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Isolation analysis of tissue fatty acids by ultramicro-ozonolysis in conjunction with thin-layer chromatography and gas-liquid chromatography*

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SUMMARY

Apparatus and technique for the detailed analysis of natural mixtures of fatty acids by a combination of thin-layer chromatography (TLC), gas-liquid chromatography (GLC), and reductive ozonolysis are described.

The fatty acids, as methyl esters, are separated on the basis of differences in unsaturation and chain length by the application of TLC using chromatoplates coated with silver nitrate–silica gel followed by GLC. Each group of fatty acid esters is analyzed by reductive ozonolysis by an ultramicromethod.

The general procedure is demonstrated with pure methyl esters and mixtures thereof, and it is applied to the analysis of the fatty acids of the liver phospholipids of rats fed a fat-free diet. The detection of endogenous 4,7,10,13-eicosatetraenoic acid of the palmitoleic acid family is reported.

Identification of peaks on a gas-liquid chromatographic chart is generally made on the basis of their carbon numbers (1) or from direct reference to known standards. The limitations of this method are well recognized, especially for the minor components of the mixture, but further identification is frequently impossible because of the small amounts of the components. The problem is further compounded by technical difficulties in the recovery of minute amounts of substances from the effluent gas of a fractionation by gas-liquid chromatography (GLC) in sufficient amounts and purity for further analysis. A confirmatory analysis of peaks on a GLC chart and spots on a thin-layer chromatography (TLC) plate, for which the above comments also generally apply, is especially important because the analysis is based on separations by virtue of a difference in general physical properties. Thus, a spot or a peak does not necessarily represent a single compound.

As a contribution toward solution of these problems, an ultramicro-ozonolysis technique is described for the identification of unsaturated fatty acids. Its use is demonstrated on standard and natural mixtures of fatty acids (as their methyl esters) isolated by GLC and TLC.

EXPERIMENTAL METHODS

Materials. Highly purified methyl linoleate and oleate were obtained from The Hormel Foundation, Austin, Minn. Methyl linolenate and methyl arachidonate of > 99% purity were prepared as described by Privett and Nickell (2).

Methyl decanoate was obtained from Matheson Company, Inc., Norwood, Ohio, and purified by fractional distillation. Those fractions that appeared to be pure by GLC analysis were combined. The combined product was stirred vigorously under high vacuum at room temperature until the condensate of evaporated methyl decanoate collected in a trap in dry ice was free of short-chain impurities. Usually, it was necessary to treat the freshly distilled methyl decanoate for about 24 hr to remove the last traces of short-chain impurities.

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Methylene chloride, Fisher Reagent, was used as a solvent for catalytic reduction without further purification.

Pentane was purified as follows. First, it was saturated with ozone at about −20°; then it was shaken with small amounts of concentrated sulfuric acid in a separatory funnel until the acid layer remained clear. Finally, it was washed with distilled water, dried, and distilled.

Fatty acids of rat liver phospholipids were obtained from a group of animals fed a fat-free diet for six months. The fat was extracted by macerating the pooled livers in a Servall Omni-Mixer under nitrogen three times with 200 ml of chloroform-methanol 2:1 (v/v). The phospholipids were separated and purified by three successive acetone precipitations and fatty acid esters were prepared by heating the lipid in refluxing, dry 1 N HCl–methanol for 2 hr. The composition of the methyl esters was as follows: 16:0 = 18.4%; 16:1 = 8.0%; 18:0 = 23.4%; 18:1 = 25.0%; 18:2 = 1.6%; 20:3 = 20.6%; 20:4 = 3.0%.

Reductive Ozonolysis. The ozonization was carried out essentially as previously described (3) but on a smaller scale. A 3-ml pentane solution of ozone was prepared in the reaction flask (A, Fig. 1) immersed in a dry ice bath (−65 to −75°) by bubbling oxygen containing about 3% ozone from a Bonner generator (4) at about 100 ml/min through it for about 5 min. The solution turned a blue color and iodimetric analysis indicated that the concentration of ozone was about 0.03 N in solutions prepared in this manner. The sample (10–100 μg) was dissolved in about 100 μl of pentane and added to the pentane solution of ozone in the reaction flask (A, Fig. 1). (This flask was later used as the reaction flask for catalytic reduction.) Ozonization is virtually instantaneous under these conditions. Thus, the solvent was evaporated immediately under reduced pressure as the solution warmed up to about 0° to remove the excess ozone and dissolved oxygen. The final traces of solvent were removed by means of a mechanical pump.

Catalytic reduction of ozonides was carried out with the apparatus shown in Fig. 1; flask A contains the ozonides. Usually two samples were reduced, one in methylene chloride for the analysis of the long-chain fragments and the other in methyl decanoate for the analysis of the short-chain fragments.

General Procedure. About 0.5 ml of solvent and 10 mg of Lindlar catalyst (5) are added to the reaction flask (A, Fig. 1) in a bath at 0°. When methylene chloride is used as the solvent, the system is flushed with nitrogen and then filled with hydrogen. About 20 min is allowed for completion of the reaction, during

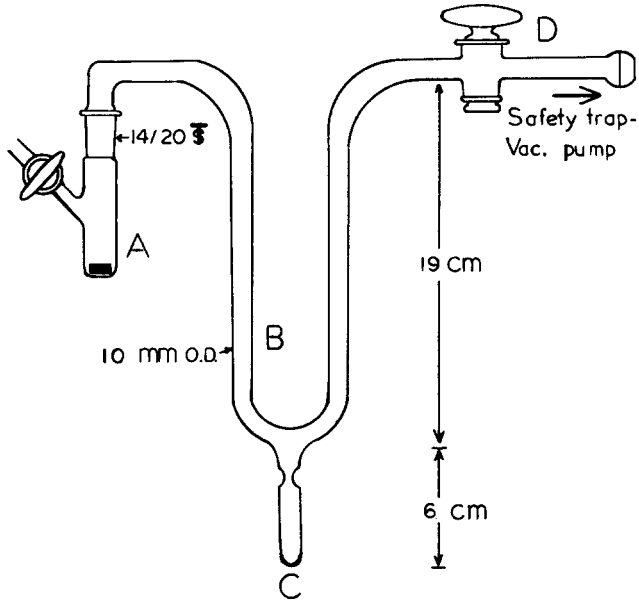

FIG. 1. Apparatus for reductive ozonolysis. A, reaction flask; B, U-tube trap; C, collection bulb.

which the solution is stirred vigorously with a magnetic stirrer. After the reduction is complete, the catalyst is removed by filtration and washed with about 1 ml of fresh solvent. The solution is reduced to a volume of about 100 μl by blowing a stream of nitrogen over it, and analyzed by GLC as described below.

A somewhat different procedure is used for reductions in methyl decanoate. After the system (Fig. 1) is flushed with nitrogen, it is evacuated for about 5 min by means of a mechanical pump and then filled with hydrogen. Twenty minutes is allowed for completion of the reduction. Then a Dewar flask containing liquid nitrogen is raised around the U tube (B, Fig. 1) and the reaction flask (A, Fig. 1) is cooled in a dry ice bath for several minutes to solidify the sample. The system is then evacuated with a mechanical pump, the stopcock (D, Fig. 1) is closed, and the reaction flask is allowed to warm up to room temperature by removing the dry ice bath. As the reaction flask warms up, some methyl decanoate evaporates. About 50 μl of methyl decanoate is allowed to condense in a near arm of the U tube; then the liquid nitrogen is lowered until only bulb C is immersed in it. The U tube (B, Fig. 1) is gently warmed with an infrared lamp to melt the methyl decanoate, which then runs down the U tube and is collected with the aldehyde fragments of the reduction in bulb C. Bulb C is sealed off at the constriction and usually kept in dry ice until the sample can be analyzed. The analysis should be performed without undue delay, however, because even at low

temperatures free aldehydes are not stable for long periods.

The GLC analysis of the fragments was performed with a flame ionization instrument (Model 609, F & M Scientific Corp., Avondale, Pa.) on both nonpolar columns (20% silicone on chromosorb W) and polar columns (15% ethylene glycol succinate polyester on chromosorb W). The silicone phase used in these columns consisted of the residue from the molecular distillation of Dow Corning Silicone Fluid 200 (viscosity 10 cs) (6). A complete analysis can usually be obtained on the silicone column; the polyester column is used mainly to distinguish simple aldehydes from ester aldehyde fragments when their identification is questionable. Frequently, a complete analysis can be made in either methylene chloride or methyl decanoate, depending on the chain length of the fragments. It is advisable to identify all fragments, however. The sensitivity and an example of the results that may be expected from the method are illustrated by the GLC analysis of the fragments from methyl linoleate shown in Fig. 2. The GLC analysis is usually carried out at 200° as well as 125° because the long-chain aldesters have a tendency to tail and give skewed peaks with the silicone phase at low temperatures.

Gas-Liquid Chromatography. The isolation and analysis of methyl esters was carried out with an F & M Scientific Corp. Model 500 instrument equipped with a thermal conductivity detector and a 1/4-in x 8-ft column packed with 15% ethylene glycol succinate polyester phase on chromosorb W at 190°. The collection of methyl esters was carried out by bubbling the effluent gas from a splitter (F & M Scientific Corp.) attached to the end of the column in front of the detector through about 5 ml of chloroform in test tubes. The splitter passed 15% of the gas through the detector for simultaneous analysis. The tubes and port needles of the splitter were changed manually as each fraction emerged from the column. The capillary outlet tube of the original splitter was replaced with an 1/8 in. o.d. copper tube of the same length to permit an unrestricted flow of gas, and it was maintained at 250°, 60° above the temperature of the column, to eliminate condensation in it. By means of this technique, recoveries of the order of 90% of the split sample were obtained on as little as 1 mg of sample. The recovery of material was checked with methyl linoleate by comparing the GLC analysis of equivalent aliquots of the sample before and after collection.

Thin-Layer Chromatography. The isolation of fatty acids as their methyl esters was carried out using silver nitrate-coated plates, a technique shown by Morris (7) and de Vries (8, 9) to be useful for the separation of fatty acid esters.

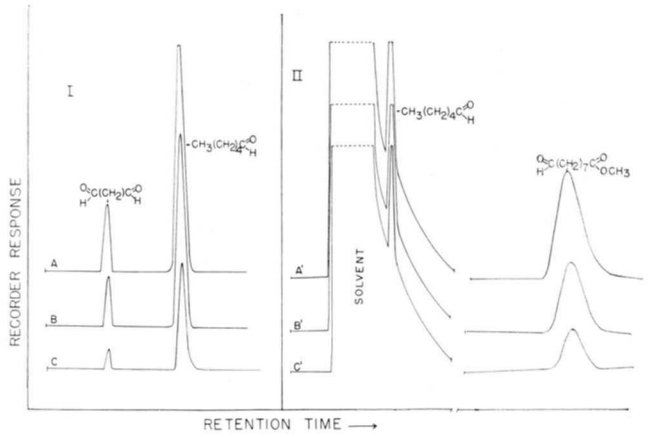

FIG. 2. Analysis of methyl linoleate by reductive ozonolysis. Gas-liquid chromatography of aldehydic fragments on a 12 ft x 1/4 in-column of 20% silicone on chromosorb W at I, 125° in methyl decanoate; II, 200° in methylene chloride. A and A′, B and B′, and C and C′ carried out on 100, 50, and 25 μg, respectively.

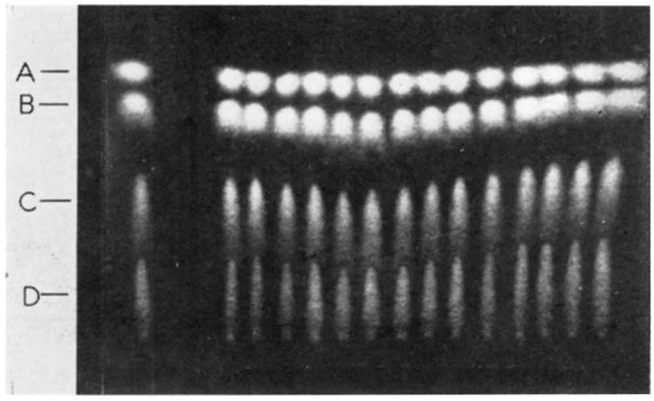

FIG. 3. Separation of a standard mixture of methyl esters for preparative purposes on a 20 x 20-cm chromatoplate by $AgNO_3$-TLC. A, methyl oleate; B, methyl linoleate; C, methyl linolenate; D, methyl arachidonate.

In this study, plates were prepared by spreading a fine slurry of silver nitrate, Silica Gel G (according to Stahl, Brinkman Inc., Great Neck, N. Y.), and water in a ratio of 1:4:10, respectively, with an applicator made out of plexiglass, in layers of about 0.3 mm. The plates were heated in an oven for 2 hr at 110° and stored in the dark until used. Before being used, each plate was developed with diethyl ether to move much of the contaminant organic matter in the adsorbent layer to the top of the plate. The separation of a standard mixture of methyl esters and a typical application for preparative purposes is demonstrated in Fig. 3. The solvent used for this fractionation consisted of 20% diethyl ether in petroleum ether.

The spots on this plate were detected by spraying the plate with 2,7-dichlorofluorescein, and then viewed and photographed under ultraviolet light. A standard mixture of methyl esters may be chromatographed in a marked-off area of the plate as shown in Fig. 3 to serve as a reference for the separation.

The spots were scraped from the plate in a band of adsorbent and extracted with freshly distilled diethyl ether. Most of the spray reagent remained on the adsorbent. As much as 100 mg of methyl esters may be chromatographed on a 20 x 20 cm plate with little overlapping. The results in Fig. 3 show that the method separates the fatty acids, as their methyl esters, into classes based on the degree of unsaturation.

Analysis of the Fatty Acids of Rat Liver Phospholipids. The methyl esters were first fractionated by silver nitrate TLC into classes based on the number of double bonds as shown in Fig. 4. Each class of esters was recovered and further purified by rechromatography on additional plates. The monoenoic fraction consisted of a mixture of 16 and 18 chain-length esters and was further fractionated by GLC into these components.

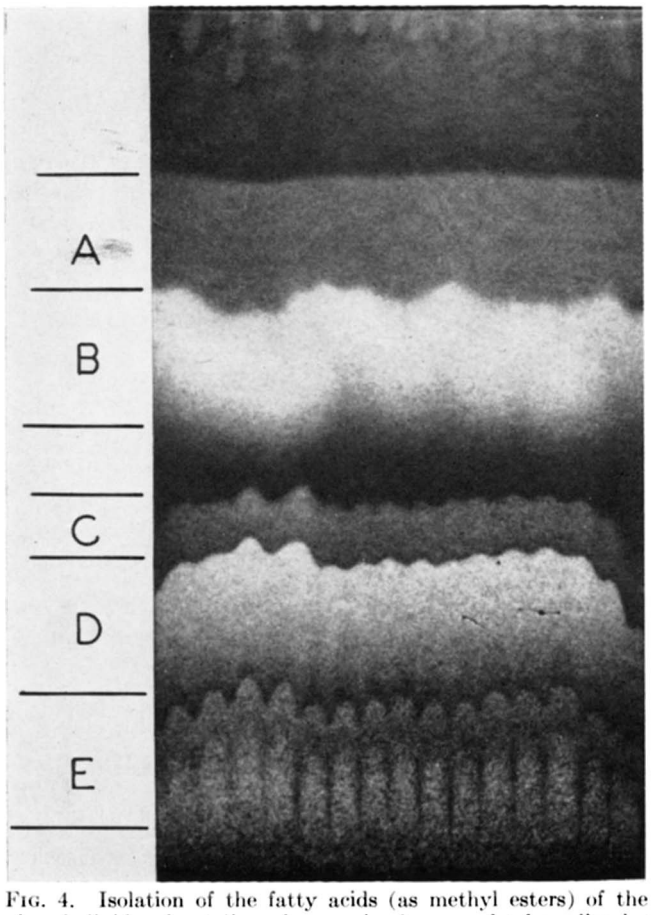

FIG. 4. Isolation of the fatty acids (as methyl esters) of the phospholipids of rat liver from animals on a fat-free diet by $AgNO_3$-TLC. A, saturated; B, monoenes; C, dienes; D, trienes; E, tetraenes.

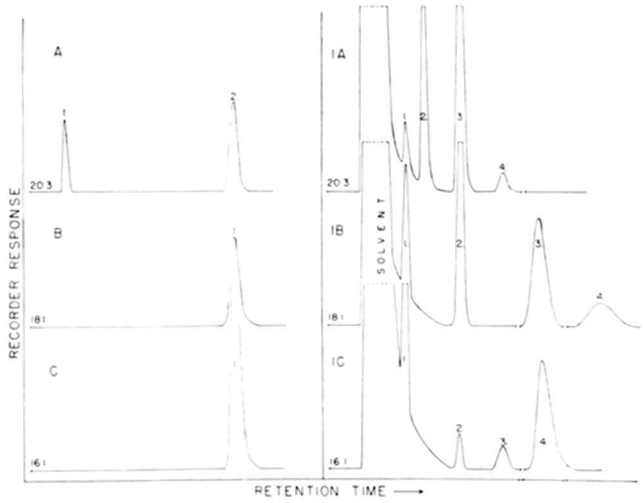

FIG. 5. Structural analysis of the 20:3, 18:1, 16:1 fatty acids isolated from the phospholipids of rat liver. A,B,C: 12 ft x 1/4 in-column of 20% silicone on chromosorb W at 125°; solvent, methyl decanoate. 1A, 1B, 1C: same column at 200°; solvent, methylene chloride.
20:3 A: 1, malonaldehyde; 2, heptaldehyde. 1A: 1, heptaldehyde; 2, methyl pentanoate-5-al; 3, pelargonyl aldehyde; 4, methyl heptanoate-7-al.
18:1 B: 1, heptaldehyde. 1B: 1, heptaldehyde; 2, pelargonyl aldehyde; 3, methyl nonanoate-9-al; 4, methyl undecanoate-11-al.
16:1 C: 1, heptaldehyde. 1C: 1, heptaldehyde; 2, pelargonyl aldehyde; 3, methyl heptanoate-7-al; 4, methyl nonanoate-9-al.

The GLC analysis of the final preparations showed that the 16 carbon chain monoene fraction was 98% pure; the impurity in this preparation was methyl palmitate. No impurities could be detected in the 18 carbon chain monoene fraction by GLC. The diene fraction (Fig. 4) consisted of about 88% methyl octadecadienoate and 12% methyl eicosatrienoate; the triene fraction about 99% methyl eicosatrienoate; and the tetraene fraction 97% methyl eicosatetraenoate with 3% methyl eicosatrienoate as an impurity.

Each fraction was analyzed by reductive ozonolysis as described above. The GLC chromatograms of the analysis of the fragments are presented in Fig. 5 and 6.

The peaks in the curves in Fig. 5 and 6 were identified by reference compounds and by graphical means based on the log plot of the retention times. Supplementary analyses (not shown) were made on an ethylene glycol succinate column to confirm the assignment of peaks 4 and 5 in Fig. 6 1A and 1B, respectively, to pelargonyl aldehyde, which pairs with the methyl pentanoate-5-al (peak 4, Fig. 6 1B) to establish the identification of the impurity as methyl 5,8,11-eicosatrienoate. This analysis also showed the absence of any methyl hexanoate-6-al, which would arise with

TABLE 1. IDENTIFICATION OF FATTY ACIDS OF RAT LIVER PHOSPHOLIPIDS BY REDUCTIVE OZONOLYSIS

Fraction	Chain Length	Component	Aldester	Aldehyde	Fatty Acid (As Methyl Ester)
Monoene	16	Major	Methyl nonanoate-9-al	Heptaldehyde	9-Hexadecenoate (palmitaleate)
		Minor	Methyl heptanoate-7-al	Pelargonyl aldehyde	7-Hexadecenoate
Monoene	18	Major	Methyl nonanoate-9-al	Pelargonyl aldehyde	9-Octadecenoate (oleate)
		Minor	Methyl undecanoate-11-al	Heptaldehyde	11-Octadecenoate
Diene*	18	Major	Methyl octanoate-8-al	Heptaldehyde	8,11-Octadecadienoate
		Minor	Methyl nonanoate-9-al	Hexaldehyde	9,12-Octadecadienoate (linoleate)
Triene	20	Major	Methyl pentanoate-5-al	Pelargonyl aldehyde	5,8,11-Eicosatrienoate
		Minor	Methyl heptanoate-7-al	Heptaldehyde	7,10,13-Eicosatrienoate
Tetraene*	20	Major	Methyl pentanoate-5-al	Hexaldehyde	5,8,11,14-Eicosatetraenoate (arachidonate)
		Minor	Methyl butanoate-4-al	Heptaldehyde	4,7,10,13-Eicosatetraenoate

* Note that fragments from the 20:3 in these preparations (Fig. 6) are not included but show that this compound is the 5,8,11 isomer.

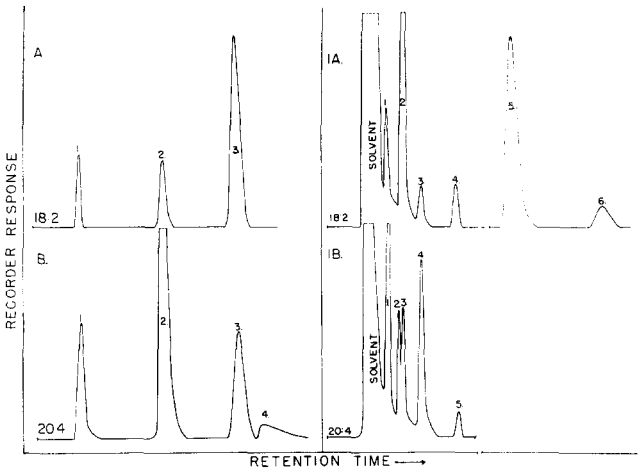

FIG. 6. Structural analysis of the 18:2 and 20:4 fatty acids isolated from the phospholipids of rat liver. A and B: 12 ft x 1/4 in-column of 20% silicone on chromosorb W at 125°; solvent, methyl decanoate. 1A and 1B: same column at 200°; solvent, methylene chloride.
18:2 A: 1, malonaldehyde; 2, hexaldehyde; 3, heptaldehyde. 1A: 1, hexaldehyde; 2, heptaldehyde; 3, methyl pentanoate-5-al; 4, pelargonyl aldehyde; 5, methyl octanoate-8-al; 6, methyl nonanoate-9-al.
20:4 B: 1, malonaldehyde; 2, hexaldehyde; 3, heptaldehyde; 4, methyl butanoate-4-al. 1B: 1, hexaldehyde; 2, heptaldehyde; 3, methyl butanoate-4-al; 4, methyl pentanoate-5-al; 5, pelargonyl aldehyde.

pelargonyl aldehyde from methyl 6,9-octadecadienoate had it been present.

The results of the GLC analysis of the fragments are summarized in Table 1.

DISCUSSION

Since reductive ozonolysis, as well as TLC and GLC, can be applied on a microgram scale, isolation analysis of fatty acids can be carried out on less than 1 mg of lipid. In fact, we are routinely making analyses on the lipid from about 2 ml of blood in a bioassay of the interconversion of fatty acids of the rat in connection with studies on essential fatty acid (EFA) metabolism.

Several positional isomers of monoenoic, dienoic, and trienoic fatty acids have been isolated from EFA-deficient rats via preparative GLC and liquid–liquid partition chromatography by Fulco and Mead (10). More recently, Schlenk, Sen, and Sand (11), in studies on the interconversions of monoenoic acids, also demonstrated the formation of positional isomers of monoenoic and dienoic acids in the carcass fat of rats via preparative GLC in conjunction with structural analysis.

The presence of the 4,7,10,13-eicosatetraenoic acid isomer of arachidonic acid in EFA-deficient animals is not unexpected because it is a member of the palmitoleic family of acids (12). A complete report on the nutritional aspects of these and related findings will be published separately.

It should be mentioned that, during the course of the development of the procedure, each step was checked for possible alteration of polyunsaturated fatty acids that might lead to artifacts by tests with methyl arachidonate.

The reductive ozonolysis technique of structural analysis described here is well suited to the identification of positional isomers because it is essentially free of side reactions (3). As such, even minor components may be analyzed with confidence. It is important, however, that all fragments of the analysis be identified and matched together to identify the parent compound. It is also important, especially for application on an

ultramicroscale (< 100 μg), that all solvents and reagents be of the utmost purity and that all glassware be thoroughly cleaned.

Little attention was given to the analysis of malonaldehyde in this study. However, should there be any question of the divinylmethane rhythm in the polyunsaturated fatty acids, the dialdehyde fraction should be analyzed separately (3).

No attempt was made in the present study to use the method for quantitative analysis, but it is evident that, with further refinement, it may be used for this purpose.

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