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A NEW FLUORIMETRIC PROCEDURE FOR THE DETECTION AND QUANTITATION OF ORGANIC COMPOUNDS IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A new procedure for the detection and quantitation of organic compounds in thin-layer chromatography is described in which the formation of fluorescent derivatives is induced by thermal treatment of the chromatoplates in the presence of ammonium hydrogen carbonate, and the fluorophores formed are quantitated on the plate by spectrofluorimetry. By using the detecting agent in the vapor phase, a complete, uniform reaction and a regular background are obtained. This procedure offers the advantages of convenience, sensitivity, versatility, stability of the fluorophores and of using an inexpensive, non-toxic reagent that is easy to handle.

INTRODUCTION

The quantitative evaluation of compounds separated by thin-layer chromatography (TLC) can be accomplished by a variety of procedures either directly on the plate or on material extracted from the adsorbent. The latter procedure usually requires relatively larger amounts of compound and is rather cumbersome.

Besides visual observation and measurement of the areas of spots, several other techniques, including transmittance, reflectance, fluorescence and fluorescence quenching, can be used for the *in situ* evaluation. All but fluorescence are indirect methods in which the background of the plate determines the sensitivity and accuracy of the procedure. Light scattering, spot deformation, light attenuation, etc., are frequently encountered.

Regardless of the method used, it is necessary in most instances to form an adequate derivative before or after separation on the chromatoplate. The conditions for forming the derivatives on the chromatoplate are far from ideal, as it is difficult to spray the reagent evenly over the plate. The unevenness results in a non-uniform background and a poorly reproducible process. Moreover, the reaction takes place in a dry or nearly dry state because of immobilization of the compound.

We have attempted to overcome some of the drawbacks of quantitative TLC by applying the reagent in vapor form. We have found that heating the chromatoplates in the presence of ammonium hydrogen carbonate allows the formation of

fluorescent derivatives from many kinds of organic compounds. Besides its usefulness for the detection of such compounds, this procedure can be used for subsequent spectrofluorimetric quantitation of the chromatograms. This system obviates the limitations of spraying the reagent into the chromatoplate and a complete, uniform reaction (and background) is obtained, permitting a more accurate quantitation to be performed.

EXPERIMENTAL

Compounds and reagents

More than 60 different organic compounds have been studied. Adequate standard solutions were made with the substances obtained from the suppliers and were kept refrigerated.

All solvents used in the chromatographic separation were of analytical or spectroquality grade. Ammonium hydrogen carbonate powder was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Nitrogen and carbon dioxide were obtained from Big Three Industries (Houston, Texas, U.S.A.).

Adsorbents

The following adsorbents (TLC grade) were used: silica gel (Woelm, Eschwege, G.F.R., and E. Merck, Darmstadt, G.F.R.); alumina in three different varieties (acidic, neutral and basic) (Woelm); Florisil (Floridin Co., Tallahassee, Fla., U.S.A.); Kieselguhr G (Merck); cellulose powder MN 300 (Macherey, Nagel & Co., Düren, G.F.R.); polyamide powder (Woelm).

Preparation of the chromatoplates

The adsorbent was suspended in ethanol and was distributed over the glass plates with a spreader (Desaga, Heidelberg, G.F.R.) at a thickness adjusted to 250 μm. After air drying, the chromatoplates were scored into twelve lanes, each of which was 10 mm wide, the lanes being 5 mm apart. In an attempt to remove any contaminants present, the chromatoplates were "washed" with methanol by continuous development for at least 6 h. Prior to their use, the plates were activated at 120° for 60 min (except in the case of cellulose and polyamide powder).

Application of the samples

All compounds studied in a particular series were applied in the same volume (usually in 20-μl samples). The samples were applied with a 25-μl Hamilton microsyringe equipped with a repeating dispenser (Hamilton, Reno, Nev., U.S.A.). Each sample was applied as a band of closely spaced dots ($N = 6$), 10 mm wide and perpendicular to the long axis of the twelve lanes described above.

When a suitable solvent system was available for the separation of all of the components present in a particular series, the compounds were allowed to migrate through the chromatoplate. A heterogeneous mixture of lipids, resembling the normal plasma lipid profile, was separated on silica gel by development first with methanol up to 3 cm from the origin and, after drying, with the solvent system trimethylpentane–diethyl ether–glacial acetic acid (75:25:2, by vol.). Cholesteryl esters were fractionated into individual components by continuous development with carbon

disulfide on silica gel. Steroid hormones were separated on silica gel chromatoplates using dichloromethane–acetone (90:10, by vol.) as a solvent system. Serial dilutions of epinephrine were prepared and 20-μl samples were applied to the chromatoplates.

Detection

The chromatoplates were transferred to a plate rack and placed inside a sealed tank of 4-gal capacity containing 6 g of ammonium hydrogen carbonate in the bottom. Alternatively, ammonium hydroxide was used instead of the ammonium hydrogen carbonate reagent. In a few experiments, the effect of heating alone and in the presence of nitrogen and carbon dioxide was also studied.

The tank thus prepared was placed inside an oven and heated to temperatures ranging between 110° and 150° for a variable period of time (from 2 to 12 h), according to the type of adsorbent and the nature of the compounds to be determined. Thereafter, the chromatoplates, already containing the fluorescent derivatives, were removed from the reaction chamber and protected from light and dust until their evaluation.

Fluorescence measurements

The fluorescent products were analyzed and measured *in situ* with a Model MK-1 spectrofluorimeter using a stabilized xenon lamp and an attachment for TLC scanning (Farrand Optical Co., New York, N.Y., U.S.A.). The scanning was carried out in the direction of the chromatographic development, along the longer axis of the score lanes, at a speed of 20 mm/min with a slit 14 mm long and 1 mm wide. Usually, the instrument setting was at the 1 range with a gain between 40 and 100%.

A strip-chart recorder was used to register the deflections observed. Peak areas were determined by planimetry and/or triangulation. In a selected number of cases, electronic integration was also used.

RESULTS

General characteristics

Heating in the presence of ammonium hydrogen carbonate induces the formation of fluorescent derivatives from most organic compounds separated on chromatoplates containing inorganic adsorbents (silica gel, alumina, Florisil, Kieselguhr). Polyamide and cellulose powder layers give a strong fluorescence themselves and are therefore unsuitable for the detection of the majority of such compounds. Most commercial silica gel or alumina TLC plates cannot be used owing to the high background produced by the reaction of the organic binder used in them. Owing to the unspecific response of the organic compounds when treated with this reagent, it is necessary to purify the adsorbent and to eliminate the organic contaminants as far as possible. This is especially necessary in the case of Florisil and silica gel.

The fluorescent derivatives are readily detected by examination under UV light (365 nm), where they show a greenish blue fluorescence. Regardless of the nature of the compound, all present very similar fluorescence characteristics. Fig. 1 shows an example of the fluorescence response given by the different plasma lipid families and by a series of steroid hormones separated on silica gel and heated at 130° for 10 h in the presence of ammonium hydrogen carbonate. Fig. 2 shows a chromatogram corre-

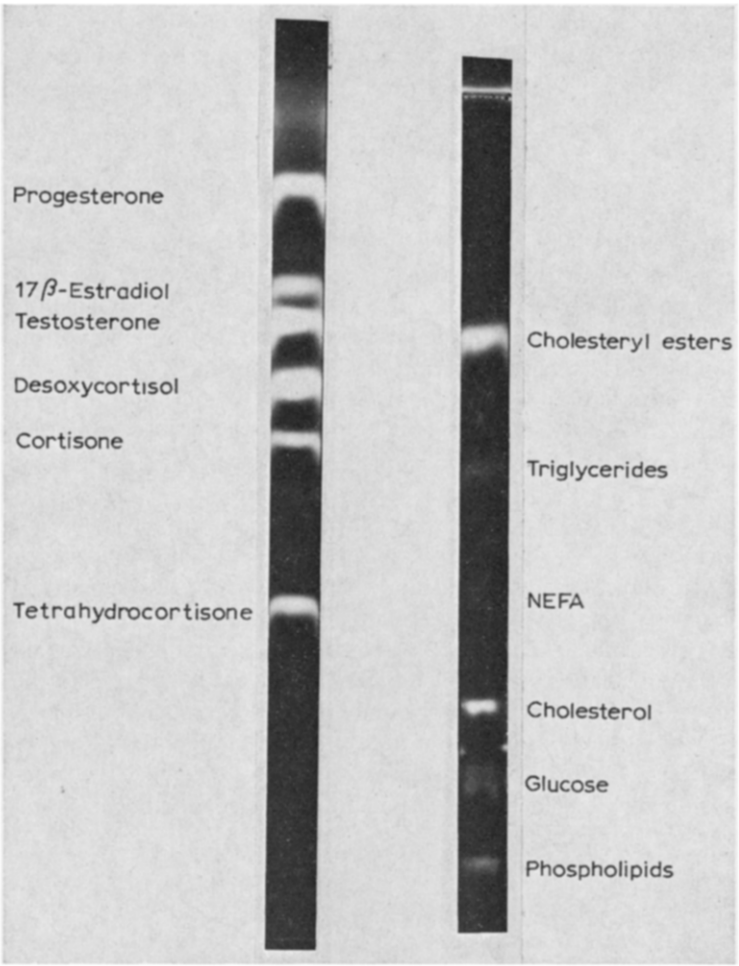


Fig. 1. Chromatogram of steroid hormones (left lane) and plasma lipid classes (right lane) separated on silica gel and viewed under ultraviolet light (365-nm lamp) after treatment with ammonium hydrogen carbonate at 130°.

sponding to the fractions obtained in the analysis of an isopropanol extract corresponding to 2 μl of human plasma.

To elicit the formation of the corresponding fluorescent derivative, each compound requires a particular time and temperature. Sugars react very rapidly and at relatively low temperatures; on silica gel or alumina plates, they show strong fluorescence after heating for 1 h at 100°. On the other hand, amino acids and purine and pyrimidine bases require heating at higher temperatures and for longer periods of time. In the analysis of plasma lipids, heating at 110° for 1 h induces fluorescence in the bands corresponding to the cholesterol and cholesteryl esters but produces a negligible response in the other lipid classes. On the other hand, heating at 150–160° for 10 h produces a very strong response for triolein but induces quenching of the

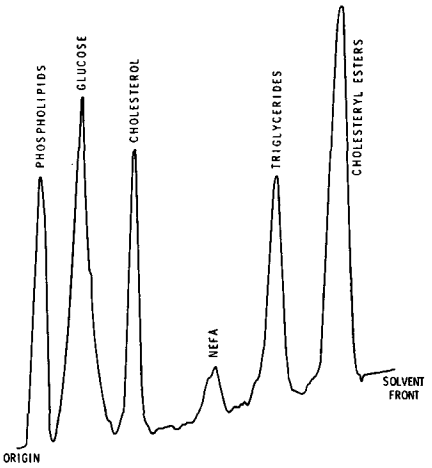


Fig. 2. Chromatogram corresponding to plasma lipids separated on silica gel chromatoplates and rendered fluorescent by treatment at 130° for 10 h in the presence of ammonium hydrogen carbonate. Sample size: 2 μl. Scanning: excitation, 380 nm; emission, 455 nm.

cholesterol and cholesteryl ester bands. For most determinations in which specimens containing between 0.5 and 5 μg of a compound are used, heating at 120–130° for 10 h gives satisfactory results. Usually, the fluorescence reaction is carried out overnight under the control of an electric timer.

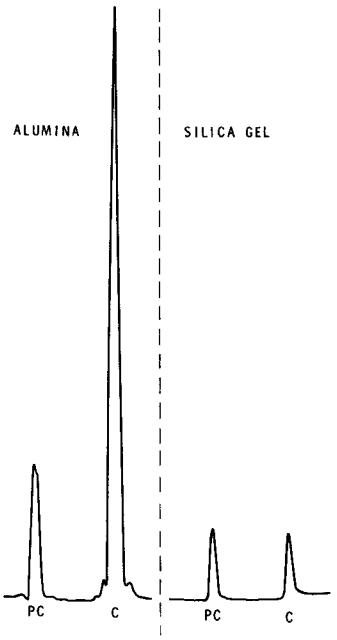
The fluorophores obtained are stable for many weeks and do not appear to be affected to a significant extent by moisture, air, etc.

Effect of adsorbent

Alumina appears to be more effective than silica gel in inducing the formation of fluorescent derivatives when the chromatoplates are heated in the presence of ammonium hydrogen carbonate. Florisil and Kieselguhr follow in decreasing order of activity. The response obtained for identical amounts of phosphatidylcholine and cholesterol (1 μg) is larger on alumina than on the silica gel plates (Fig. 3); in this experiment, each chromatoplate was measured under the same setting.

In general, alumina produces a greater differential response among the different types of compounds than does silica gel. Both cholesterol and phosphatidylcholine exhibit a stronger response on the alumina than on the silica gel plate owing to the higher efficiency of the former adsorbent, but, at the same time, it is evident that cholesterol shows a much stronger response than does the phospholipid on alumina (Fig. 3). On the other hand, both compounds show very similar responses when analyzed on a silica gel chromatoplate.

The relative responses obtained within each series of compounds according to the type of adsorbent used are given in Table I. On silica gel, there is in general a more uniform type of response within each set than there is on alumina. An exception is the case of sucrose, which on silica gel plates gives a lower response than glucose and ribose but shows an intensity similar to theirs when reacted on an alumina chromatoplate.



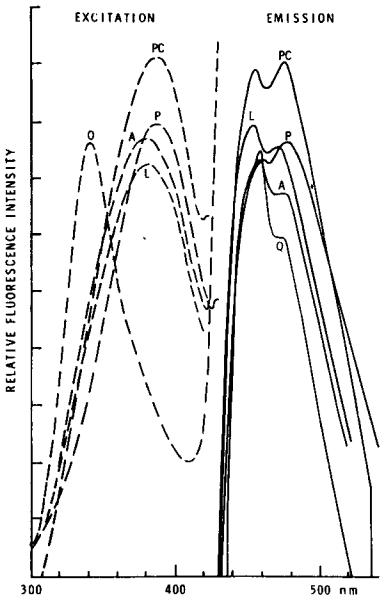


Fig. 3. Peak responses of 1-μg amounts of phosphatidylcholine and cholesterol separated on alumina and on silica gel, after treatment at 130° for 10 h in the presence of ammonium hydrogen carbonate. Both chromatoplates were measured consecutively with the spectrofluorimeter at identical settings ($\times$1 and 40% gain).

Fig. 4. *In situ* excitation and emission spectra (uncorrected) of some compounds on silica gel chromatoplates treated at 130° for 10 h in the presence of ammonium hydrogen carbonate. A = Aspartic acid; L = lactose; P = progesterone; PC = phosphatidylcholine; Q = quinine.

TABLE I

RELATIVE RESPONSE OF IDENTICAL AMOUNTS OF COMPOUND ACCORDING TO TYPE OF ADSORBENT

The measurements were made under the same spectrofluorimeter setting for each compound and each adsorbent. No comparison can thus be established between different sets or between the response for the same compound according to type of adsorbent.

Compound	*Peak area (mm²)*		*Compound*	*Peak area (mm²)*	
	Silica gel	*Alumina*		*Silica gel*	*Alumina*
Lipids (1 μg)			*Sugars (1 μg)*		
Cholesteryl linoleate	300	480	Ribose	1190	463
Oleic acid	330	50	Glucose	870	516
Cholesterol	197	944	Sucrose	200	487
Phosphatidylcholine	229	190			
Steroids (1 μg)			*Amino acids (0.1 μM)*		
Cortisone	650	600	Glycine	300	160
Tetrahydrocortisone	580	120	Phenylalanine	900	80
Testosterone	710	630	Tyrosine	520	260
Androsterone	90	160			

Characteristics of the fluorescent derivatives

Regardless of the type of compound applied, the *in situ* fluorescence characteristics of the corresponding derivatives are very similar, all showing an excitation maximum around 380 nm and emission maximum at 455 and/or 475 nm (Fig. 4). Quinine is the only exception in that it presents an excitation maximum in the 340-nm region. The excitation and emission maxima of 53 compounds are given in Table II. Each compound was applied to a silica gel chromatoplate and heated at 130° for 10 h in the presence of ammonium hydrogen carbonate.

Effect of heating alone and in the presence of nitrogen

Heating alone induces the formation of fluorescent derivatives in some of the compounds applied on thin layers of inorganic adsorbents. However, the yield is lower, in general, than that obtained for the same compounds when treated in the presence of ammonium hydrogen carbonate. The fluorescent characteristics of 30 organic compounds applied on a silica gel plate and kept at 130° for 10 h are listed in Table II.

Heating in the presence of nitrogen produces an effect qualitatively very similar to that obtained when heating with ammonium hydrogen carbonate. In a selection of 30 different compounds, the excitation and emission maxima are very similar to those obtained after ammonium hydrogen carbonate treatment (Table II). The only difference appears to be the presence of more shoulders around the 445–450-nm region in a certain number of compounds treated with nitrogen.

TABLE II

EXCITATION AND FLUORESCENCE MAXIMA OF SEVERAL ORGANIC COMPOUNDS HEATED AT 130° FOR 10 h ON SILICA GEL PLATES IN THE PRESENCE OF DIFFERENT REAGENTS

Figures in parentheses indicate excitation maxima that are not well defined and correspond to the wavelength used in the determination of the emission spectrum. The figures in bold type correspond to the peak of maximum intensity. The figures in italic type indicate a shoulder or plateau.

No.	*Compound*	NH_4HCO_3		N_2		*Heat alone*	
		Excitation (nm)	*Emission (nm)*	*Excitation (nm)*	*Emission (nm)*	*Excitation (nm)*	*Emission (nm)*
	Lipids						
1	Cholesterol	390	**455** 475				
2	Cholesteryl linoleate	380	**450** 470	385	**456** 475	385	456 **473**
3	Elaidic acid	383	455 *475*	(380)	**454** 470	395	458 **477**
4	Triolein	380	455 *475*	385	**453** 472	380	**455** 473
5	Phosphatidylcholine	390	457 **475**	400	**450** 468	380	450 *470*
	Steroids						
6	Progesterone	388	460 479				
7	Cortisone	386	458 **478**	375	455 472	380	455 475
8	Tetrahydrocortisone	385	458 *475*	360	455 470	380	455 *475*
9	Testosterone	386	*460* 478				
10	Testosterone glucuronide	396	458 **476**				
11	Δ^4-Androstene-3,17-dione	398	460 **478**				
12	Androsterone	380	**456** 475	(380)	**455** 472	380	455 *472*

(Continued on p. 650)

TABLE II *(continued)*

No.	Compound	NH_4HCO_3		N_2		Heat alone	
		Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)	Excitation (nm)	Emission (nm)
13	17β-Estradiol	375	457 *475*	358	455 **470**	365	458 475
14	Stigmasterol	394	*460* 480	385	*455* 475	385	460 **475**
15	Lanosterol	380	455 **475**				
16	Deoxycholic acid	382	**458** 475	(375)	**453** 470	380	455 *472*
17	Chenodeoxycholic acid	(385)	**455** 470	(385)	**457** 474	380	**457** 474
18	Cholecalciferol (vitamin D_3)	390	*457* 476				
19	Hecogenin	362	**435** 450				
	Sugars						
20	Ribose	385	**456** 474	385	**455** 472	(390)	460 **478**
21	Glucose	385	**454** 472	385	454 **472**	385	**454** 470
22	Fructose	380	**454** 472				
23	Sucrose	380	**458** 474	380	**454** 472	380	**458** 475
24	Lactose	380	**454** 472				
25	Glycerol	385	**456** 474				
	Amino acids						
26	Glycine	370	445 *470*				
27	Serine	380	450 *470*				
28	Valine	385	456 *475*	(375)	458 475	380	455 *470*
29	Glutamic acid	(385)	**458** 475	(375)	459 475	385	455 *470*
30	Aspartic acid	380	450 *470*				
31	Arginine	380	458 *475*	375	459 *475*	385	458 *470*
32	Phenylalanine	380	452 *470*	375	**459** 475	375	455 *470*
33	Tyrosine	382	458 **475**				
34	Tryptophan	382	454 *472*	306/380	457 *475*	310/385	450 *470*
35	Methionine	385	452 *470*				
	Amino acid derivatives						
36	Iodotyrosine	377	459 474	(375)	**456** 473	(385)	**454** 470
37	Diiodotyrosine	(390)	**458** 475	(375)	**455** 472	(385)	**457** 474
38	Triiodothyronine	(385)	**458** 476	(375)	**457** 474	(385)	**458** 475
39	Epinephrine	380	456 474	(375)	**454** 470	380	**455** 475
40	Metanephrine	385	458 476	(375)	455 **473**	(395)	455 **470**
41	5-Hydroxyindolacetic acid	(385)	**458** 476	(375)	**455** 473		
42	D-Leucylglycine	385	455 *475*				
	Purine and pyrimidine derivatives						
43	Cytosine	395	450 *475*	(380)	**457** 472	390	450 *470*
44	Cytidine	380	450 *475*	(385)	**456** 472	380	455 *472*
45	Thymidine	380	456 474				
46	Deoxyadenosine	390	456 *474*				
47	AMP	385	450 *477*	(380)	**457** 472	385	**458** 475
48	Xanthine	(380)	**455** 472				
	Miscellaneous						
49	Morphine	370	**458** 476	355	**457** 475	390	**455** 472
50	Codein	380	**456** 475				
51	Propoxyphene·HCl (Darvon)	(380)	458 475				
52	Pentazocine (Talwin)	370	**445** 472	355	**455** 472	385	457 **473**
53	Quinine	342	**458** 475				

Heating in the presence of carbon dioxide also produces fluorophores which, on visual observation, exhibit characteristics very similar to those obtained with ammonium hydrogen carbonate.

Treatment with ammonium hydroxide also induces the formation of fluorescent derivatives but the yield appears to be lower and the background higher than those obtained after ammonium hydrogen carbonate treatment.

Quantitative measurements

For all compounds tested, there appears to be a linear relationship between the square root of the peak area and the logarithm of the corresponding amount of compound. This linear response extends over a fairly broad range of concentrations.

Lipids. The calibration curves for phosphatidylcholine, oleic acid, triolein, cholesterol and cholesteryl linoleate are given in Fig. 5. Heating at 130° for 10 h in the presence of ammonium hydrogen carbonate gives a linear response between 0.5 and 80–100 μg. At higher concentrations, quenching begins to appear, particularly with cholesterol and cholesteryl linoleate; a lower temperature or a shorter period of treatment is required.

Within the cholesteryl ester class, the degree of unsaturation of the fatty acid does not significantly affect the intensity of the response. Cholesteryl stearate, oleate and linoleate give practically the same response for each concentration applied and show identical calibration curves (Fig. 6). However, this is not the case for triglycerides or non-esterified fatty acids, for which the fluorescence intensity given by the unsaturated fatty acids is higher than that of the corresponding saturated homologues.

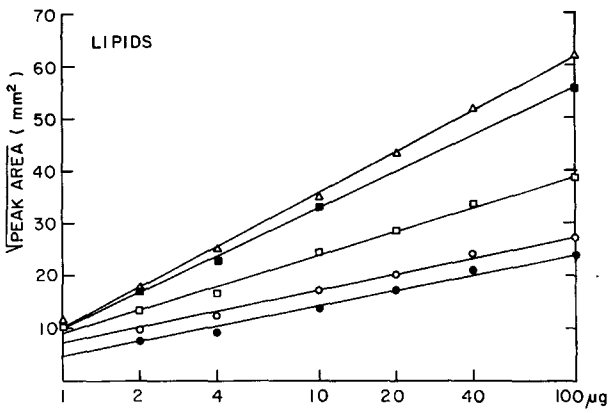


Fig. 5. Calibration curves for different types of lipids separated on silica gel–alumina (2:1) and treated at 130° for 10 h in the presence of ammonium hydrogen carbonate. All samples were measured under identical spectrofluorimeter settings: excitation, 380 nm; emission, 455 nm; range, ×1 (50% gain). △—△, Cholesteryl linoleate; ■—■, oleic acid; □—□, cholesterol; ○—○, phosphatidylcholine; ●—●, triolein.

Steroids. The steroid hormones also give a linear relationship between the logarithm of concentration and square root of the corresponding peak areas. The calibration curves for a series of corticosteroids separated on silica gel chromatoplates are shown in Fig. 7.

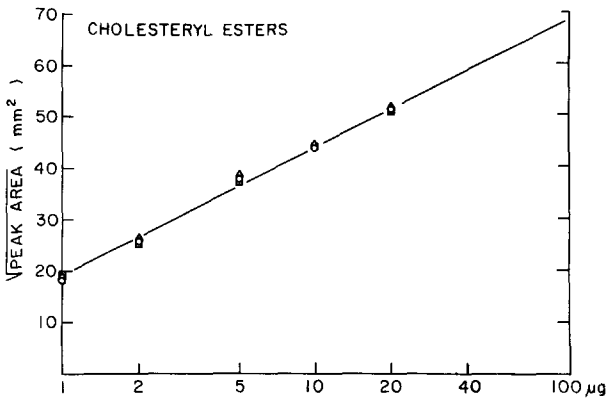


Fig. 6. Response obtained with three types of cholesteryl esters differing only in the degree of unsaturation of the acyl chain. Separation: continuous development with carbon disulfide. Detection: heating at 130° for 10 h in the presence of ammonium hydrogen carbonate. Measurement: excitation, 380 nm; emission, 455 nm; range, ×1 (90% gain). □—□, Cholesteryl stearate; ○—○, cholesteryl oleate; △—△, cholesteryl linoleate.

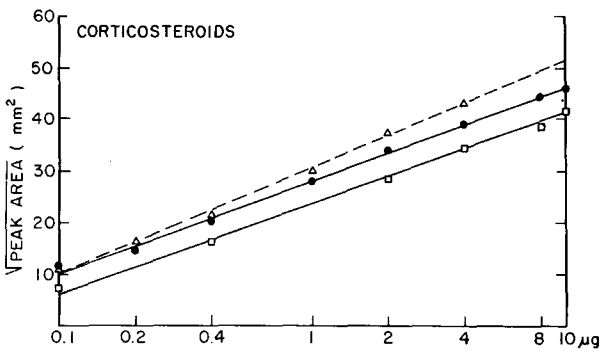


Fig. 7. Calibration curves for steroid hormones separated on silica gel and treated at 130° for 10 h in the presence of ammonium hydrogen carbonate. Spectrofluorimetric measurement: excitation, 380 nm; emission, 475 nm; range, ×0.3 (50% gain). △---△, Progesterone; ●—●, cortisone; □—□, tetrahydrocortisone.

Catecholamines. Both epinephrine and norepinephrine show very strong fluorescence after treatment in the presence of ammonium hydrogen carbonate (Figs. 8 and 9).

Sensitivity

For lipids, steroids and sugars, 0.1 µg of a compound can be easily detected. Catecholamines exhibit a much stronger response and can be detected and quantitated at the low nanogram level.

Analysis of the fluorophores

The fluorophores produced in the reaction are not totally extracted from the adsorbent by any of the organic solvents employed. After extraction with chloroform,

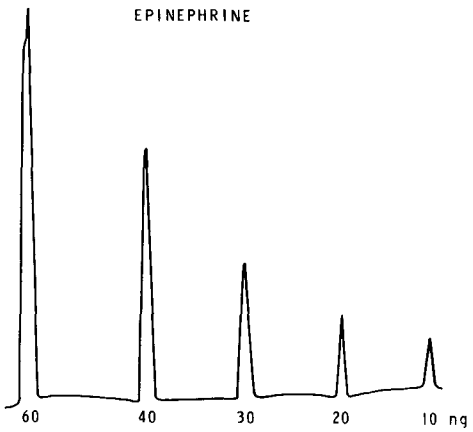


Fig. 8. Peak responses of nanogram amounts of epinephrine on alumina chromatoplates. Detection: heating at 130° for 10 h in the presence of ammonium hydrogen carbonate. Spectrofluorimetric measurement: excitation, 380 nm; emission, 475 nm; range, ×0.3 (90% gain).

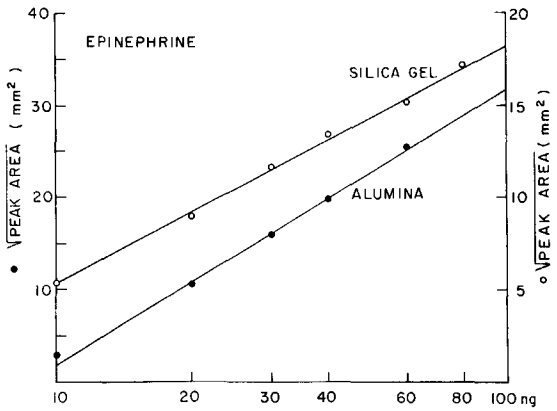


Fig. 9. Calibration curves for epinephrine on two different types of adsorbent. Conditions as in Fig. 8.

the fluorophores corresponding to galactose and corticosterone treated with ammonium hydrogen carbonate on silicagel plates show an excitation maximum at 380 nm and an emission maximum at 440 nm. The fluorophores corresponding to the same compounds treated by heating alone show similar excitation and emission maxima.

After methanol extraction, the fluorophores can be fractionated into at least four different bands by development with dichloromethane on silica gel chromatoplates.

Preliminary data from infrared spectra indicate that these types of fluorophores present bands in the 1050–1150, 1600, 1700–1750 and 3680 cm^{-1} regions.

DISCUSSION

TLC is a very convenient, simple, sensitive and inexpensive procedure for the qualitative analysis of organic and inorganic compounds. However, the quantitation of the separated components is far from being adequate. Quantitation by TLC can be carried out either after elution of the compounds from the plate and subsequent

use of conventional chemical methods, or directly on the plate using spot area measurements, photodensitometry or, less commonly, fluorescence.

For elution techniques, the substance must be completely extracted from the adsorbent or at least a known and reproducible fraction must be recovered[1]. Furthermore, the substance must be obtained in the pure state. These criteria are not met in most methods. In our experience, when working with less than microgram amounts of compounds, the recovery is usually very low and variable. In general, the percentage of loss increases with the reduction in sample size. These phenomena have not been satisfactorily explained[2].

Of the direct methods, the qualitative densitometric determination of the chromatoplates has certain limitations. In general, there is a limited range in which the relationship between the densitometric peak area and the amount of substance in a chromatographic spot is linear. The correct centering of the spot against the scanning slit is difficult and, in addition, the moisture content of the adsorbent can vary significantly and appreciably affect the precision of the measurement[3].

Transmission, reflection and fluorescence quenching are indirect methods in which the background of the plate determines the sensitivity of the procedure. In fluorescence measurements, where the background should ideally appear dark to the instrument, most of the sources of error that occur in the indirect methods (ambiguous relationship between sample concentration and light attenuation, spot deformation, light scattering, etc.[4]) are eliminated.

Measurements utilizing fluorescence are carried out by determining the intensity of light of a wavelength different to that of the illuminating beam. Fluorescent techniques are more sensitive than photodensitometric techniques owing basically to a reduction in background noise[5].

Regardless of the system used, it is usually necessary to convert the compounds into suitable derivatives. In the conventional procedure used for the detection and quantitation of thin-layer chromatograms, the reagent is applied in the form of spray over the plate. Often a corrosive agent such as sulfuric or chromic acid is used, with subsequent heating or charring at elevated temperature. It is difficult to spray the plate evenly, especially when viscous reagents are used, and there is no guarantee that the reagent will penetrate completely into the layer or into the dried compound. Moreover, the reaction often takes place in a dry or nearly dry state[6].

In the present work, we have attempted to overcome most of the limitations encountered in conventional photodensitometry of chromatoplates sprayed with the detecting reagent. We have developed a detection procedure by using the vapor phase to ensure a completely uniform reaction and, at the same time, to induce the formation of fluorescent derivatives with most organic compounds. This procedure combines the advantages of a direct measurement, fluorescence, and of a complete and uniform reaction all over the chromatoplate.

When examining the application of ammonium hydrogen carbonate to the detection of several organic compounds, we found that, in addition to cholesterol and cholesteryl esters, which reacted very readily, other members of the lipid group (triglycerides, fatty acids, phospholipids) could also be converted into fluorescent derivatives. On evaluation of the procedure with about 60 different organic compounds, including most of the different chemical groups and structures, we have found that ammonium hydrogen carbonate has extensive applications as a detection reagent.

Touchstone and co-workers tried to overcome the problem of spraying by impregnating the silica gel layers with phosphomolybdic acid[7], sulfuric acid[8] or ammonium hydrogen sulfate[9]. Walker[10] used ammonium sulfate incorporated into the silica gel; on heating, the thermally unstable ammonium sulfate decomposes into volatile ammonia and sulfuric acid. Impregnation of the plates with the charring reagent has several disadvantages, *e.g.* some limitation to the type of solvent system that can be used or interference with the separation of certain components.

Touchstone *et al.*[9] reported the appearance of different visible colors with a number of steroids and lipids after heating them on silica gel impregnated with ammonium hydrogen sulfate. In addition, the steroids showed fluorescence when excited with a 360-nm lamp. The lipids produced no fluorescence after heating (cholesterol showed little fluorescence). No color was produced by heating the lipids at 140°. The conditions used by Touchstone *et al.* are different from those employed in the present study to detect the same type of compounds. All lipid classes show strong fluorescence, the temperature necessary to achieve the formation of the derivative not exceeding 150° (usually around 130°). Cholesterol and cholesteryl esters show fluorescence after heating for 60 min at 110°. Steroids react very readily and show fluorescence in the ammonium hydrogen carbonate system after heating at 110° for 60–120 min. At the same time, both groups of compounds can tolerate the thermal treatment at 130° for many hours without significant quenching phenomena when applied in amounts up to 40–100 μg. Another difference in the mechanism of reaction and the type of derivative obtained is suggested by the analysis of the fluorescence characteristics in the two systems. The C_{21} steroid compounds treated with ammonium hydrogen sulfate appear to have different excitation and emission maxima, while after treatment in the presence of ammonium hydrogen carbonate all show remarkably similar excitation and emission maxima. This is rather surprising, taking into account the structural and functional differences that exist between the different groups and among the individual compounds. These differences suggest that the mechanism involved in the formation of the fluorescent derivatives in the presence of ammonium hydrogen carbonate is different from the case using ammonium hydrogen sulfate. In the latter system, the reaction is due mainly to the effect of the sulfuric acid liberated in the decomposition of the reagent and the fluorescence observed corresponds to the initial stages of the charring reaction.

Although there is no mention of the time of persistence of the fluorescent derivative in the ammonium hydrogen sulfate procedure, one would expect a relatively rapid fading of the compound formed and a strong effect of moisture on the life and characteristics of the derivative. In our procedure, the fluorescent derivatives obtained are stable for many weeks and do not appear to be significantly affected by moisture, air, etc.

Truppe and co-workers used the procedure of Touchstone and co-workers to induce fluorescence with cholesteryl esters and cholesterol[11], and, while this work was in progress, they reported its extension to triglycerides and phospholipids as well[12,13]. Their procedure involves the use of a modified silica gel layer by the incorporation of ammonium hydrogen sulfate. In our system all adsorbents remain unmodified and the established developing systems can be used. The detection agent is applied after the separation has been completed and therefore no interference in the chromatographic process occurs.

Truppe *et al.*[11] found only a limited range of linearity between the concentration and fluorescence intensity for a given period of heating. Thus, in the case of cholesterol, in order to obtain a linear response between 1 and 4 μg, it is necessary to heat the plate at 150° for 25 min; for the range between 0.2 and 1.2 μg, heating at 150° for 45 min is required and when the range extends between 0.032 and 0.192 μg, it is necessary to heat the plate for 70 min at 150° in order to obtain a linear response. The treatment with ammonium hydrogen carbonate appears to present a much broader range of linearity. For the different lipid families, a linear response between 1 and 100 μg is obtained. An important difference between the types of responses obtained in most photodensitometric procedures and the method described in this paper is that in our procedure the relationship between amount and response is not direct. Within rather narrow limits, the photodensitometric methods usually define a linear relationship between the amount of compound and the peak area. In our method, a linear relationship is established between the square root of the peak area and the logarithm of the amount of compound present. This relationship was found empirically and resembles the equation given by Purdy and Truter[14] in 1962 for the quantitative determination of compounds separated by TLC. They stated that "in thin-film chromatography, the square root of the area of a spot is a linear function of the logarithm of the weight of the material present". In our procedure, it is the square root of the peak area which is a function of both the spot area and fluorescence intensity, the parameter that bears a direct relationship to the logarithm of the amount of compound present.

Despite the versatility of the fluorimetric procedure described here, it is evident that the conditions necessary to elicit fluorescence with the different compounds are not identical. The time and temperature required depend to a certain extent on the type of organic compound and the kind of functional groups it contains and the type of adsorbent with which it interacts. Alumina appears to be the adsorbent that gives the fluorescent derivatives most rapidly and the greatest sensitivity when used in conjunction with the ammonium hydrogen carbonate reagent.

On the other hand, silica gel induces a more uniform response among the different types of compounds. Although in certain instances the sensitivity achieved is lower than in the case of alumina (owing probably to a higher fluorescent background), it appears to be a more suitable adsorbent for the determination of natural samples constituted by a mixture of heterogeneous compounds. Moreover, the layers are not affected by the treatment and the plates can be measured again if necessary many weeks (even months) later.

The yellow colour and the fluorescence characteristics of lipofucsin pigments are thought to be the result of the reaction between certain amino groups and malonaldehyde. The products obtained after thermal treatment in the presence of ammonium hydrogen carbonate show characteristics remarkably similar to those of human neuronal lipofucsin, which has an absorption peak at 375 nm and fluorescent peaks at 440–460 nm (ref. 15). Malonaldehyde is produced by many organic compounds when subjected to the action of physical agents, for example, during the γ-irradiation of amino acids such as arginine, glutamic acid, methionine and homocystine[16], glycerol[17], galactose[18] and others[19]. Malonaldehyde, and other aldehydes, can then give rise to a Schiff base type of compound. The Schiff bases derived from glycine, valine and leucine show intense blue fluorescence in aqueous solutions with an excitation maximum

at 370 nm and an emission maximum at 450 nm. The chromophoric system RN=CH–CH=CH–NH–R, which contains six π-electrons, could explain the fluorescence properties of these derivatives. Infrared spectra of these compounds contain a band of the C=N band at 1650–1655 cm^{-1} and a band of the C=C band in the 1610–1620 cm^{-1} region[20]. The similarities between several physicochemical characteristics of the products derived from the degradation, via malonaldehyde, of different types of organic compounds and by the fluorescent derivatives obtained after thermal treatment on thin-layer plates in the presence of ammonium hydrogen carbonate should be noted.

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