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# A NEW PROCEDURE TO ANALYZE FREE FATTY ACIDS

# APPLICATION TO 20-mg BRAIN TISSUE SAMPLES

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## SUMMARY

Fatty acids were analyzed by a new method which involved their isolation from hexane extracts of serum or brain tissue in aqueous potassium hydroxide (10 μl) and methylation directly in this solution with methyl iodide. The resulting fatty acid methyl esters were partitioned into ethylene chloride (25 μl) and were quantitated by gas—liquid chromato-

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**graphy. The procedure was documented by comparison with conventional methylation reactions on serum fatty acids. This method, which avoids thin-layer chromatography and which measures individual free fatty acid concentrations in 20-mg brain tissue samples, should be of particular value for examining regional free fatty acids in brain following ischemia and trauma.**

## INTRODUCTION

The conventional method for separation of free fatty acids (FFA) from other lipid components in brain tissue extracts is thin-layer chromatography (TLC) [1—4]. However, TLC procedures usually require sizable quantities of tissue, prolonged isolation time, and can result in a low recovery of FFA (e.g. see ref. 5). Furthermore, the variability in the quality of separation of FFA from di- and triglycerides on TLC plates from different suppliers may result in erroneously high FFA concentrations [5]. In order to measure FFA concentrations in small brain tissue samples we developed an alternative method for their separation and methylation.

The present procedure integrates previously developed techniques [6, 7] for extraction of FFA and methyl esters with a new method for FFA methylation. We separated FFA from other lipids in a hexane extract by concentrating them in a small volume of an aqueous base [6]. To avoid the back-extraction of FFA into a non-aqueous medium, which is required for some methylation reactions, we utilized methyl iodide to methylate FFA directly in this basic solution. The resulting fatty acid methyl esters were partitioned into a small volume of ethylene chloride [7] and were quantitated by gas—liquid chromatography (GLC).

In the present report we compare our methyl iodide methylation procedure with: (1) a conventional method using boron trifluoride ($BF_3$) [8]; and (2) a previous method developed by our laboratory using trimethyl ($\alpha,\alpha,\alpha$-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) [6] on total saponifiable serum fatty acids. Documentation of the methyl iodide procedure was initially obtained with serum because its relatively high concentration of polyunsaturated fatty acids enabled a test of whether our methylation procedure altered double bonds. Furthermore, the greater availability of serum versus brain tissue permitted macrochemical analyses thus avoiding the inherent difficulties associated with trace measurements. Having demonstrated the quality of our method with serum, we then successfully carried out a "trace analysis" of FFA concentrations on 20-mg samples of cortical gray matter of cat brain.

## EXPERIMENTAL

### *Materials*

Organic solvents and reagents were of reagent grade quality and were purchased from Fischer Scientific (Pittsburgh, PA, U.S.A.) with the following exceptions: $BF_3$—methanol (14%, w/v), and standards pentadeconic acid and Fat and Oil Reference Mix No. 6 from Applied Sciences Labs. (State College, PA, U.S.A.) and PUFA 1 and PUFA 2 from Supelco (Bellefonte, PA, U.S.A.);

TMTFTH from Regis (Morton Grove, IL, U.S.A.); hexane was HPLC grade quality. Glassware was soaked in methanolic potassium hydroxide (15%, w/v) and rinsed well to remove lipid contamination. All glass stoppers and glassware were handled carefully to prevent skin lipid contamination.

*Saponification and extraction of fatty acids in serum*

*Comparison of boron trifluoride and methyl iodide methylations.* Fatty acids in fresh pooled human serum (0.8 ml) were saponified with 4.0 ml of 15% methanolic potassium hydroxide by incubation in a heating block for 45 min at 65°C. The solution was cooled, and 4.0 ml of 1.5 *M* phosphoric acid were added. Following mixing, 1.6 ml of water and 40 ml of hexane were added, and the mixture was shaken vigorously by hand for 2 min to extract the fatty acids. Following centrifugation (500 *g*), most of the hexane phase was removed to a conical centrifuge tube, and the fatty acids were extracted with 1.0 ml of 0.5 *M* potassium hydroxide in water. Following centrifugation (500 *g*) for 1 min most of the lower potassium hydroxide—water phase was removed to another tube. The solution was then acidified with 1.5 *M* phosphoric acid, and 2.0 ml of water and 5.0 ml of ethylene chloride were added. The fatty acids were extracted by shaking vigorously for 2 min. Following a 1-min centrifugation (500 *g*) most of the ethylene chloride layer was removed to another tube that served as the common source of fatty acids for the methylations described below. Multiple aliquots (0.1 ml) of this solution were flushed with nitrogen and stored at −10°C. On the day of assay, six tubes (for each assay) were removed from the freezer and carefully evaporated to dryness under nitrogen at 65°C. Ethylene chloride (45 μl) was added to those tubes that were carried through the boron trifluoride methylation procedure described below. To the tubes that were carried through the methyl iodide methylation procedure described below, 10 μl of 0.5 *M* aqueous potassium hydroxide were added and the tubes vortexed to dissolve the fatty acids.

*i. Boron trifluoride methylation.* Following vortex mixing, boron trifluoride (0.5 ml of 14% $BF_3$ in methanol) was added. The solution was mixed by manual shaking for 15 sec and then incubated for 10 min at 65°C in a stoppered tube. Following cooling, 1.5 ml of water were added, the tube was vortexed for 30 sec and centrifuged for 2 min at 500 *g*. A 10-μl syringe was primed with ethylene chloride and 5 μl of the ethylene chloride layer were injected into the gas chromatograph.

*ii. Methyl iodide methylation.* N,N-Dimethylacetamide (DMA) (50 μl) and 30 μl of methyl iodide were added with mixing, and the mixture was incubated in a hood for 10 min at 65°C. Pyridine (60 μl) was then added with mixing, and the mixture was again incubated for 10 min at 65°C. Following cooling, 0.5 ml of 0.1 *M* phosphoric acid and 25 μl of ethylene chloride were added and the fatty acid methyl esters were extracted by vortexing for 30 sec. The sample was centrifuged for 2 min (500 *g*), and most of the upper phase was removed. Water (4 ml) equilibrated with ethylene chloride was added. The upper walls of the tube and lower phase were washed by suspending the lower phase six times with a vortex mixer. Following centrifugation for 2 min (500 *g*), most of the upper phase was removed. This wash step was repeated. A 10-μl syringe was primed with ethylene chloride, and the lower phase was drawn up.

All of the lower phase (5—10 μl) can be removed for injection into the gas chromatograph. Removal of a small amount of the upper phase did not affect the analysis.

*Comparison of TMTFTH and methyl iodide methylations.* Fresh pooled human serum (1.0 ml) (different batch than described in *Comparison of boron trifluoride and methyl iodide methylations*) was saponified as described above with adjustments in the reagents for the larger volume of serum. Following extraction of fatty acids into hexane, six 5.0-ml aliquots for the TMTFTH procedure described below and six 1.7-ml aliquots for the methyl iodide procedure were removed to 5-ml conical centrifuge tubes, flushed with nitrogen, and stored at −10° C.

*i. TMTFTH methylation.* The tubes containing fatty acids in 5.0 ml of hexane were extracted with 10 μl of TMTFTH according to the procedure of MacGee and Allen [6]. A 2.5-μl sample—methyl acetate (treated with anhydrous sodium carbonate) sandwich was injected into the gas chromatograph.

*ii. Methyl iodide methylation.* The tubes containing fatty acids in 1.7 ml of hexane were extracted with 10 μl of 0.5 *M* potassium hydroxide in water. The tubes were shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed using a Pasteur pipet and vacuum (drawing off liquid followed by air drying of the interface). The methylation was carried out as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

*Brain tissue analysis*

*Animal preparation.* An adult conditioned cat was anesthetized with pentobarbital, 30 mg/kg, intravenously. The right femoral artery was catheterized with P.E. No. 90 polyethylene tubing, and the free end was attached to a Statham strain gauge transducer to record blood pressure and drive a cardiotachometer. Blood pressure and heart rate were recorded on a Brush Gould polygraph. The cat was intubated and ventilated with a Harvard respirator. The rectal temperature was continuously monitored, and body temperature was maintained at 38.0 ± 1° C with a heating pad.

Periodic blood samples were withdrawn and analyzed for pH, $pO_2$, and $pCO_2$ (Corning Model 168 blood gas analyzer). The adequacy of the animal's physiologic status throughout a 30-min control period and during in situ head freezing was verified by the recording of cardiovascular function and analysis of blood samples.

*Brain tissue fixation by in situ freezing and tissue sampling.* A bottomless plastic cup was attached to the surgically exposed calvarium. The cup was filled with liquid nitrogen, rapidly freezing the underlying skull and brain. Brain metabolites of rats [9] and cats [10] fixed by this method fall well within their normal ranges as defined by other methods. We have confirmed the validity of this method for the study of stable metabolic states in brains of monkeys [11] and cats [12].

The entire cat brain was frozen after 15 min of treatment with liquid nitrogen. After head freezing, the cat was decapitated and the frozen head stored at −80° C. The head was subsequently cut into 5—7 mm thick coronal

blocks using a band saw in a −20°C walk-in freezer. Tissue sampling and weighing procedures were accomplished in a −20°C glove box cryostat. A 2-mm stainless-steel trochar was used to sample cortical gray matter from the crowns of the gyri lateralis, suprasylvius, and ectosylvius. The tissue plugs were weighed on a Roller-Smith precision balance.

*Extraction of FFA from brain tissue*

*i. Precision study.* For the precision study on brain tissue (Table V), a tissue sample (159.6 mg) was homogenized by sonication in 3.2 ml of normal saline (0.9%) in a 50-ml centrifuge tube. Immediately, 12.0 ml of methanol, 25 ml of hexane, 4.0 ml of 1 *M* phosphoric acid and a known amount (9.068 μg) of the internal standard, pentadecanoic acid, were added to the homogenate. The mixture was shaken vigorously for 1 min and centrifuged (500 *g*). The hexane phase was then washed twice with 16.0 ml of 0.1 *M* phosphoric acid. Most of the hexane phase was removed to a volumetric flask and the volume was adjusted to 25 ml with hexane (2 ml). Then five 3.13-ml aliquots of the hexane extract (each equivalent to 19.98 mg brain tissue) were pipeted into individual tubes and 10 μl of aqueous 0.5 *M* potassium hydroxide were added. The tubes were shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed using a Pasteur pipet and vacuum (drawing off liquid followed by air drying of the interface). The tubes were carried through the methyl iodide procedure as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

*ii. General procedure.* The general procedure for extraction of FFA from brain tissue involved homogenization of 20 mg of tissue by sonication in 400 μl normal saline in a glass centrifuge tube. Immediately 1.5 ml methanol, 4.0 ml hexane, 0.5 ml of 1 *M* phosphoric acid and a known amount of internal standard (1.136 μg pentadecanoic acid) were added to the homogenate. The mixture was shaken vigorously for 1 min and centrifuged (500 *g*). Most of the lower phase was removed and 2.0 ml of 0.1 *M* phosphoric acid were added and the hexane phase was washed for 1 min and centrifuged. This wash step was repeated. Most of the hexane phase was removed to another tube and extracted with 10 μl of 0.5 *M* potassium hydroxide in water. The tubes were then shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed as previously described. The methyl iodide methylation was then carried out as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

*Blanks*

Blanks for the methyl iodide procedure were obtained on 400 μl of 0.9% saline using pentadecanoic acid as the internal standard and the procedure described above (*Extraction of FFA from brain tissue, General procedure*).

*Gas—liquid chromatography*

GLC was performed with either a Bendix 2600 gas chromatograph (Bendix, Ronceverte, WV, U.S.A.) or a Perking-Elmer Sigma 1 microprocessor-controlled computing gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.). Both instruments have flame ionization detectors and temperature programming. Two 182.9 cm × 6.35 mm (4 mm I.D.) glass columns were packed with 10% Silar 10 C on 100—120 mesh Gas-Chrom Q (Applied Science Labs.). The

temperatures of the injector and detector were 280°C and 250°C, respectively, for both instruments. The temperature programme for both instruments was 130°C to 220°C at 2°C/min. Peak areas and retention times for the Bendix instrument were determined with a Hewlett-Packard 3309A reporting integrator. Fatty acid methyl esters were identified on the basis of their retention times as compared to standards of known composition (Fat and Oil No. 6, PUFA 1, PUFA 2).

*Calculations*

The individual fatty acid concentrations for brain tissue were corrected by subtracting the corresponding blank values. The paired *t*-test was used to determine statistically significant differences between serum fatty acids analyzed by different methods.

## RESULTS

*Serum studies*

There were no significant differences in serum fatty acid concentrations or in the precision of the measurements analyzed by the methyl iodide procedure as compared with the conventional $BF_3$ or TMTFTH methods (Tables I and II).

TABLE I

COMPARISON OF BORON TRIFLUORIDE AND METHYL IODIDE METHYLATION PROCEDURES FOR ANALYSIS OF FATTY ACID CONCENTRATIONS OF POOLED HUMAN BLOOD SERUM

Values are means of normalized peak areas ± standard deviation (S.D.) from six samples each for $BF_3$ and methyl iodide procedures. Different batches of pooled human blood serum were used for the analyses in Table I and Table II.

| Fatty acid | $BF_3$ | Methyl iodide |
|---|---|---|
| 16:0 | 25.62 ± 0.26 | 25.34 ± 0.24 |
| 16:1 | 2.73 ± 0.22 | 2.54 ± 0.20 |
| 18:0 | 4.79 ± 0.26 | 4.64 ± 0.22 |
| 18:1 | 33.58 ± 0.23 | 33.75 ± 0.37 |
| 18:2 | 27.49 ± 0.25 | 28.05 ± 0.47 |
| 20:4 | 5.36 ± 0.29 | 5.69 ± 0.28 |

TABLE II

COMPARISON OF TMTFTH AND METHYL IODIDE METHYLATION PROCEDURES FOR ANALYSIS OF FATTY ACID CONCENTRATIONS OF POOLED HUMAN BLOOD SERUM

Values are means of normalized peak areas ± S.D. from six samples each for TMTFTH and methyl iodide procedures. Different batches of pooled human blood serum were used for the analyses in Table I and Table II.

| Fatty acid | TMTFTH | Methyl iodide |
|---|---|---|
| 16:0 | 24.15 ± 0.21 | 24.46 ± 0.22 |
| 16:1 | 4.36 ± 0.23 | 4.47 ± 0.32 |
| 18:0 | 5.38 ± 0.20 | 5.38 ± 0.20 |
| 18:1 | 32.17 ± 0.21 | 31.99 ± 0.28 |
| 18:2 | 27.20 ± 0.23 | 27.13 ± 0.25 |
| 20:4 | 6.74 ± 0.20 | 6.58 ± 0.20 |

A reaction time study determined that the methylation with methyl iodide was 90.0%, 94.7%, and 100% complete after 2.5, 5, and 10 min of incubation, respectively, at 65°C (data not shown). Further incubation for 15 or 30 min did not alter the results. In addition, a negative test with moist starch paper indicated that the pyridine destruction of excess methyl iodide was complete after 10 min of incubation at 65°C. FFA methyl esters were stable in ethylene chloride extracts for as long as seven days (Table III).

TABLE III

STABILITY OF FFA METHYL ESTERS FROM POOLED HUMAN BLOOD SERUM PREPARED BY THE METHYL IODIDE PROCEDURE

Values are normalized peak areas. The pooled human serum used for the data in this table is different from that presented in Tables I and II.

| Fatty acid | Day 0 | Day 1 | Day 2 | Day 3 | Day 7 |
|---|---|---|---|---|---|
| 16:0 | 24.60 | 24.26 | 24.54 | 24.35 | 24.37 |
| 16:1 | 4.76 | 5.17 | 4.41 | 4.66 | 4.52 |
| 18:0 | 4.66 | 4.44 | 4.75 | 4.60 | 4.80 |
| 18:1 | 31.86 | 32.16 | 31.91 | 31.79 | 31.76 |
| 18:2 | 27.41 | 27.47 | 27.52 | 27.85 | 27.75 |
| 20:4 | 6.70 | 6.50 | 6.88 | 6.74 | 6.80 |

*Brain tissue*

The blank concentrations of palmitic, oleic, stearic, arachidonic and docosahexaenoic acids are presented in Table IV. These blank values were subtracted from the corresponding brain tissue fatty acid concentrations.

The free fatty acid concentrations in cortical gray matter of cat brain tissue as determined by the methyl iodide procedure are presented in Table V. A typical chromatogram of FFA in a 19.98-mg sample of cat brain cortex is presented in Fig. 1.

TABLE IV

METHYL IODIDE PROCEDURE BLANKS

Values are means ± S.D. expressed as nmol for six blanks.

| Fatty acid | Blank value |
|---|---|
| 16:0 | 0.936 ± 0.139 |
| 18:0 | 0.533 ± 0.092 |
| 18:1 | 0.249 ± 0.055 |
| 20:4 | 0.052 ± 0.015 |
| 22:6 | 0.112 ± 0.056 |

## DISCUSSION

The results of the present study demonstrate that fatty acids can be successfully methylated with methyl iodide following their extraction from serum with aqueous potassium hydroxide. This new method uses non-adsorbing techniques, small-size glassware, and small amounts of reagents and solvents for

TABLE V

FREE FATTY ACID CONCENTRATIONS IN CORTICAL GRAY MATTER OF CAT BRAIN DETERMINED BY THE METHYL IODIDE PROCEDURE

Values are means ± S.D. expressed as nmol/g of five aliquots (3.13 ml per aliquot) of a hexane extract of brain tissue (19.98 mg per aliquot). See Experimental for description of sample handling.

| Fatty acid | Concentration (nmol/g wet weight) |
|---|---|
| 16:0 | 115.90 ± 10.97 |
| 18:0 | 86.83 ± 6.54 |
| 18:1 | 116.12 ± 8.07 |
| 20:4 | 28.29 ± 3.86 |
| 22:6 | 56.74 ± 8.80 |
| Total | 403.87 ± 31.54 |

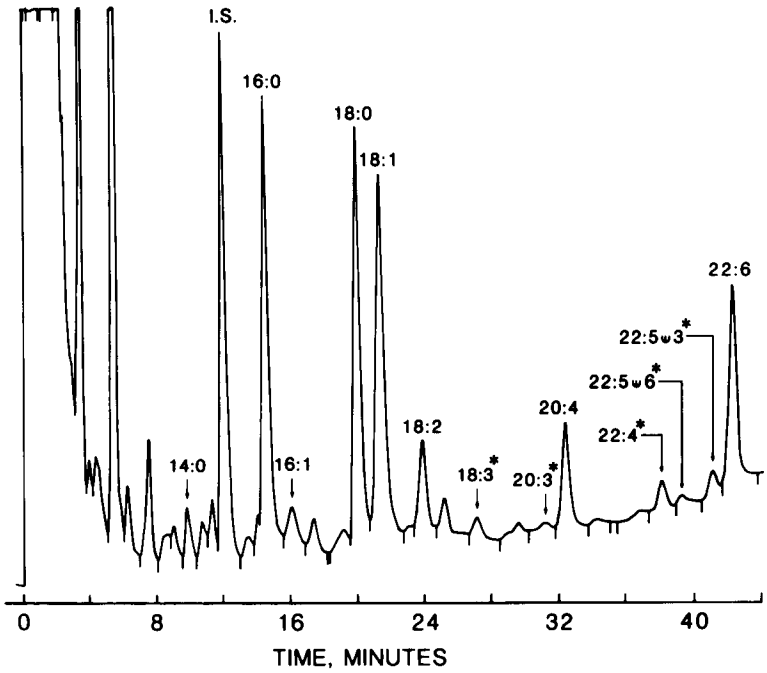


Fig. 1. Chromatogram of fatty acid methyl esters derived from a 19.98-mg sample of anterior cortical gyri of the cat brain. Fatty acids were extracted and methylated with methyl iodide as described in Experimental. FFA were identified on the basis of their retention times as compared with standards (see *Materials*). I.S. is the internal standard (pentadecanoic acid). The concentration of the 22:6 FFA appears to be variable between brain regions in the cat [13]. * = Tentative identification.

isolating FFA and preparing their methyl esters. Loss of FFA by irreversible adsorption, numerous steps utilizing different glassware, transfer of solutions, and evaporation or drying is thus eliminated.

Fatty acid methylation with methyl iodide yields results comparable to a conventional methylation method using $BF_3$ (Table I). In addition, similar results were obtained with the methyl iodide procedure as with our previously

described TMTFTH procedure (Table II). FFA concentrations can be successfully determined on 20 mg of brain tissue with this new method. Furthermore, the analysis time is short, about 1 h from homogenization of brain tissue to injection of the concentrated fatty acid methyl esters into the gas chromatograph. Recovery of most or all of the methyl esters is a necessary requirement when measuring FFA in small tissue samples. In addition, polyunsaturated fatty acid concentrations are not altered by the methyl iodide reaction. This method will enable an examination of FFA alterations in specific brain loci following ischemia, head trauma, and other insults which produce focal injury.

Other methods for esterification of FFA have certain disadvantages. Diazomethane has been used to esterify fatty acids [14]. However, this compound's explosive and toxic characteristics have discouraged its use [15]. In addition, diazomethane has occasionally produced artifacts and it appears to have a slower rate of esterification [15]. The commonly used $BF_3$—methanol reagent [8] does not yield quantitative recoveries of fatty acid methyl esters with small samples (50—150 mg) [15].

Our method is a modification of methods described by Grunert and Bassler [16], Gehrke and Goerlitz [17] and Johnson and Wong [18]. Grunert and Bassler [16] used methyl iodide over solid potassium carbonate at 90°C for fatty acid esterification which, when compared with classical procedures, was their method of choice. Gehrke and Goerlitz [17] described a silver salt—methyl iodide procedure which requires preparation of the silver salts of fatty acids with silver nitrate, evaporation of water, followed by an 8-h incubation with methyl iodide. Our method, which is considerably less complex and time-consuming than either of these procedures, completely methylates fatty acids in 10 min with methyl iodide in 0.5 *M* aqueous potassium hydroxide at 65°C with N,N-dimethylacetamide as a catalyst. Excess methyl iodide is then destroyed with pyridine.

We previously described that aqueous bases could be used for the quantitative extraction of free fatty acids from other lipids in the hexane phase including TMTFTH, trimethylphenylammonium hydroxide, tetramethylammonium hydroxide, potassium carbonate and potassium hydroxide [6]. In the present study, the TMTFTH methylation procedure, which is a more straight-forward method, was initially our method of choice. However, 7 or 8 $\mu$l of the TMTFTH extract of the hexane phase had to be injected into the gas chromatograph to obtain adequate methyl ester peaks. This resulted in a large solvent response that obscured resolution of the internal standard and made quantitation difficult. In addition, this large volume of TMTFTH led to a bothersome blank with a complex of peaks at the same retention times as palmitic acid and other fatty acids of interest. Thus, this method was unsatisfactory for the "trace analysis" of FFA concentrations in small quantities of brain tissue.

For quantitation of FFA in 20 mg of tissue, blanks have to be analyzed with the methyl iodide procedure. These blanks contain significant amounts of the fatty acids of interest (Table IV). A small amount of these fatty acids may arise from desorption from the GLC system, i.e., "memory effects" [6], etc. However, the probable source is trace impurities in the reagents. Fatty acid con-

tamination from glassware was likely eliminated by soaking with methanolic potassium hydroxide as described under *Materials*. This blank is the factor which limits the size of the tissue sample which can be used for the analysis.

A stability study demonstrated no changes in the concentrations of methyl esters extracted into ethylene chloride for at least one week at room temperature (Table III) in agreement with our previous findings [7]. Thus, a large number of samples can be prepared on one day and the chromatography performed on subsequent days.

In summary, the present new method utilizes a non-adsorbing technique for isolation of FFA from other lipids in a hexane extract of brain tissue, i.e., extraction with aqueous base. The FFA are then methylated with methyl iodide directly in this medium, and their methyl esters are partitioned into ethylene chloride and quantitated by GLC. This method, in which FFA concentrations in 20-mg brain tissue can be analyzed will enable a measurement of regional FFA in brain following such insults as ischemia and trauma.

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