strictly according to this rule. For example, Sipos and Ackman[4] reported a response factor of 0.69 for CHOL with an ionizable carbon content of 82.3% and a factor of 1.03 for tristearin with an ionizable carbon content of 72.7%.

Copper(II) sulphate-treated rods

A comparison of the areas of the eleven classes of lipids analysed on untreated and copper(II) sulphate-treated Chromarods clearly shows that this treatment improves the responses of all the compounds except PC and CHOL, for which there was a slight reduction in response compared with that obtained on untreated rods at all scan speeds. The greatest effect of the copper(II) sulphate treatment was noted with FA and TAG. At a scan speed of 2.13 mm/sec the increase in area for HC was marginal but at a speed of 3.14 mm/sec it doubled. Increasing the speed further to 4.17 did not increase the response of this compound. The effect of increasing the scan speed was similar to that observed with untreated rods. However, the change was more uniform than that noted with untreated rods. As with untreated rods, PC gave the highest response at a speed of 3.14 mm/sec, so that this speed appears to be the most suitable for the analysis of phospholipids. For other compounds a speed of 4.17 was ideal. It can be seen that with the exceptions already noted, all the lipids gave uniform responses on treated rods at a scan speed of 4.17 mm/sec. Hence, one could dispense with response factors altogether under this set of conditions. The standard deviation was well below 10% for most of the compounds analysed. The peak areas were measured manually and could probably be improved by use of an electronic integrator.

The utility of the method was verified by analysing synthetic mixtures of lipids. Table II gives the results for the analysis of five such mixtures, where correction factors were applied only to the peak areas for PC and CHOL. These were calculated from the values given in Table I as the area for 10 μg of HC divided by the area for 10 μg of the compound being analysed. The results agreed well. SE and WE, which have high R_F values, gave broad-based peaks resulting in smaller areas. This was due to band broadening, common to all forms of chromatography.

A reduction in response was observed at the next higher scan speed available with the instrument (5.45 mm/sec, by using speed gear No. 55). The values could probably be further improved by more precise selection of the scan speed, that is, if more speed gears in the vicinity of 40 were available or, as suggested by Ackman[12], if the gear drive is replaced with a stepping motor.

How copper(II) sulphate helps in optimizing the response of compounds that which are structurally dissimilar is not clear. Obviously, copper(II) sulphate helps more uniform ionization of the sample which might partly be lost on the untreated rods by simple volatilization/pyrolysis. During the preparation of copper(II) sulphate-treated rods, it was observed that at a scan speed of 4.17 mm/sec, the one most frequently used, the heat absorbed by the rod from the flame was not uniform, with the result that the top portion of the rods (in relation to the flame) was not charred properly. It is therefore reasonable to assume that the sample present on this part of the rod is not completely volatilized and ionized during the usual run on untreated rods. However, a re-run with the rods, even at high sensitivity settings, did

TABLE II

QUANTITATIVE ANALYSIS OF SYNTHETIC MIXTURES ON COPPER(II) SULPHATE-TREATED CHROMARODS

Abbreviations as in Table I. Experimental conditions: sample amount 2–5 μg per component; solvent, *n*-hexane–chloroform (84:11), the latter containing 5% of isopropanol and 0.5% of formic acid. Iatroscan operating conditions: hydrogen flow-rate, 160 ml/min; air flow-rate, 2000 ml/min; scan speed, 4.17 mm/sec (speed gear No. 40); recorder sensitivity, 100 mV; chart speed, 12 cm/sec. Correction factors were applied to the area for CHOL (1.73) and PC (1.10). These were computed as area for 10 μg of HC/area for 10 μg of compound using the values given in Table I.

Mixture		*Composition (wt.-%)*				
		HC	*ME*	*F Alc*		*PC*
1A	Actual	16.1	32.8	25.6		25.5
	Exptl.					
	1	16.4	34.9	23.5		25.2
	2	15.7	34.5	24.9		24.9
	3	15.6	36.3	23.6		24.5
	4	15.8	34.1	25.8		24.3
	5	16.6	33.9	24.9		24.6
	Mean	16.0	34.7	24.5		24.7
	Standard deviation	0.40	0.53	0.86		0.31
1B	Actual	10.2	41.4	32.3		16.1
	Exptl.					
	1	10.3	43.8	31.8		14.1
	2	10.4	43.0	32.1		14.5
	3	10.0	43.0	31.6		15.4
	4	10.3	42.4	31.6		15.7
	Mean	10.2	43.0	31.8		14.9
	Standard deviation	0.16	0.45	0.20		0.65
		WE	*ME*	*TAG*	*DAG*	*MAG*
2A	Actual	17.5	28.6	23.5	15.5	14.9
	Exptl.					
	1	14.2	27.2	23.4	17.5	17.7
	2	14.5	28.2	23.9	16.3	17.1
	3	14.5	29.1	22.9	16.4	17.1
	4	13.4	29.2	23.8	15.7	17.9
	Mean	14.1	28.4	23.5	16.5	17.4
	Standard deviation	0.45	0.80	0.39	0.65	0.36
2B	Actual	12.6	20.6	33.8	22.3	10.7
	Exptl.					
	1	9.7	20.7	35.4	24.3	9.9
	2	9.6	19.5	35.6	24.5	10.9
	3	9.2	21.8	34.3	23.9	10.8
	4	10.0	20.1	36.2	22.6	11.1
	5	10.3	19.4	36.9	23.0	10.4
	Mean	9.8	20.3	35.7	23.7	10.6
	Standard deviation	0.35	0.60	0.54	0.71	0.43

TABLE II (*continued*)

Mixture		*Composition (wt.-%)*			
		SE	*TAG*	*FA*	*CHOL*
3	Actual	19.4	30.0	14.1	36.5
	Exptl.				
	1	17.2	32.7	15.4	34.7
	2	17.2	32.4	15.0	35.4
	3	15.7	32.4	15.4	36.5
	4	16.7	33.4	16.8	33.1
	5	15.7	33.2	16.0	35.1
	Mean	16.5	32.8	15.7	35.0
	Standard deviation	0.67	0.47	0.62	1.10

not show residual material on the rods except at high sample loadings. This means that some of the sample escapes detection owing to delayed volatilization, as the rod has moved away and the ionized sample is not within each of the detector. When the untreated rod enters the flame it burns with a faint bluish green flame, whereas the treated rod becomes red hot and burns with a uniform glow (visible more clearly at lower scan speeds). Obviously, heat transfer to the treated rod is much more rapid and uniform.

Some years ago, a flame-ionization phenomenon was reported[20] in which organometallic compounds produced an ionization response three to five orders of magnitude greater than that observed for other organic compounds. This flame was later developed[21,22] into a selective and sensitive GC detector and the enhanced metal response with this hydrogen atmosphere flame-ionization detection (HAFID) was attributed to a charge-transfer process within the flame[23] with the negatively polarized collector electrode. Some similar, related process might be operating in the FID system of the Iatroscan with the treated rods. The copper could react with the organic compound in some way at the high temperature of the flame, effectively becoming an organometallic compound, giving an increased response. If this happens, other structural features of organic compounds would become secondary and all would give uniform responses irrespective of their structural dissimilarities. Among other things, the HAFID flame is doped with a small amount of silane. In the Iatroscan the whole operation takes place on the silica gel and so a comparison of FID on copper(II) sulphate-treated silica gel rods with HAFID may not be unreasonable.

Certain structural features appear to be necessary for this reaction with copper. For example, cholesterol is insensitive to copper(II) sulphate treatment whereas steryl esters respond. Carbonyl groups, in general, appear to favour the reaction. From Table I, it can be seen that the scan speeds of 2.13 and 3.14 mm/sec, the increase in response for stearic acid on treated rods was three-fold. A similar increase occurred with TAG. The presence of three such groups in TAG may be responsible for the increased response of this compound compared with WE or ME at lower scan speeds. With PC, this effect seems to be nullified by the presence of the phosphorus atom. As a better response was obtained for this compound at lower scan speeds, volatility may take precedence over any other factor for this highly polar compound. When

analysing PC it was observed that the point where the sample was spotted (there was no migration in the solvent system used) turned white after combustion. Obviously the copper content at this point is considerably reduced after combustion.

The FID response to organic compounds in a gas chromatograph has been thoroughly investigated[24–26]. The FID system in the Iatroscan can not be expected to function identically with that in a GC. In GC, the sample is already in the gaseous state at a high temperature and is presented to the detector at a relatively much slower rate (compare the chart speed of 1 cm/min used in GC with the 12–24 cm/min speed used with the Iatroscan), so that quantitative ionization and hence quantitative detection are possible. On the Chromarod the sample is in the solid or liquid state and at ambient temperature until it enters the flame. This is burnt all at once and the signal comes as a very sharp pulse. Simple breakdown of the sample can compete with ionization, with the result that the number of ions collected is not necessarily proportional to the amount of sample analysed.

The copper(II) sulphate treatment does not alter the basic chromatographic properties of the Chromarods. The R_F values of all the eleven classes of lipids studied were the same on both untreated and treated Chromarods. In fact, the treatment remedies some of the minor defects of untreated Chromarods; thus, better baseline stability and the slight tailing observed for polar compounds such as PC an F Alc (in the solvent system described) on the untreated rods was absent on the treated rods. However, tailing of FA was present and a small amount of formic acid was necessary to correct for this.

One major practical advantage of the treated Chromarods lies in the uniformity of the properties of such rods. Rod to rod variation is a problem faced by all workers with untreated Chromarods, so that a time-consuming, initial grouping of rods prior to analysis is necessary. We have not encountered a single instance where this was necessary with treated rods. The migration characteristics are remarkably consistent when developed in a chamber lined with filter-paper on three sides. Copper(II) sulphate treatment also overcomes another often reported difficulty. On an untreated rod the solvent front is rarely visible except at the beginning of development. Hence development by time rather than by distance is practised by most workers. On treated rods, owing to their brown colour, the solvent front is clearly visible, which is of practical utility. The normal cleaning process (we use overnight immersion in concentrated sulphuric acid) removes copper(II) sulphate from the rods.

A set of rods were used on average for 25–30 scans. Usability beyond this range was not tested. After every ten scans a mixture of wax ester and methyl ester was separated to test the rods. At the end of the day the rods were stored in a humidified atmosphere in a desiccator. Immersion in distilled water works well for untreated rods, but for treated rods this treatment reduces the copper content of the rods (as observed by reduced peak areas) after a few immersions. When a 10% solution of copper(II) sulphate was used for impregnation, the results were less reproducible. Concentrations less than 5% were not tested. Salts of tin may be suitable alternatives for copper(II) sulphate.

The linear range was also not affected by the copper(II) sulphate treatment. Fig. 1 shows a plot of peak area *versus* amount of sample spotted. Some divergence was noted with sample amounts exceeding 7 μg. The optimum range appears to be 2–5 μg per component.

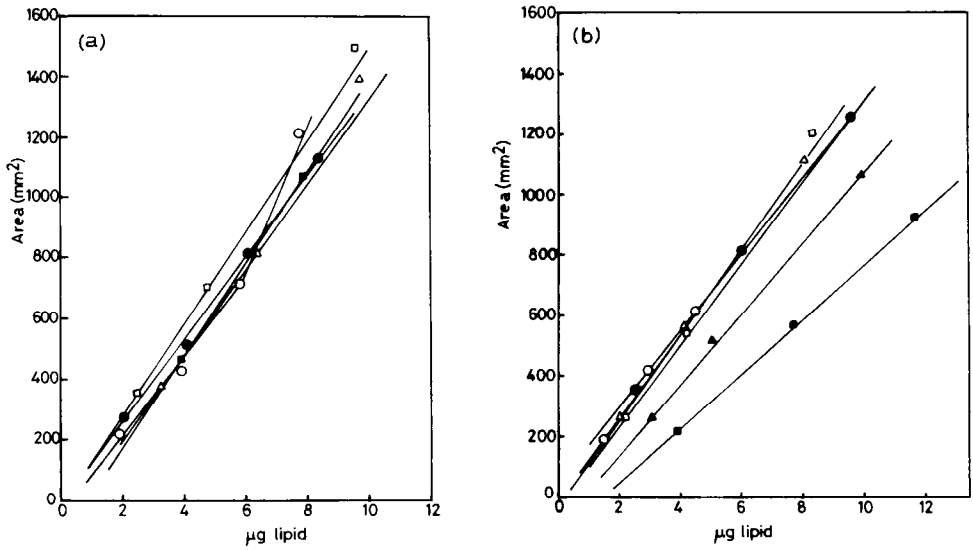

Fig. 1. Plot of area (mm^2) *versus* amount of lipid loaded on Chromarod S-II impregnated with 5% copper(II) sulphate solution. The figure is divided into two to avoid severe overlap of the points. (a) ○, HC; ●, SE; □, WE; ■, ME; △, TAG. (b) ○, FA; ●, F Alc; □, DAG; ■, CHOL; △, MAG; ▲, PC.

The Chromarod–FID system has many advantages. The whole operation is very simple and rapid. Various lipids can be quantitated in a single determination. A maximum of ten samples can be handled simultaneously. Compounds not amenable to GC can be conveniently analysed. As applied to lipids, this may also be achieved by high-performance liquid chromatography, but not without recourse to complicated gradient elution systems. Most of the irregularities associated with the quantitative aspects of "Iatro-scanning" can be corrected by treatment of the rods with copper(II) sulphate so that reliable and reproducible data can be generated.

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