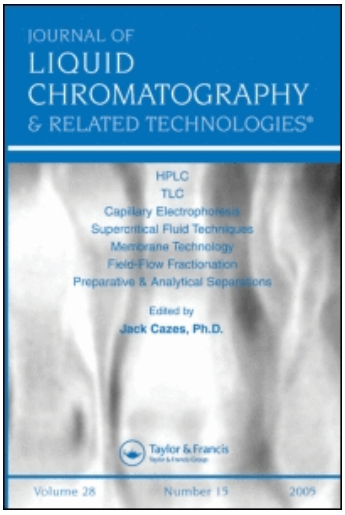

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Determination of Free Fatty Acids by Diphasic-Two Dimensional TLC-Fluorescence Spectrodensitometry

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DETERMINATION OF FREE FATTY ACIDS BY DIPHASIC-TWO DIMENSIONAL TLC-FLUORESCENCE SPECTRODENSITOMETRY

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ABSTRACT

A sensitive method for the determination of fatty acids is presented. Free fatty acids (20pg-1μg), were first covalently reacted in a displacement reaction with a 70 μM solution of the fluorescent probe, 4-bromomethyl-6,7-dimethoxy-coumarin (Br-Mdmc) and the fluorescence-labelled fatty acid esters were separated in a diphasic-two dimensional high performance thin layer chromatographic system (diphasic-2D-TLC). This system consisted of a reversed phase C18 layer (2 x 10 cm) interfaced with a $AgNO_3$-modified silica gel layer (10 x 10 cm). Aliquots of the reaction mixture were streaked onto the C18 layer, and the plates developed in the first dimension using acetonitrile-acetone-methanol-water (60:20:10:10, v/v/v/v) (solvent 1) as the mobile phase. Development in this dimension gave separation based on the number of carbons. Following the first development, the plates were dried and the silica gel layer impregnated with a saturated solution of $AgNO_3$ in methanol. The plates were then predeveloped in the second dimension in chloroform-ethyl acetate-acetonitrile (90:8:2, v/v/v)(solvent 2) to the plate interphase, and developed in solvent 2 in the second dimension. Development in the $AgNO_3$-modified silica gel, allowed separation based on the number of double bonds. The fluorescent bands were scanned with a Shimadzu CS-9000 spectrodensitometer in the fluorescent mode, using 352 nm as the excitation wavelength and a cut-off filter at 400 nm. Baseline resolution was obtained for all 15 fatty acids tested. The lower limit of detection was 300 fmol, with linear detector response extended to 0.13 pmol. This method provides a sensitive means of analysis of fatty acids present in biological systems.

INTRODUCTION

The coupling of $AgNO_3$-modified silica gel and reversed-phase layers in a single thin layer chromatography plate (TLC), for the separation of fatty acids, was originally described by Bergelson *et al.* (1). This reversed-phase layer, rather than being a bonded reversed-phase adsorbant, it consisted of a dodecane-impregnated silica gel. The latter impossed serious limitations on its applications due to the lack of reproducibility, poor resolution, and displacement of the dodecane during development in the $AgNO_3$-modified silica gel.

The labelling of fatty acids with the fluorescence probe, 4-bromomethyl-7-methoxycoumarin (Br-Mmc), followed by separation of the Br-Mmc esters by high performance liquid chromatography (HPLC) has been described (2-4). This method of analysis provided both, a carboxylic acid-specific mode of labelling, thus excluding aliphatic components that lack the carboxylic acid moiety, i.e.: fatty aldehydes, fatty alcohols. And high sensitivity.

In this study we report the separation of 4-bromomethyl-6,7-dimethoxycoumarin (Br-Mdmc)-derivatized fatty acid esters by diphasic-2D-TLC. In contrast to the original method described by Bergelson *et al.*, the reversed-phase layer utilized in this study consisted of a 2 x 10 cm C18-bonded silica layer rather than dodecane impregnated; and the sample is first applied and developed in the reversed-phase C18 layer rather than in the $AgNO_3$-modified silica gel layer. A unique advantage of a system with these characteristics, as compared to gas liquid chromatographic (GLC), or HPLC analysis of fatty acids, will be that the distribution of both endogenous fatty acids intrinsic to a biological system; and of the incorporated exogenous radiolabelled fatty acids to the same system, could be determined by a combination of fluorescence spectrodensitometry and digital scanning or X-ray film autoradiographic analysis, repectively.

MATERIALS AND METHODS

Reagents

The fatty acid standards lauric (12:0), myristic (14:0), myristoleic (14:1), palmitic (16:0), palmitoleic (16:1), stearic (18:0), icosanoic (20:0), docosanoic (22:0), oleic (18:1), linoleic (18:2), linolenic (18:3), icosatetraenoic (18:4), icosatrienoic (20:3), arachidonic (20:4), docosatrienoic (22:3), docosatetraenoic (22:4), docosapentaenoic (22:5), and docosahexaenoic (22:6) acids, were purchased from Sigma Chemical Co (St. Louis, MO). Radiolabelled [1-^{14}C]-lauric, [1-^{14}C]-myristic, [^{3}H]-myristoleic, [1-^{14}C]-palmitic, [^{3}H]-palmitoleic , [1-^{14}C]-stearic, [1-^{14}C]-icosanoic, [1-^{14}C]-docosanoic, [^{3}H]-oleic, [1-^{14}C]-linoleic, [1-^{14}C]-linolenic, [^{3}H]-icosatetraenoic, [^{3}H]-icosatrienoic, [U-^{3}H]-arachidonic, [1-^{14}C]-docosatrienoic, [1-^{14}C]-docosatetraenoic, [1-^{14}C]-docosapentaenoic, and [1-^{14}C]-docosahexaenoic fatty acids were purchased from Amersham Corp. (Arlington Heights, IL). Analtech diphasic plates (Unibond RP_{18}/silica gel, 10 x 12 cm,

250 μm thickness) were obtained from Analtech Inc. (Newark, DE, U.S.A.). Plastic glove bags were obtained from Instruments for Research Inc.(Cheltenham, PA). Solvents were EM Science chromatographic grade. Inorganic salts were from J.T. Baker (Phillisburg, N.J.) and of the highest purity available.

Fluorescence labelling

Free fatty acids were covalently linked in a displacement reaction to the fluorescence probe, 4-bromomethyl-6,7-dimethoxycoumarin (Br-Mdmc) following a modification of the procedure described by Dunges *et al.* (5): 2 x 10^{-5} to 1 μg of the fatty acid standards were placed in 1 x 10 cm reaction vials, and evaporated to dryness. Then 150 μl of a 70 μM solution of Br-Mdmc in acetone, containing 2 mg of Na_2CO_3 were added and the vials placed in a heating block at 50^0C for 30 min . The fluorescence-labelled Br-Mdmc fatty esters were then applied to Analtech diphasic plates.

Diphasic-two dimensional TLC

This system consisted of a reversed-phase C18 layer (2 x 10 cm) interfaced with a $AgNO_3$-modified silica gel layer (10 x 10 cm). One to five μl aliquots of the reaction mixture were streaked onto the C18 layer, the plates dried, predeveloped in $CHCl_3$-MeOH (1:1, v/v), and developed in the first dimension using CH_3CN-$(CH_3)_2CO$-MeOH-H_2O (60:20:10:10, v/v/v/v)(Solvent 1) as the mobile phase. Development in this dimension allowed separation based on the number of carbons. Following development in the first dimension, the plates were thoroughly dried, the silica gel layer impregnated with a saturated solution of $AgNO_3$ in methanol, to the interphase with the reversed-phase C18 layer, predeveloped in $CHCl_3$-$CH_3CH_2C00CH_3$-CH_3CN (90:8:2, v/v/v)(Solvent 2) in the second dimension to the plate interphase, and developed in Solvent 2. Development in the $AgNO_3$-modified silica gel layer allowed separation based on the number of double bonds. The resulting fluorescent bands were scanned in a Shimadzu CS-9000 spectrodensitometer in the fluorescence mode, using 352 nm as the excitation wavelength, and a cut-off filter at 400 nm. Baseline resolution was obtained for all the 15 fatty acids tested. The lower limit of detection was 300 fmol, with linear detector response extended to 0.13 pmol. The fluorescence labelled fatty acid esters were stable for up to 2 weeks when plates were kept in the dark. The diphasic 2D-TLC chromatograms corresponding to the radiolabelled fatty acids were either scanned in a Berthold two-dimensional digital autoradiograph; or exposed to X-ray film for 24 hr. Radioactive labelling of the fatty acids was also aimed to establish the reaction efficiency since the unreacted fatty acid and/or decomposition product(s) would be detected following scanning with the 2D-digital autoradiograph. The specific activities of the radiolabelled fatty acids applied to the plates were

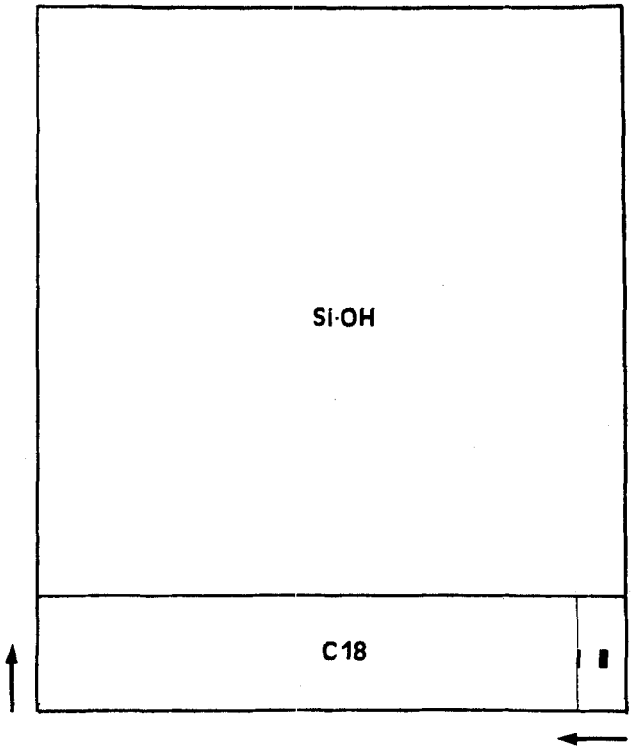

FIG. 1: Schematic representation of the diphasic two-dimensional TLC plate.

such as to compensate for the different efficiencies of ^{3}H and ^{14}C, to produce an equivalent detector response (either on the X-ray film or on the 2D-digital autoradiograph), and to produce fluorescence below the limit of detection.

In order to investigate wheather any oxidative degradation occurred during the process of separation of the Br-Mdmc unsaturated fatty esters, the manipulation of the plates, including predevelopment, drying, development, and impregnation with the $AgNO_3$ reagent, was conducted in plastic glove bags. The atmosphere inside the bags was equillibrated with nitrogen gas. Solvents were also purged with nitrogen.

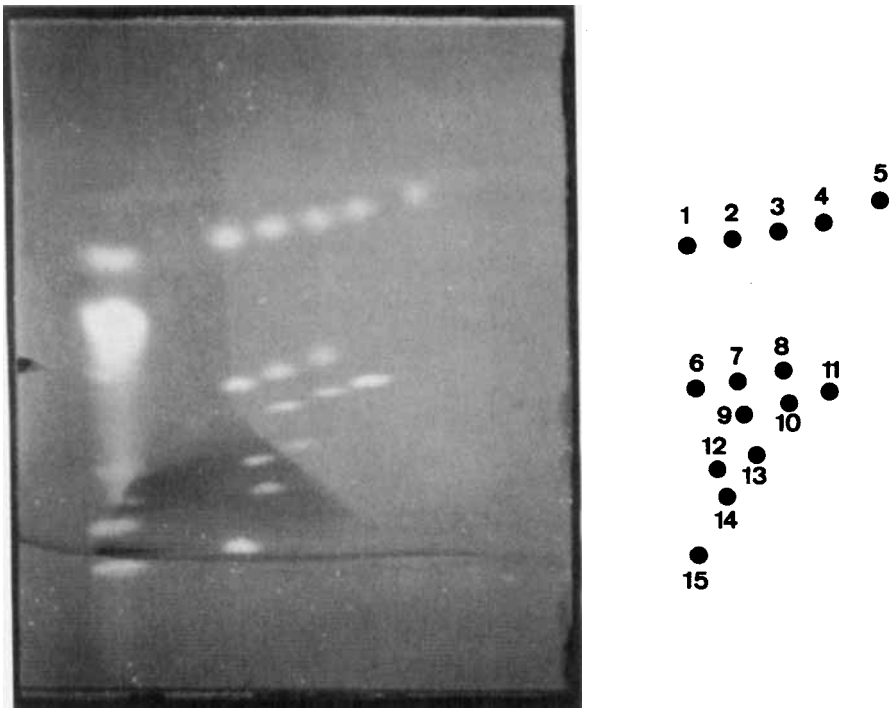

FIG. 2: 2D-chromatogram of Br-Mdmc-derivatized fatty acids.The numbers on the right panel correspond to the following fatty acids: **1**: 14:0; **2**: 16:0; **3**: 18:0; **4**: 20:0; **5**: 22:0 ;**6**: 16:1; **7**: 18:1; **8**: 20:1; **9**: 18:2; **10**: 20:2; **11**: 22:2; **12**: 18:3; **13**: 20:3; **14**: 20:4; and **15**: 22:6

RESULTS AND DISCUSSION

Fig. 1 depicts an schematic representation of the diphasic two-dimensional plate. The Br-Mdmc-derivatized fatty acids were applied to the C18 layer and developed in the first dimension. The plate was then turned 90^0 and developed in the second dimension, as indicated by the arrows. (see Materials and Methods for experimental details). Fig 2 shows a 2D-chromatogram of the Br-Mdmc-derivatized fatty acids following UV excitation at 365 nm. Development in the first dimension, resulted in separation based on the number of carbons and/or double bonds. That is, fatty acids containing longer methylene chains (18:0; 20:0; 22:0), were retained most by the C18 stationary phase; and those containing shorter methylene chains (12:0; 14:0), migrated closer to the solvent front (less retained by the stationary phase). This effect can be attributed to London

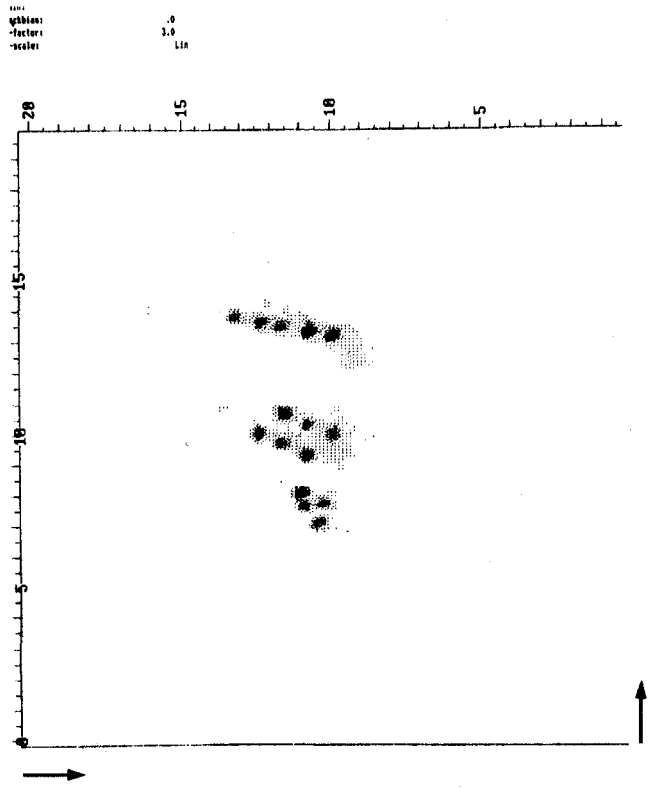

FIG. 3: 2D-radiochromatogram of the Br-Mdmc-derivatized radiolabelled fatty acids. The Br-Mdmc-derivatized radiolabelled fatty acids correspond to those separated in Fig. 2. Sample application and development in the first dimension went from the opposite side of the plate as compared to that of Fig 2., as indicated by the arrows.

forces interactions (non-permanent dipoles) between the hydrophobic methylene chains of the fatty acids, and the octadecyl chains of the stationary phase. At the same time, the faster migration of the unsaturated fatty acid derivatives could be caused by solvation of the stationary phase by the mobile phase, exposing the octadecyl groups to the more polar unsaturated fatty acid derivatives. Consequently, as a result of this dual mode of interaction, fatty acids containing longer methylene chains and higher number of double bonds, tended to overlap with those fatty acids containing shorter methylene chains and lower number of double bonds. The diagram of Fig 2 clearly depicts

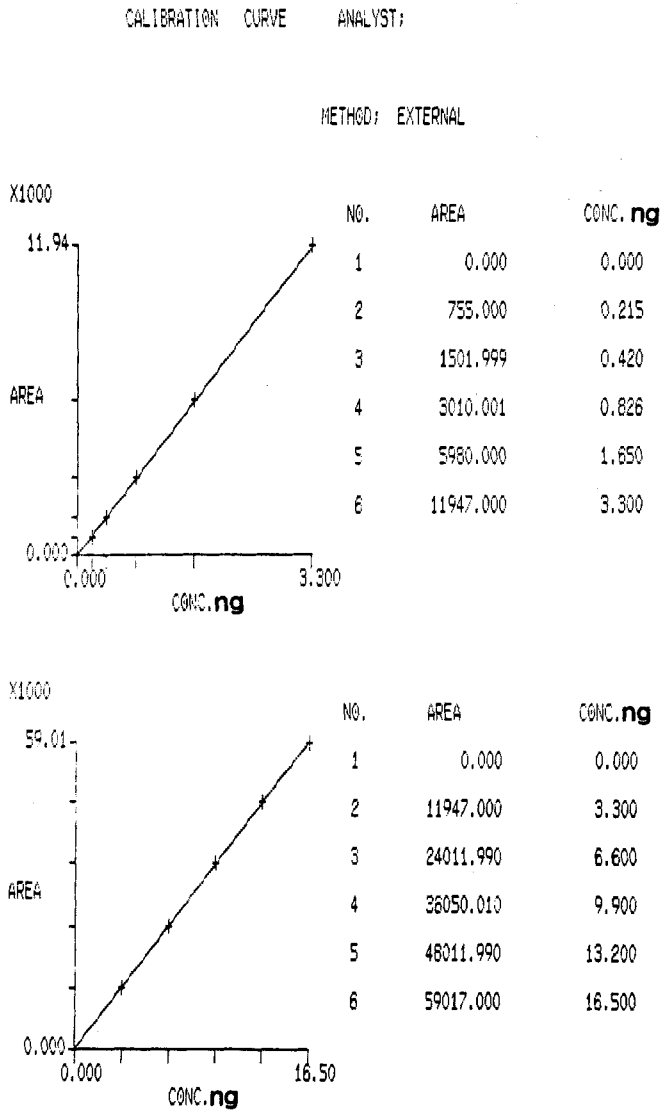

FIG. 4: Calibration plots corresponding to the Br-Mdmc-derivatized fatty acid esters. Each point represents the mean of 4 different experiments.

this effect for the following overlaps: 1/6/15; 2/7/9/14; 3/8/10; 4/11. This problem was resolved by developing the Br-Mdmc-derivatized fatty acids in the second dimension. Interaction between the π orbitals of the double bonds corresponding to the unsaturated fatty acids, and the Ag^+ ions present in the $AgNO_3$-modified silica gel, resulted in faster migration of the saturated fatty acid derivatives (1,2,3,4,5), followed by the mono (6,7,8), di (9,10,11), and polyunsaturated (12,13,14,15) fatty acid derivatives. Due to the hydrophobic effect, the fatty acid derivatives containing short methylene chains would tend to interact more with the polar silanyl groups of the silica gel, than those containing longer methylene chains, resulting in the faster migration of the latter. This effect is also illustrated in the diagram of Fig. 2 : 5>4>3>2>1.

Fig 3 shows a radiochromatogram of the Br-Mdmc-derivatives of the radiolabelled fatty acid esters. Since no radiactive material remains at the origin, or appears anywhere else in the plate, it can be concluded that the reaction yield is quantitative and that no oxidative degradation occurred during the process of separation, respectively. The concentration of the different Br-Mdmc-derivatized radiolabelled fatty acids applied to the diphasic-2D TLC system could be such as to fall below the limit of detection, when analyzed in the fluorescent mode (0.3 nmol); but detectable by the 2D-autoradiograph scanner. Thus, the application of such a mixture of Br-Mdmc-derivatized radiolabelled fatty acids (preferably the saturated and/or monounsaturated ones) along with the Br-Mdmc-derivatives from an unknown sample, could be utilized for identification purposes.

Analysis of the Br-Mdmc-derivatized fatty acids by spectrodensitometry at 352 nm in the fluorescence mode, resulted in a lower limit of detection of 300 fmol, with linear detector response extended to 0.13 pmol (Fig. 4). This method provides a sensitive means for the analysis of fatty acids present in biological systems.

ACKNOWLEDGMENTS

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