Charring With Sulfur Trioxide for the Improved Visualization and Quantitation of Thin Layer Chromatograms

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Abstract

Continuous and uniform exposure to an atmosphere of dry SO_3 effectively chars chromatograms with convenience, safety and speed. The sandblasted, concave surface of a heat resistant glass lid is coated with fuming sulfuric acid. The lid encloses the thin layer chromatography plate while it is heated by a hot plate. Cholesterol, oleic acid and triolein show SO_3 char and char-fluorescence detection limits much lower and carbon yields more nearly equal than those reported to be obtained with conventional spray char reagents. Liquid reagent does not touch the layer. It remains clean, dry and permanent. Freedom from excess acid lends stability to the intensity of char-fluorescence spots. Because of lower temperature requirements, SO_3 char probably can be extended to include substances which are too volatile for acid spray char. A guide to the selection of conditions for the detection, identification and quantitation of char, char-fluorescence and char-color spots is given. Additional areas of investigation with SO_3 as well as other reagent fumes are indicated.

Introduction

When destructive reagents can be tolerated, char techniques are widely accepted and used to visualize colorless components separated by thin layer chromatography (TLC). In the most common procedure, layers are sprayed with an oxidizing liquid and subsequently heated. Sulfuric, nitric and chromic acids or mixtures of these are generally used (1–11). Heating is accomplished by an oven, hot plate or open flame. During the process, most relatively nonvolatile organic substances are partially oxidized to give carbon spots which are visible against a light-colored inorganic adsorbent layer.

These techniques are highly sensitive and have great quantitative potential when combined with densitometry (3,8,12–16). Nelson and Booth (17) report detection limits of 0.5–5 μg for lipids when char spots are viewed under visible light and a fivefold increase in sensitivity when char-fluorescence spots are observed under UV light. Downing (18) increased carbon yield and reduced the influence of molecular structure and weight on conversion to carbon (8,12,14–16).

But uniform spraying of reagent to effect maximum carbon yield without damage to the chromatogram is difficult. The analyst must protect laboratory furnishings and equipment as well as himself from the aerosols of powerful oxidizing agents. Ovens, particularly door gaskets, suffer rapid deterioration. Reagent impurities or droplets of the reagent itself will discolor or mottle the charred layer. Failure to remove excess acid from charred layers causes the loss of char-fluorescence spot intensity and the degradation of layers by moisture in the air.

Attempts by others to remedy some of the difficulties and hazards associated with conventional spray char techniques have resulted in a number of modifications. At the expense of a slightly darker charred layer background, methyl orange was added to sulfuric acid spray reagent so that more uniform coverage could be assured (19). Hot layers were sprayed so that sufficient carbonizing reagent could be applied without diffusive enlargement of spots (20). Layers were modified by the incorporation of sulfuric acid in the adsorbent slurry before application to plates (21). High temperatures were used to generate sulfuric acid in layers by the thermal decomposition of ammonium sulfate applied as a noncorrosive aqueous spray (22). In 1966, Jones et al. (23) reported successful chars with sulfuric acid generated on layers by exposing them first to sulfuryl chloride or sulfur trioxide vapor and then to steam.

As early as 1963, Suryaraman and Cave (24) found that fuming sulfuric acid was superior to concentrated sulfuric acid for charring. They used a pad of glass wool to coat a sandblasted Pyrex plate with pyrosulfuric acid containing 20–23% free sulfur trioxide, lightly pressed it against the layer, and heated the two plates in an oven at a temperature of 150–180 C. In our laboratory, this technique resulted in very hygroscopic charred layers which were so badly mottled by contact with liquid reagent that carbonized sample spots could scarcely be detected. But, if pyrosulfuric acid actually was a superior char reagent, it seemed likely that free sulfur trioxide might be even better. To test this idea and to eliminate contact mottling, the sandblasted flat plate was replaced by a concave one. This simple innovation provided a new method for treating thin layer chromatograms with a variety of reagent fumes. With sulfur trioxide from pyrosulfuric acid, it has resulted in a greatly improved and much more useful char technique.

In this improved technique, the sandblasted, concave surface of a heat resistant glass lid is coated with fuming sulfuric acid. The lid encloses the TLC plate while it is heated by a hot plate in an atmosphere of free sulfur trioxide. Liquid acid does not touch the layer. Continuous and uniform exposure of the layer to dry SO_3 is assured. A wide range of selected and controlled process conditions can be used, but there are four, rather than the usual three, variables. In addition to char acid strength, time and temperature, layer thickness is introduced as a fourth variable. The object of this study was to investigate the scope and limitations of SO_3 char for the visualization and quantitation of sample components separated by TLC.

Materials

Chemicals

Merck No. 7731 Silica Gel G and Baker Analyzed 30–33% fuming sulfuric acid were used. Distilled-in-glass normal hexane, chloroform and diethyl ether were prepared by Burdick and Jackson Laboratories,

Inc., Muskegon, Michigan. The chloroform and ether were preserved with ethanol, shipped, and subsequently stored in brown bottles under nitrogen. Cholesterol, oleic acid and triolein reference standards were obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. These lipids and chloroform spotting solutions of them prepared in brown bottles with Teflon-lined screw caps were kept refrigerated in the dark except when actually in use.

Equipment

The 66 × 66 × 1 mm glass TLC plates were purchased from J. Melvin Freed, Inc., Perkasie, Pennsylvania. A local glass shop supplied the 20 cm square TLC plates in matched sets of uniform thickness. Each set was cut from a single sheet of 14 oz picture glass using a jig pattern and system of individual plate identification suggested by R.L. Squibb (private communication).

A Brinkmann standard mounting board and adjustable applicator were employed. A 43¼ × 7⅞ × 7/32 in. glass platform plate was used on the mounting board. The original 135 × 8 × 8 mm guide bar of the applicator was replaced with a 5¼ × 5/32 × 7/16 in. guide bar of polished stainless steel.

Figure 1 shows the fume-treating apparatus selected for this study. Essential parts include a hot plate, heat-conducting metal block, block cover plate, TLC plate platform and fuming reagent lid. A Corning No. PC-100, 1,320 watt electric hot plate was used. The top surface was ground flat and lapped smooth by a local machine shop. The same shop made the metal components for the isothermal hot block mounted on top. This one consists of a 10½ in. square × 1½ in. thick block of tempered aluminum assembled between 10½ in. square × ¼ in. thick aluminum plates. Specifically spaced heat leak channels were incorporated into our design of the block to remove the symmetrical temperature gradient of the hot plate. It was also equipped with a −10 to 260 C thermometer for measuring the nominal fume-treating temperature and a Fenwal No. 17100–10 thermoswitch. The thermoswitch is wired to control the hot plate heating element and indicator light. The cover plate is 12 in. square, and the platform plate is 8 in. square. Both were cut from a sheet of heat resistant glass approximately 3/16 in. thick. A Sunbeam Model No. FPM medium size electric frypan cover (mold mark J-2071) was used for the lid. The concave surface of this lid was heavily sandblasted by the machine shop.

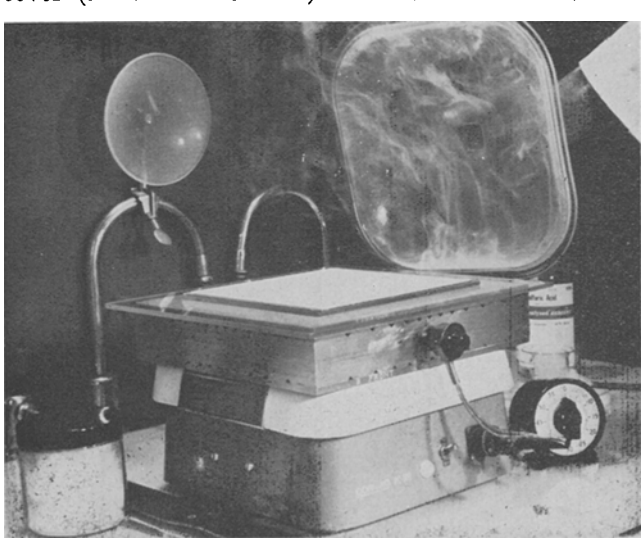

FIG. 1. Fume-treating apparatus with SO_3 char about to begin.

For densitometry, a Photovolt Automatic Scanning TLC Densitometer was modified for use. The basic instrument consisted of a Light Source Unit (Model 52-C) with TLC Stage Equipment, Multiplier-Photometer (Model 520-A), and Varicord Recorder (Model 42B). Improved guide bars were installed for the TLC Stage, the 0.5 × 4 mm collimating slit of Blank et al. (14) was used for the light source, and the standard rates of plate scan as well as chart drive were doubled.

Experimental Procedures

In one series of tests, microchromatograms containing cholesterol, oleic acid and triolein were used to compare SO_3 fume char detection limits with the acid spray char data of Nelson and Booth (17). They were also used for a preliminary study of the effect of process variables on SO_3 fume char results. For these tests, 66 mm square layers of Silica Gel G were spread according to Hofmann (25,26) with applicator gate settings of 250, 500, 750, and 1,000 μ. After spotting, all miniature layers were developed to the top with hexane, ether and acetic acid, 70:30:1 v/v, without full tank saturation.

In the first of another series of tests, full size chromatograms containing cholesterol and oleic acid were used for a preliminary comparison of SO_3 fume char carbon yields with the acid spray char data of Downing (18). They were also used to partially define the problem of char spot fade. For these tests, the matched plate methodology of Squibb (private communication) was combined with the thin plate techniques of Hofmann (25,26) to prepare uniform 250 μ layers on four 20 cm square plates in a single spread. After pre-elution, these layers were divided into 25 tracks 5 mm wide as recommended by Squibb (27 and private communication) for automatic scanning TLC densitometry. The chromatograms were developed to the top with hexane, benzene, ether and acetic acid, 25:25:50:1 v/v, after full layer-solvent vapor equilibration in a filter paper lined tank.

For all tests, activated layers were purified by multiple or flow-through pre-elution at right angles to the intended direction of development. These were edge stripped to remove impurities prior to storage in glass tanks or reactivation, or both, immediately before use. Benzene, ether and acetic acid, 50:50:2 v/v, was used for pre-elution, and 45 min at 130 C was used for reactivation. Spotting solutions were applied with disposable micropipettes. Immediately before visualization, each layer was dried to remove the developing solvent and any surface moisture.

The dried layers were charred with SO_3. This was done in a well ventilated fume hood. Microplates were positioned in the center of the platform plate. For full size plates, the apparatus was protected from drafts to prevent asymmetric temperature gradients. The bench top was covered with a sheet of plate glass, and the usual precautions for safe handling of fuming sulfuric acid (28,29) were observed.

Figure 1 shows an SO_3 char about to begin. The TLC plate is on the platform plate and the char lid is ready. A loose ball of glass wool, held by corrosion resistant metal tongs, was dipped into a 50 ml beaker containing about 15 ml of fresh acid and used to coat the sandblasted concave surface of the lid. The lid is used to enclose the layer while it is heated

by the hot plate in an atmosphere of free sulfur trioxide. At no time does the liquid acid or the glass surface upon which it is smeared touch the layer. During the process, the heat-conducting block and hot plate are protected from acid by the cover plate. The platform plate prevents liquid acid contact with the TLC plate. To terminate the process, the char lid is removed from the cover plate. The liquid seal which sometimes forms between the char lid and cover plate is broken by gentle leverage applied to one corner of the lid rim. The TLC plate is retrieved with a thin-bladed spatula. After cooling, exposed glass surfaces of the TLC plate are wiped clean. A wood cabinet is used to store the plate overnight in the dark before densitometry.

Except for limitations imposed by the lack of an electronic integrator as well as instrument and track width modifications already described, the photodensitometric techniques of Downing (18) were employed. Simple triangulation with the use of straight tangent base lines was substituted for the electronic integration of peak areas.

Results and Discussion

General Remarks

Before the present study began, preliminary tests were conducted with miniature equipment. The relatively simple, small scale fume-treating apparatus was easily constructed from items normally found in most laboratories. These experiments dispelled all doubt that free sulfur trioxide was a convenient, safe and effective reagent for charring microchromatograms. With lids made from Pyrex watch glasses, very little acid was required for each test. Even an etched or ground lid surface retained sufficient acid to insure a continuous and plentiful source of fumes. Because of the direct application of fuming acid to the char lid, a small supply of this reagent could be used repeatedly without layer contamination. Unlike the aerosols from spray char, the white smoke which is formed when SO_3 combines with atmospheric moisture was able to reveal the exact location and direction of contaminated airflow. On the other hand, the colorless, dry SO_3 and the translucence of the acid wet lid used to confine it permitted the rapid evolution of char spots to be easily observed. For investigative work, miniature TLC had already earned a permanent place in this laboratory (30–32).

The present study was primarily designed to evaluate SO_3 as a new and potentially useful reagent for charring a variety of thin layer chromatograms. To accomplish this objective, a model system of chromatograms was needed. The kind of chromatograms and procedural detail of preparing and treating them with SO_3 were of secondary importance. It was only necessary that the chromatograms be properly representative of those which are customarily prepared in this as well as other laboratories for subsequent treatment by char techniques. It never was intended that treatment with SO_3 should be associated with any specific application, particular class of compounds, or special method and condition of exposure.

Cholesterol, oleic acid and triolein were not selected because these neutral lipids present any problem to the thin layer chromatographer or because any of them exhibits some unique response to SO_3. On the contrary, they are quite amenable to the classical techniques of TLC, well understood by the academic as well as the industrial community, and respond to SO_3 in a manner which seems generally characteristic of most organic compounds. The fume-treating apparatus was a convenient, safe and effective method of exposure. The use of these particular lipids was largely influenced by the commercial availability of pure reference standards and adequate coverage by the literature.

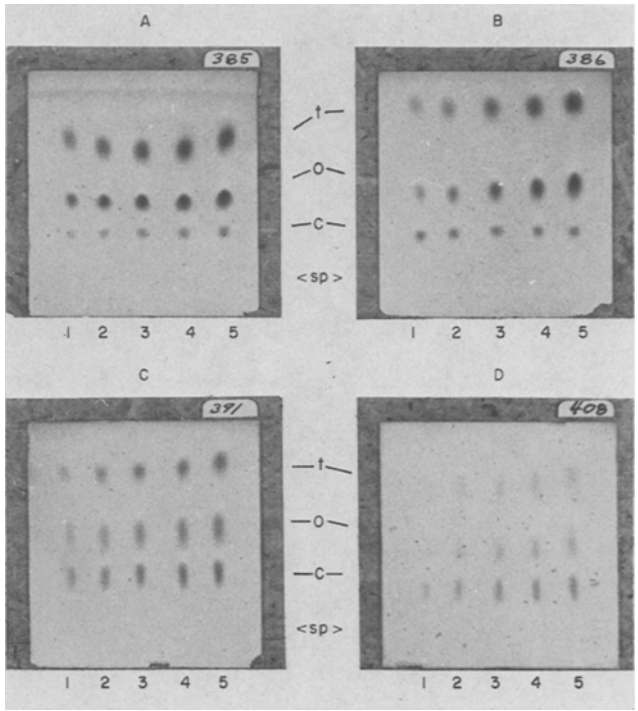

FIG. 2. Experiments with microchromatograms of neutral lipids. Silica Gel G on 66×66×1 mm glass plates; spotted with triolein (t), oleic acid (o) and cholesterol (c); developed with hexane, ether and acetic acid, 70:30:1 v/v; charred with 30–33% pyrosulfuric acid fumes for 30 min. A, Detection, 180 C block and 250 μ layer with: 1-t,o,c = 1,1,0.1 μg; 2-t,o,c = 2,2,0.2 μg; 3-t,o,c = 3,3,0.3 μg; 4-t,o,c = 4,4,0.4 μg; 5-t,o,c = 5,5,0.5 μg. B, Relative carbon yield, 180 C block and 250 μ layer with: 1-t,o,c = 0.5,0.5,0.5 μg; 2-t,o,c = 1,1,0.5 μg; 3-t,o,c = 2,2,0.5 μg; 4-t,o,c = 3,3,0.5 μg; 5-t,o,c = 4,4,0.5 μg. C, Effect of layer thickness, 180 C block and 1,000 μ layer with: 1-t,o,c = 0.5 μg ea; 2-t,o,c = 1.0 μg ea; 3-t,o,c = 1.5 μg ea; 4-t,o,c = 2.0 μg ea; 5-t,o,c = 2.5 μg ea. D, Effect of temperature, 60 C block and 1,000 μ layer with lipid loads same as C.

Experiments With Microchromatograms

In 1967, Nelson and Booth (17) reported the sensitivity of a conventional spray char reagent for detecting the three lipids of interest. They sprayed layers with 55% sulfuric acid containing 0.6% potassium dichromate and heated them for 20–30 min in a forced draft oven at about 185 C. Their limits of detectability under UV and visible light were, respectively: cholesterol, 0.1 and 0.5 μg; oleic acid, 1 and 5 μg; and triolein, 1 and 5 μg. The results obtained when these amounts of the three lipids were charred with SO_3 are pictured in Figure 2A.

In Figure 2A, each spot was charred through the entire thickness of the layer. Every char spot had a very slight halo of char-fluorescence. All spots and halos were easily seen from the front or the back of the plate. Chromatograms 1 and 5 represent the char-fluorescence and char spot detection limits reported by Nelson and Booth. Detection limits with SO_3 char are clearly much lower. Figure 2A also shows that charring with SO_3 reduces the effect of lipid class on carbon yield. This result is further defined in Figure 2B.

In Figure 2B, the tenfold difference between the carbon yield of cholesterol and the other two lipids, which is implied by the 0.5 :5.0 :5.0 μg char spot detection limits of Nelson and Booth, is virtually erased. When spot size and density as well as the weight fraction of carbon in each lipid is considered, these chromatograms show that all three lipid classes approach equal carbon yields. Equal carbon yields are important if direct quantitation of char spots is to be accomplished without the use of reference standards or calibration factors.

Under the same conditions used for Figures 2A and 2B, as little as 1–10 nanograms of each lipid could be detected. Even with these small loads, the transverse profile of spots (33,34) appeared to be unaltered by lipid migration to or SO_3 concentration on the surface of the layer. Each spot could be seen as well by char as by char-fluorescence while viewing the layer from the front or back of the plate. When layers were similarly exposed to SO_3 at room temperature, as little as one nanogram of cholesterol and 10–100 nanograms of the other two lipids could still be detected by front and back fluorescence. The high sensitivity and nicely graded spots of Figures 2A and 2B suggested that the effect of thicker layers, shorter times and lower temperatures might be profitably studied. Miniature layers spotted with a single series of five mixed standard solutions were used.

Figures 2C and 2D show two of the plates from the study of SO_3 char process variables. These plates indicate the effect of layer thickness and char temperature on visibility of the lipid spots. In every chromatogram, each spot was charred clear through the 1,000 μ layer and easily seen from the front and back when the plate was viewed under white or UV light. With lower char temperatures and shorter intervals of exposure to SO_3, conversion to carbon continued to decrease and was substantially arrested, first for triolein and oleic acid and finally for cholesterol. It is of interest to speculate that this order of reactivity probably is responsible for the much lower detection limit of Nelson and Booth (17) and somewhat higher carbon yield of Downing (18) for cholesterol by spray char techniques.

Figure 3 is based on results from the study of SO_3 char process variables. It outlines the estimated scope of the technique and indicates the conditions which already have proved useful in this laboratory. With weaker fuming sulfuric acids (20–23% and 15–18% free SO_3), longer char times and higher char temperatures produce similar results. But the shorter

TEMP. C	TIME, minutes			
	5	10	20	30
180				
165	A (established)			
150				
135				
120	A (likely)			
105				
90				
75	A, B, & C (possible)			
60				
45	B & C (likely) ; D (established)			
RT	E & F (established)			

FIG. 3. Guide to conditions for treating layers up to 1,000 μ thick with 30–33% pyrosulfuric acid fumes. (A) Char spot quantitation; (B) char-fluorescence spot quantitation; (C) char-fluorescence spot identification; (D) char spot detection; (E) char-fluorescence spot detection; (F) char-color spot detection.

times and lower temperatures made possible by using the strongest of the three fuming acids (30–33% free SO_3) are the ones that captured our interest. Because the dry fumes are so aggressive and high temperatures are not required to evaporate excess liquid reagent from layers, SO_3 char applications probably can be extended to include compounds which lie above the normal range of volatility considered suitable for conventional spray char techniques.

Char spots of quantitative quality are obtained with SO_3 in less time and at lower temperatures than are required for conventional spray char techniques. These mild conditions exert a most favorable influence on char process efficiency (8,15,18). Evaporative losses are minimized while total conversion of organic substances as well as selectivity to carbon are maximized by the fast acting SO_3 fumes. But process efficiency is only one of the important advantages of charring with SO_3. The layers have a clean background. They are dry, virtually nonhygroscopic and permanent. Freedom from excess liquid acid lends stability to the intensity of char-fluorescence spots.

At milder conditions, many compounds acquire enhanced char fluorescence and assume char-colors. These SO_3-induced spots are qualitatively characteristic and quantitatively promising. Aside from their analytical potential, they suggest that continuous and controlled exposure to SO_3 fumes might be used to study the chemical behavior of a large variety of substances. Perhaps other useful fluorescence and color reagent fumes (35–43) could be employed in much the same way. Also, the possibility of evaluating the catalytic effect of various adsorbing media should not be overlooked. If direct spectrophotofluorometric and spectrophotometric measurements could be made during such continuous and controlled exposures, many old as well as new reactions, rates and mechanisms might be studied.

Experiments With Full Size Chromatograms

In 1968, Downing (18) described a procedure for the photodensitometric quantitation of six neutral lipids in a single chromatogram without the necessity for reference standards. Mixtures of cholesterol, oleic acid, triolein, cetyl palmitate, cholesteryl oleate and squalene were separated in 7 mm tracks. Chromatograms were sprayed with 50% sulfuric acid and charred with a hot plate. Each chromatogram was scanned in a single uninterrupted run with a Photovolt Automatic Scanning TLC Densitometer. The instrument was equipped with a 1 × 4 mm incident light slit and included an Integraph Integrator (Model 49). Normally, the relative weight percentage of each component was calculated directly from the integrator output on the recorder chart.

Downing optimized spray char efficiency by omitting dichromate from the reagent and programming the char temperature. The temperature was raised from 20 to 220 C during a period of approximately 30 min and held there for another 10 min. Equal carbon yields were obtained for all the neutral lipids except cholesterol. The peak area for cholesterol was reduced by the factor 0.66 in the calculation of results. Because of the improved spray char quantitation achieved, these results were selected as a bench mark for evaluating the quantitative capability of SO_3 char.

In the first of a continuing series of experiments, the objective was to see if SO_3 char could close the spray char carbon yield gap between cholesterol and the other neutral lipids (17,18). To simplify the

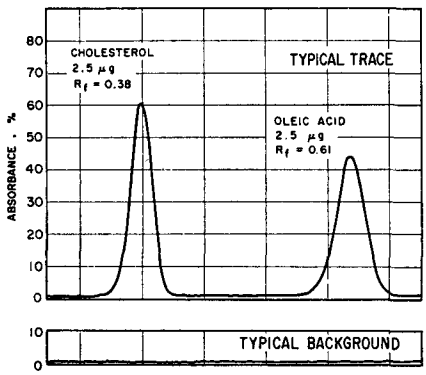

FIG. 4. Typical densitometer traces for SO_3 charred lipid chromatograms and layer background tracks.

developing step and still remain within the range of quantitatively noncontroversial R_f values (14,18), triolein was dropped from the standard solution used. Based on the study of SO_3 char process variables and the apparent success of other work (44), a 30 min exposure to SO_3 at a fixed nominal temperature of 150 C was selected for charring. Several plates were processed to give 12 replicate SO_3 charred chromatograms in the even-numbered tracks of each layer. All char spots fell within the undisputed range of R_f 0.4 to 0.8 recommended for constant carbon yield (14) and appeared to be uniformly dense through the entire depth as well as across the full width (18,27,44 and R.L. Squibb, private communication) of the track.

Figure 4 shows traces which are typical of those obtained for all tracks. Each trace represented an uninterrupted scan which included 80% of total track width (R.L. Squibb, private communication). All SO_3 char spots were well below the 1.3 limit of optical density (18). Every peak was characteristically symmetrical and well resolved. Layer backgrounds were low, regular and horizontal. When straight tangent base lines were used, no statistical difference could be found between peak areas measured with a planimeter and those obtained by triangulation. Calculations for each set of 12 traces gave similar results when the techniques of Downing were used without a correction factor to compensate for differences in carbon yield. One set of results is given in Table I. For comparison, the results reported for cholesterol and oleic acid in the publication (Sample 1 of Table I) by Downing (18) have been uncorrected by the use of his 0.66 carbon yield factor for cholesterol and are included in Table I.

Table I shows that SO_3 char can indeed narrow the gap between cholesterol and oleic acid spray char carbon yields (17,18). It also indicates how effectively more heat can be poured into the perimeter of a char block to compensate for radiant heat loss from the walls. On the other hand, Table I cannot show that the carbon yield gap has been completely closed. The 93.8% maximum purity for the cholesterol used, which is indicated by the carbon yields obtained, cannot be readily accepted. It would be more surprising if the oxidative degradation of carbon could be responsible for the lower carbon yield from cholesterol. Evaporative losses seem more probable; but it is hard to imagine that the parent compound can be the primary target as others (8,15) have implied.

TABLE I

Comparison of Lipid Carbon Yield With SO_3 Fume Char vs. 50% H_2SO_4 Spray Char[a]

Statistic, n = 12	Cholesterol	Oleic acid
Mean, wt. %	48.4 (59.4)	51.6 (40.6)
Standard deviation	0.57 (2.43)	0.57 (1.25)
Mean ± standard deviation	47.8 — 48.9	51.1 — 52.2
95% Confidence limit	48.0 — 48.7	51.3 — 52.0
95% Relative error	0.75 (3.42)	0.70 (2.57)
Estimated maximum purity, wt. %	93.8	100.0

[a] Spray char data in parentheses.

Rescanning the same set of chromatograms after an additional 40 days of storage in the dark produced an interesting new set of results. While all the other statistics in Table I remained the same, the mean weight percentages for the two components had become exactly reversed. An 8.3% loss in the total peak areas for cholesterol and a 19.6% loss in the total peak areas for oleic acid were responsible for the change. The char spots for both classes of compounds had faded, and they had faded at significantly different rates. This phenomenon would seem least of all likely to be caused by the degradation of carbon or evaporation of parent compound.

The first experiments in this series have provided one answer and raised at least two questions. They show that SO_3 char can almost close the carbon yield gap between cholesterol and oleic acid. On the other hand, they also show the need for more work on the problem of apparent evaporative loss of cholesterol as well as the problem of SO_3 char spot fade. It is expected that one or both of these problems could be related to the intermediate reaction products which were so abundantly in evidence during the first series of experiments. Other fixed char temperatures, programmed char temperatures, and additional compound classes are under investigation. The effect of time, temperature, light, air and humidity on char spot fade is being studied. Several ideas for the destruction of residual SO_3 by subsequent treatment with other reagent fumes or bakeout, or both, immediately after char are also receiving attention.

Meanwhile, it is believed that this laboratory has been privileged to witness the long-awaited demise of acid spray char. Using SO_3 char to bring a wide range of seemingly difficult and complex industrial sample types within the reach of modern direct instrumental methods has been most encouraging. A number of TLC densitometric applications already have been developed. One of these is described elsewhere (44). In all of them, charring with SO_3 has eliminated most of the disadvantages of spray char and greatly improved the visualization and quantitation of thin layer chromatograms.

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