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6. AUTHOR(S)
Dr Sidney J. Stohs

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Departments of Pharmaceutical Sciences & Pharmacology
Creighton University Health Sciences Center
California at 24th Street
Omaha, NE 68178

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We have developed a HPLC method for the simultaneous determination of four lipid peroxidation products, namely, formaldehyde, malondialdehyde, acetaldehyde and acetone. This procedure has wide-spread applicability to exposure to environmental pollutants as well as the study of various disease states. Initial studies have clearly demonstrated that the in vitro exposure of peritoneal macrophages, mitochondria and microsomes to selected PCH results in the production of reactive oxygen species as well as a decrease in membrane fluidity. Furthermore, the administration of a variety of polyhalogented cyclic hydrocarbons to rats results in an induction of DNA damage as assessed by the formation of DNA single strand breaks. We are extremely pleased with the results which we have obtained to date. The results strongly support our basic hypothesis concerning the ability of polyhalogenated cyclic hydrocarbons to induce production of reactive oxygen species which may lead to membrane damage and the subsequent manifestation of toxic symptoms following exposures.

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Production of Reactive Oxygen Species by Polyhalogenated Cyclic Hydrocarbons (PCH)

by

Dr. Sidney J. Stohs
Departments of Pharmaceutical Sciences and Pharmacology
Creighton University Health Sciences Center
California at 24th Street
Omaha, NE 68178

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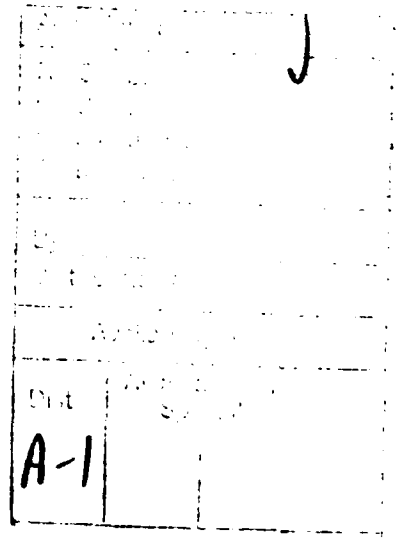


AFOSR Grant #90-0278
Dr. Sidney J. Stohs

I. INDUCTION OF LIPID PEROXIDATION AND EXCRETION OF LIPID METABOLIC PRODUCTS IN RESPONSE TO POLYHALOGENATED CYCLIC HYDROCARBONS (PCH)

One of our specific aims is the assessment of damage to tissues in response to an oxidative stress induced by polyhalogenated cyclic hydrocarbons (PCH). Lipid peroxidation is a potentially useful method for assessing oxidative damage, but to date, a highly specific, non-invasive method for assessing lipid peroxidation has not been developed. We have developed a method for simultaneously identifying formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) in urine, and have quantitated the excretion of these lipid metabolites in response to an oxidative stress induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), endrin, carbon tetrachloride and paraquat. The initial results are presented in detail in the attached manuscript which has been submitted for publication.

Urine samples of female Sprague-Dawley rats were collected over dry ice in metabolism cages. One ml aliquots of urine were derivatized with 2,4-dinitrophenylhydrazine, and extracted with pentane. The hydrazones of the four lipid metabolic products were quantitated by high pressure liquid chromatography on a Water μBondipak C_{18} column, eluting with

an acetonitrile:water system. The detector was set at 330 nm.

The identities of FA, ACT, MDA, and ACON in urine were confirmed by gas chromatography-mass spectrometry. An oxidative stress was induced by orally administering 100 μg TCDD/kg, 75 mg paraquat/kg, 6 mg endrin/kg or 0.25 ml carbon tetrachloride/100 G to rats. The urinary excretion of FA, ACT, MDA, and ACON increased relative to control animals 24 hrs after treatment with all xenobiotics (See Table 1 of manuscript). Thus, a convenient, non-invasive system has been developed for the simultaneous determination of four lipid metabolites. Furthermore, the results clearly demonstrate a marked increase in the excretion of these lipid metabolites in response to free radical-induced tissue injury. The system has wide-spread applicability to the investigation of altered lipid metabolism in disease states and exposure to environmental pollutants.

II. INITIATION OF IN VITRO PRODUCTION OF REACTIVE OXYGEN SPECIES BY ADDITION OF PCH TO PERITONEAL MACROPHAGES, MITOCHONDRIA AND MICROSOMES

We have begun to address a second major specific aim of our grant which is concerned with determining whether the direct addition of PCH to various membrane fractions and macrophages can initiate formation of reactive oxygen species such as superoxide anion. Measurement of chemiluminescence is a sensitive means of assessing the

production of reactive oxygen species, and a chemiluminometer has been purchased, and is being used.

Initial studies assessed the linearity of chemiluminescence production based on numbers of peritoneal cells used per ml in the assay system. These investigations indicated that between 5 X 10^5 and 3 X 10^6 cells per ml the production of chemiluminescence was linear (Figure 1). Subsequent studies are being conducted using 3 X 10^6 cells per ml.

The production of chemiluminescence as an index of the production of reactive oxygen species following the in vitro incubation of peritoneal cells with 100 ng/ml of either the phorbol ester PMA (positive control), TCDD, lindane and endrin are shown in Figure 2 of the attached set of data sheets. As can be seen in the figure, a time-dependent increase in produced in chemiluminescence by PMA, TCDD, lindane and endrin. The greatest production of chemiluminescence was produced by PMA. Similar studies are currently being conducted with chlordane and DDT.

Hepatic mitochondria and microsomes from rats have been isolated and similarly incubated in vitro with PMA, TCDD, lindane and endrin. The results of these initial studies are presented in Figures 3 and 4 for mitochondria and microsomes, respectively. As is observed with peritoneal macrophages, the in vitro incubation of mitochondria and

microsomes results in a time-dependent increase in the production of reactive oxygen species as assessed by the production of chemiluminescence. Similar studies will also be conducted with chlordane and DDT.

The dose-dependent effects of endrin on the in vitro production of reactive oxygen species are shown in Figure 3 for mitochondria, and Figure 5 for peritoneal macrophages. In both cases, a concentration-dependent effect is observed with endrin. Similar studies will be conducted with the other PCH. A manuscript regarding these studies is in preparation.

In an initial study, we have assessed the effect of incubating microsomal membranes in vitro with endrin, TCDD and lindane on microviscosity of the membranes. These results are presented in Figure 6. In each case, the PCH in question resulted in an increase in microviscosity which corresponds with a decrease in membrane fluidity. Thus, these initial results indicate that the in vitro incubation of PCH with microsomal membranes results in a decrease in membrane fluidity. This decrease in membrane fluidity may be either due to a physical effect of the PCH or may occur as a result of the production of reactive oxygen species. Subsequent studies will address this question.

Studies are currently under way to quantitate the superoxide anion production by peritoneal macrophage, hepatic microsomes, and hepatic mitochondria following in vitro exposure to the PCH being studied.

III. IN VIVO INDUCTION OF HEPATIC DNA DAMAGE BY PCH

We have initiated a series of studies on the induction of hepatic DNA damage in rats by the administration of selected PCH. The incidence of DNA single strand breaks has been determined by assessing the elution constants using the alkaline elution technique.

The incidence of DNA damage as produced by the administration of 3, 4.5 or 6 mg endrin/kg at 0, 12, 24, 48, and 72 hours post-treatment is presented in Figure 7. A dose-dependent increase in DNA damage was observed 12 and 24 hours after endrin administration. At 48 and 72 hours post-treatment, the incidence of DNA damage decreased relative to the 24 hour time point at all doses. The results clearly indicate that endrin induces hepatic DNA damage.

The effects of lindane, chlordane and DDT on DNA single strand breaks is presented in Figure 8. Lindane, chlordane and DDT were administered at a dose of approximately 2/3 of the LD_{50}'s. Time-dependent studies were conducted. Of these three PCH, only lindane significantly increased the

incidence of hepatic DNA damage 6 hours post-treatment. At 12 hours post-treatment, the greatest increase in DNA dam ge was induced by DDT. At 24 hours post-treatment, the incidence of DNA damage was elevated only in animals treated with DDT.

The above results indicate that a wide variety of structually dissimilar PCH are capable of inducing DNA damage. This DNA damage may in part explain toxic manifestations, as well as provide a plausible explanation for the carcinogenic potential of these compounds. Based on the studies described above, the mechanism for production of DNA damage may involve the induction of reactive oxygen species by these xenobiotics.

III. CONCLUSIONS

We have developed a HPLC method for the simultaneous determination of four lipid peroxidation products, namely, formaldehyde, malondialdehyde, acetaldehyde and acetone. This procedure has wide-spread applicability to exposure to environmental pollutants as well as the study of various disease states. Initial studies have clearly demonstrated that the *in vitro* exposure of peritoneal macrophages, mitochondria and microsomes to selected PCH results in the production of reactive oxygen species as well as a decrease in membrane fluidity. Furthermore, the administration of a

variety of polyhalogenated cyclic hydrocarbons to rats results in an induction of DNA damage as assessed by the formation of DNA single strand breaks.

We are extremely pleased with the results which we have obtained to date. The results strongly support our basic hypothesis concerning the ability of polyhalogenated cyclic hydrocarbons to induce production of reactive oxygen species which may lead to membrane damage and the subsequent manifestation of toxic symptoms following exposures.

Figure 1

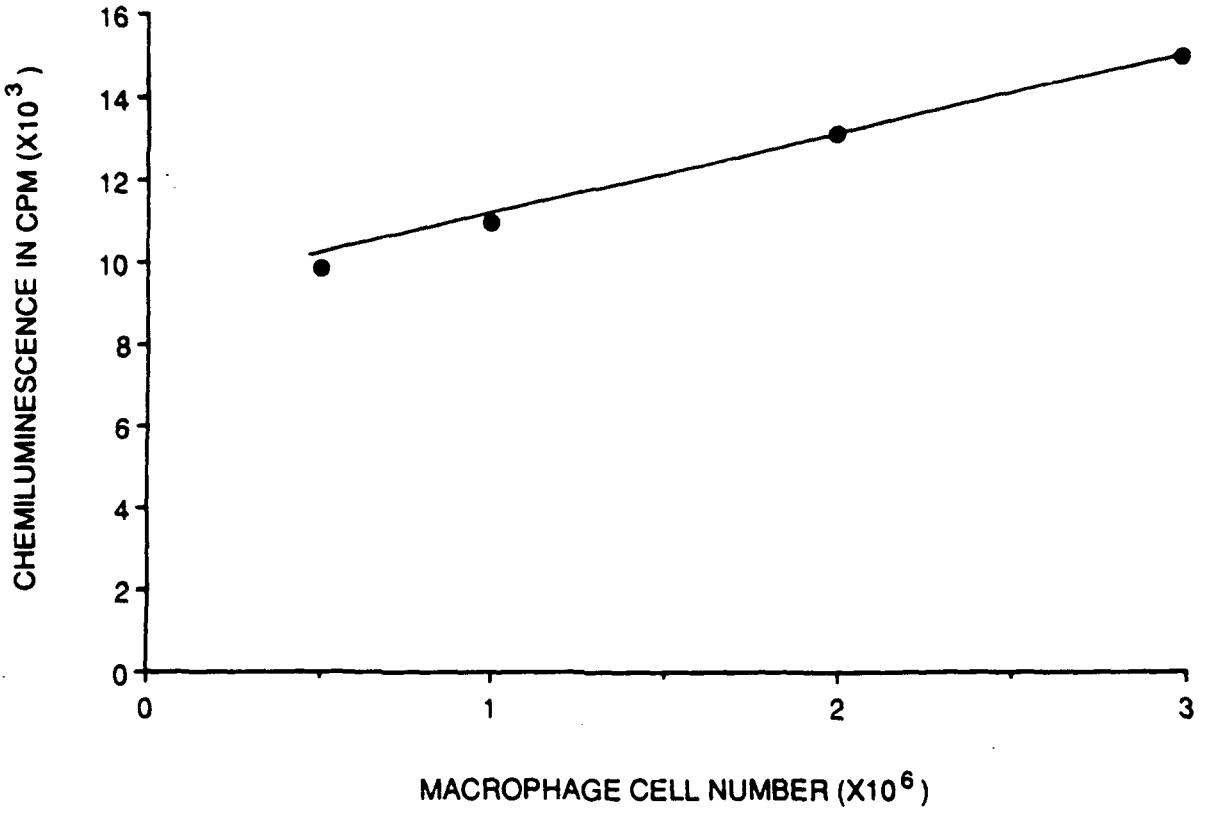


Figure 2

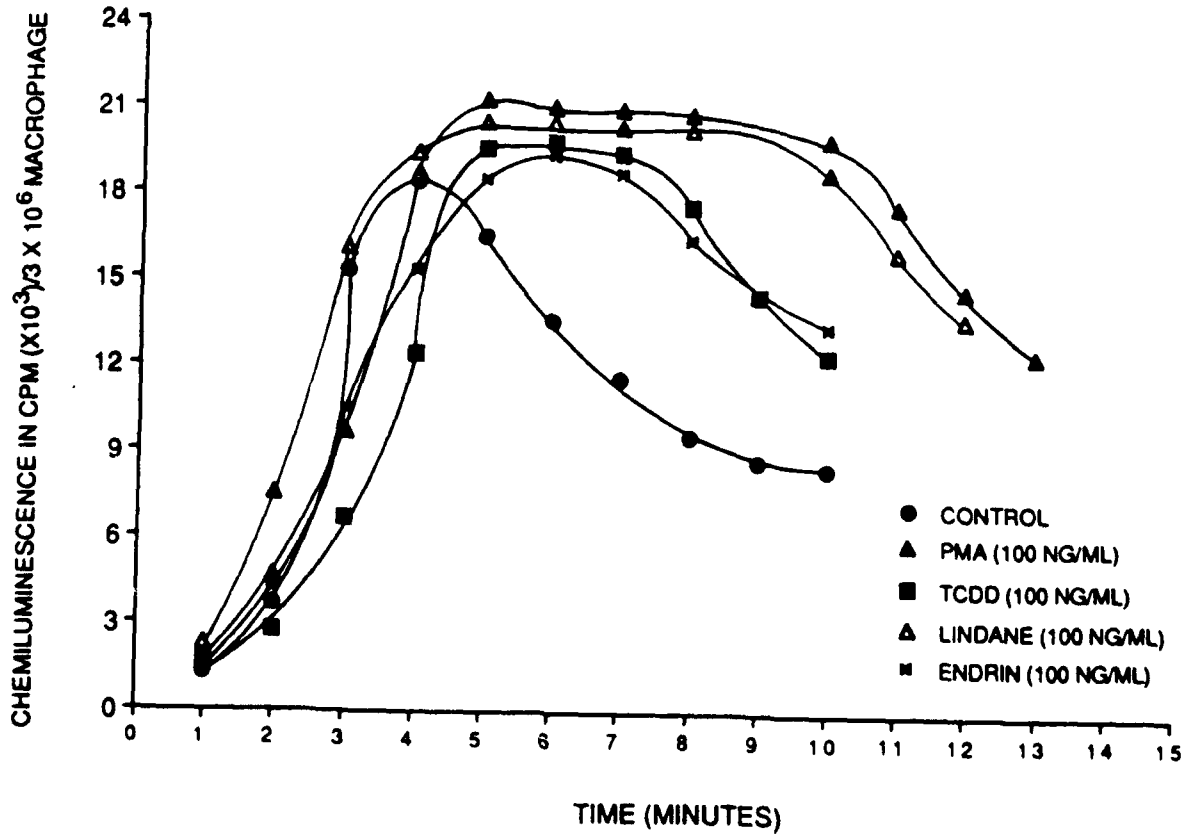


Figure 3

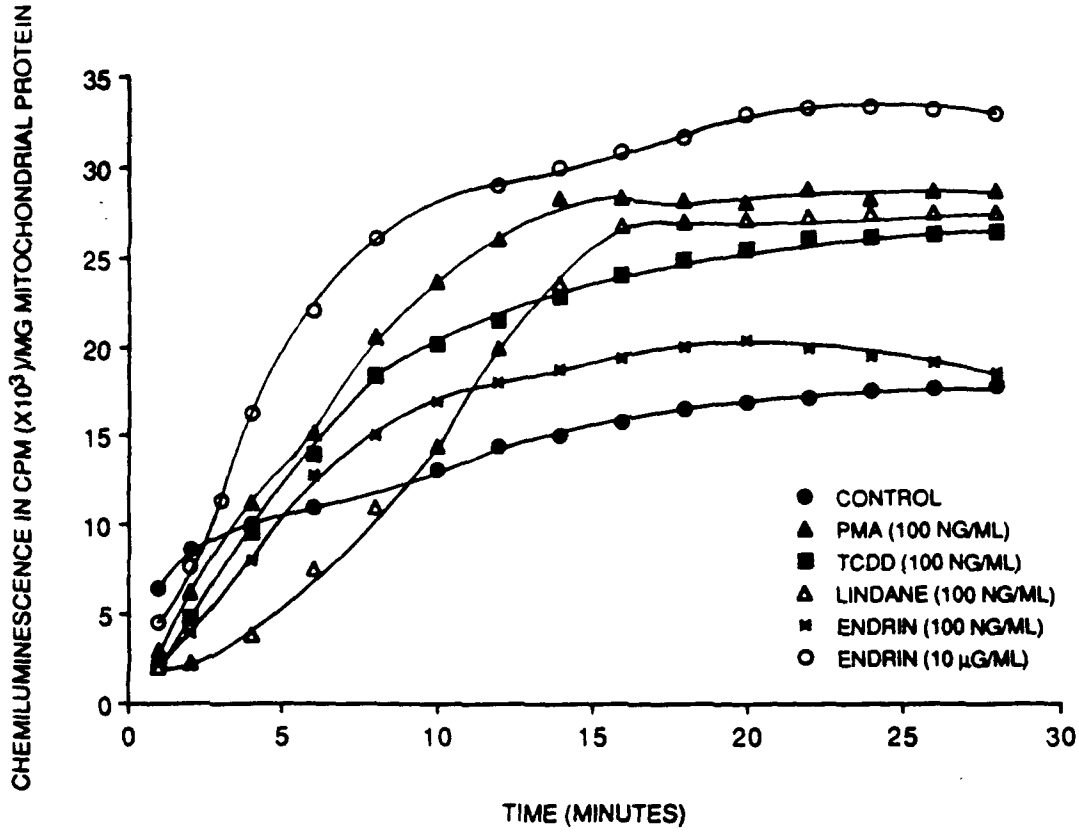


Figure 4

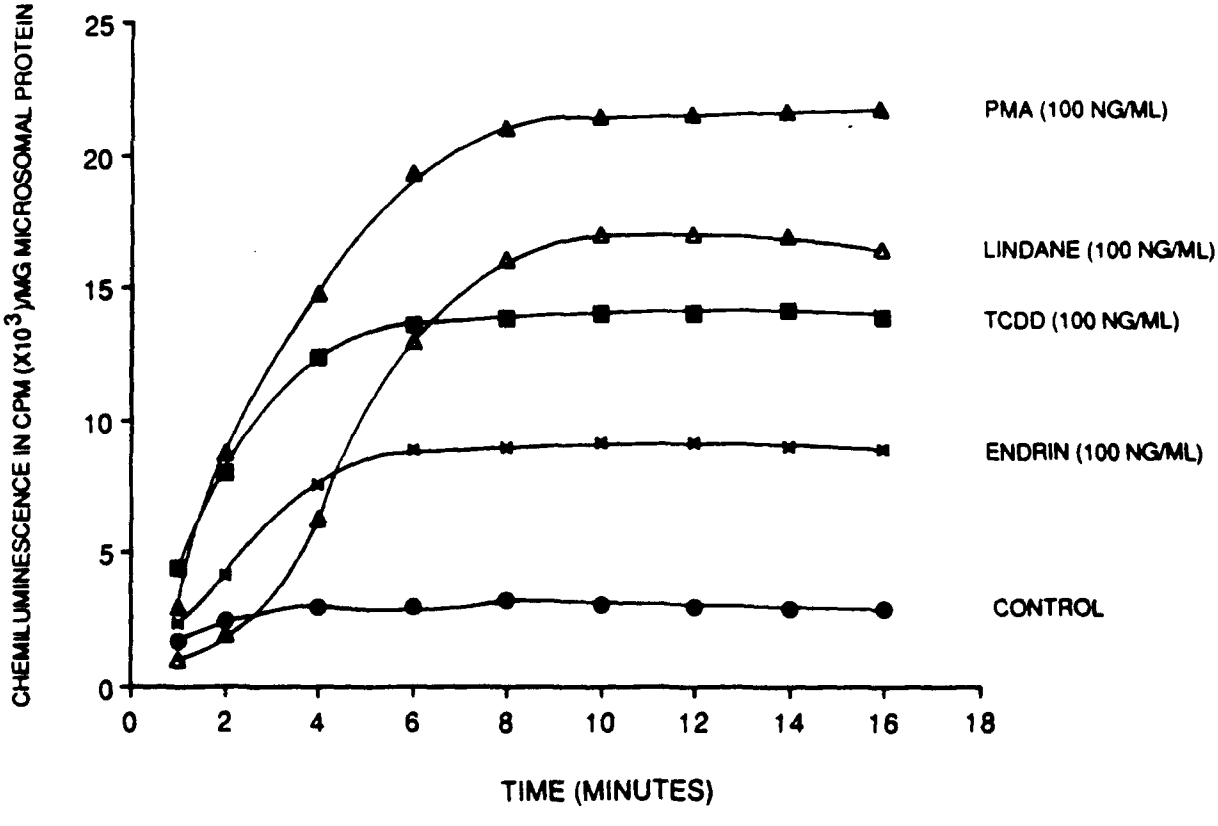


Figure 5

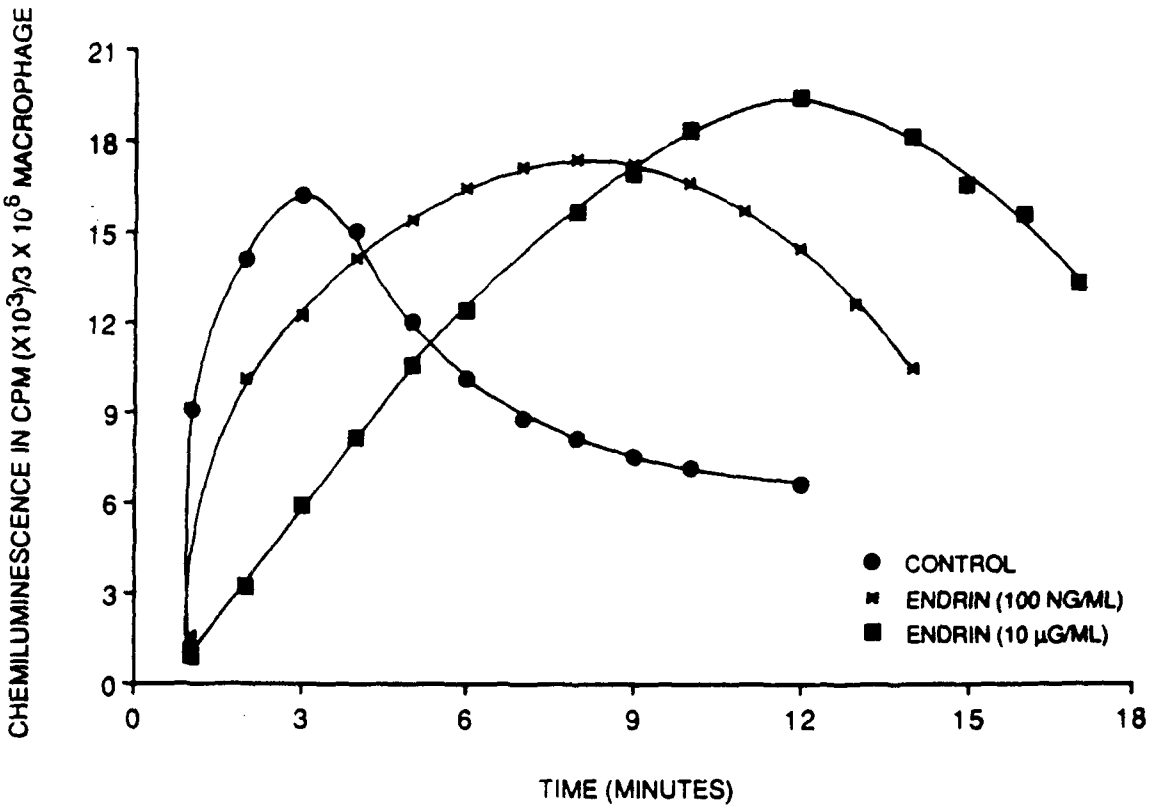


Figure 6

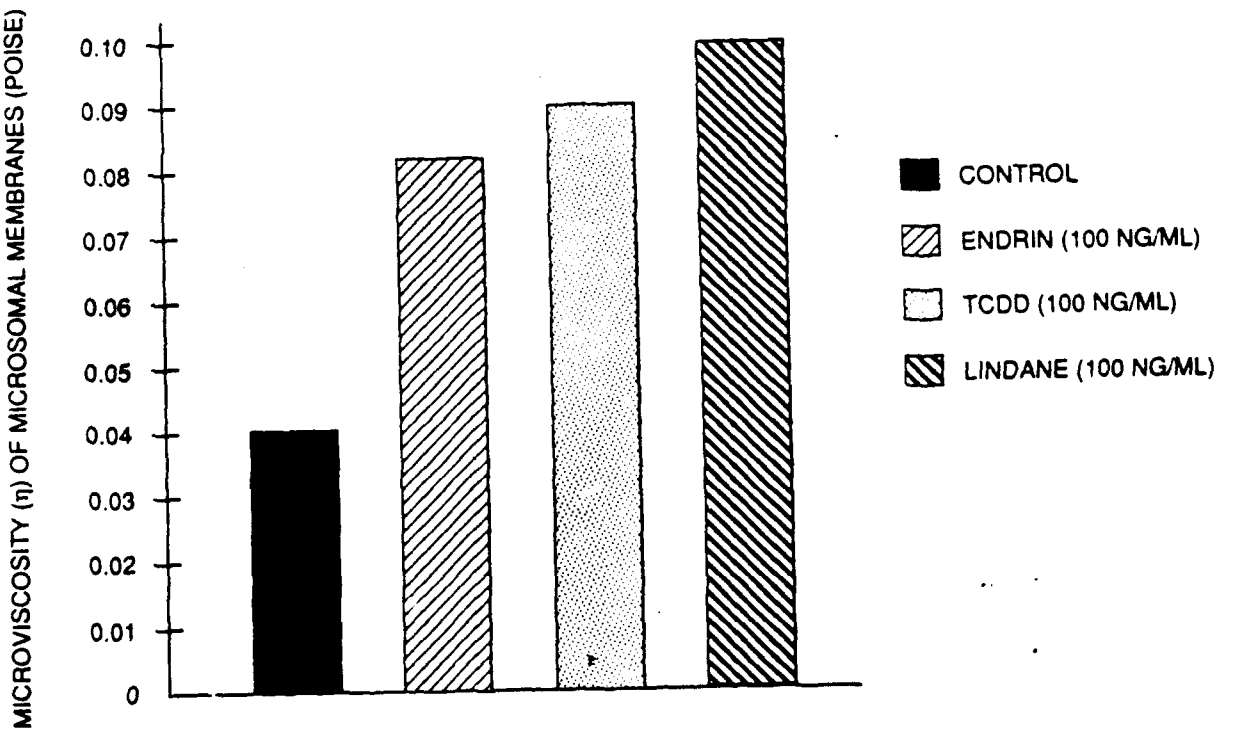


Figure 7

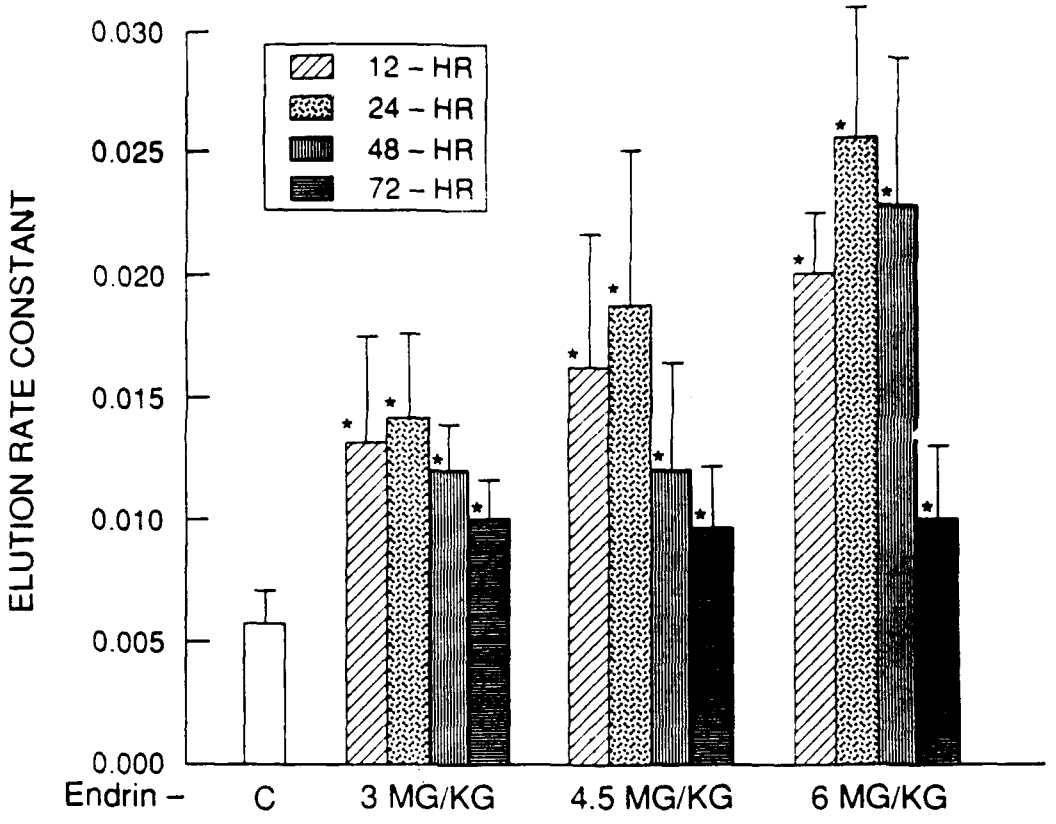
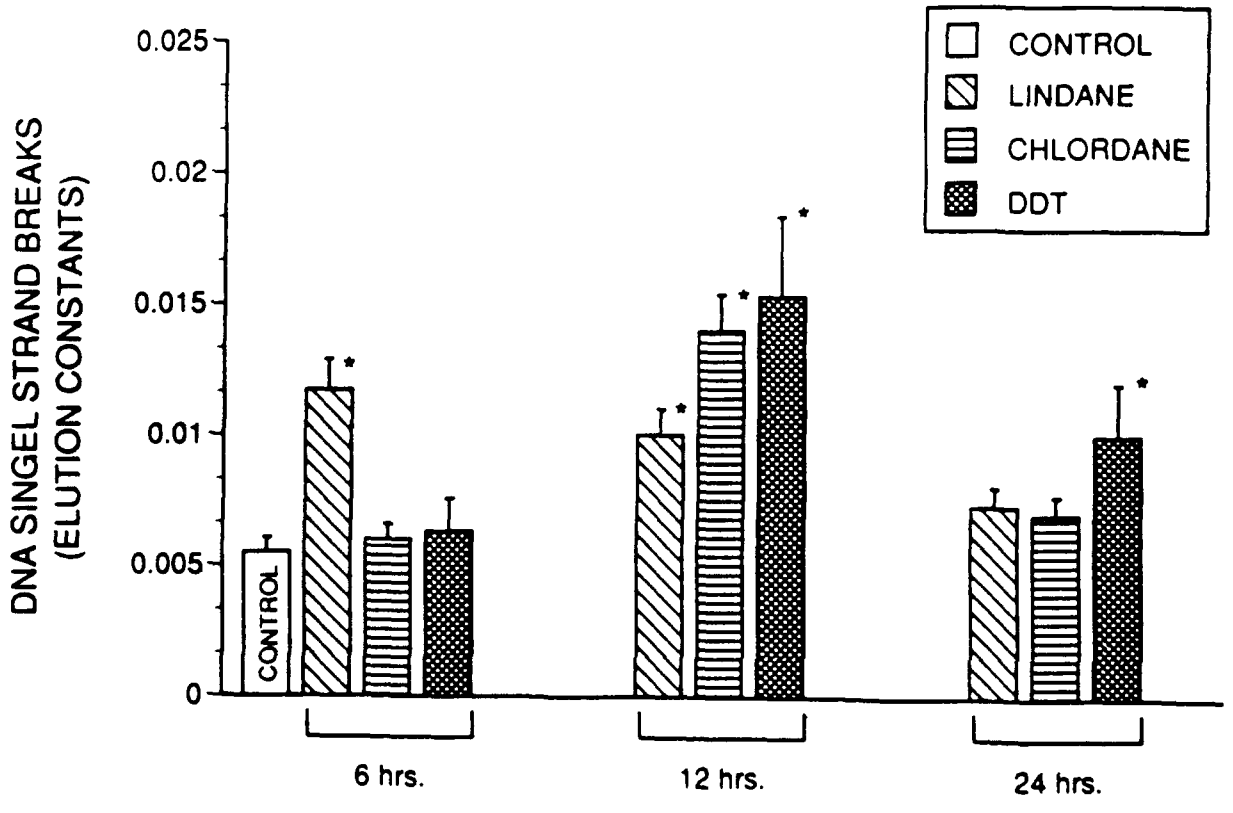


Figure 8



Excretion of formaldehyde, malondialdehyde, acetaldehyde and acetone in the urine of rats in response to 2,3,7,8-tetrachlorodibenzo-p-dioxin, paraquat, endrin and carbon tetrachloride

M.A. Shara, P.H. Dickson, D. Bagchi and S.J. Stohs

Creighton University Health Sciences Center, Omaha, NE 68178, U.S.A.

Address correspondence to: Dr. S.J. Stohs, School of Pharmacy and Allied Health Professions, Creighton University Health Sciences Center, California at 24th Street, Omaha, NE 68178 U.S.A.

Key Words: formaldehyde; acetaldehyde; malondialdehyde; acetone; high pressure liquid chromatography; 2,4-dinitrophenylhydrazine; gas chromatography-mass spectroscopy, 2,3,7,8-tetrachlorodibenzo-p-dioxin; paraquat; endrin; carbon tetrachloride.

Summary

Numerous environmental contaminants and pollutants induce oxidative stress and alter lipid metabolism. However, little information exists regarding the metabolic products of lipids which are excreted in the urine in response to oxidative stress. We have simultaneously identified formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) in urine, and quantitated the excretion of these lipid metabolites in response to chemically induced oxidative stress. Urine samples of female Sprague-Dawley rats were collected over dry ice in metabolism cages. One ml aliquots of urine were derivatized with 2,4-dinitrophenylhydrazine, and extracted with pentane. The hydrazones of the four lipid metabolic products were quantitated by high pressure liquid chromatography (HPLC) on a Waters μBondipak C_{18} column, eluting with an acetonitrile-water system. The detector was set at 330 nm. The identities of FA, ACT, MDA, and ACON in urine were confirmed by gas chromatography-mass spectrometry. An oxidative stress was induced by orally administering 100 μg 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)/kg, 75 mg paraquat/kg, 6 mg endrin/kg or 0.25 ml carbon tetrachloride/100 G to rats. The urinary excretion of FA, ACT, MDA, and ACON increased relative to control animals 24 hrs after treatment with all xenobiotics. Thus, a convenient, non-invasive system has been developed for the simultaneous determination of four lipid metabolites. Furthermore, the results clearly demonstrate a marked increase in the excretion of these lipid metabolites in response to free radical-induced tissue injury. The system has wide-spread applicability to the investigation of altered lipid metabolism in disease states and exposure to environmental pollutants.

INTRODUCTION

The peroxidation of membrane lipids is associated with a wide variety of toxicological effects, including decreased membrane fluidity and function, impaired mitochondrial and Golgi apparatus functions, inhibition of enzymes associated with various organelles including the endoplasmic reticulum, and impaired calcium homeostasis [1,2]. In many human diseases, membrane damage often occurs in an organ or tissue, which provokes lipid peroxidation and accelerates the disorder [3]. When lipid peroxides and peroxidation products accumulate, they leak from the organ or tissue into the bloodstream, and may be excreted in the urine [1,3]. Lipid peroxidation reflects the interaction between molecular oxygen and polyunsaturated fatty acids, resulting in the oxidative deterioration of the latter with the production of various breakdown products including alcohols, aldehydes, ketones and ethers [4,5].

The detection of lipid peroxidation products in the urine provides a non-invasive method of assessing lipid metabolism and oxidative stress. Ekstrom et al. [6] reported the detection of urinary malondialdehyde (MDA) after derivatization with 2,4-dinitrophenyl hydrazine (DNPH), and separation of the adducts by HPLC. Identification of the hydrazone derivative of MDA was based on HPLC retention time. Ekstrom et al. [7] also confirmed the identity of the MDA hydrazone standard by means of mass spectrometry. However, the identity of the MDA hydrazone from urine which had been derivatized with DNPH was not confirmed by mass spectrometry. Furthermore, no attempt was made to identify any of the other peaks which could be separated by HPLC. In the present study, four lipid metabolites have been identified in the urine of rats by HPLC and GC-mass spectrometry, and the effect of free radical-induced cell injury by TCDD, paraquat, endrin and carbon tetrachloride on the excretion of these metabolites has been examined.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats, weighing 140-160 g (Sasco, Inc., Omaha, NE) were used in these studies. All animals were acclimated 3-5 days prior to use. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from the Chemical Resource Program, National Cancer Institute (Bethesda, MD). TCDD was dissolved in corn oil containing 10% acetone, and was administered intragastrically at a single dose of 100 μg/kg [8]. The herbicide paraquat was dissolved in water and given orally at a single dose of 75 mg/kg [1,9]. The chlorinated cyclodiene insecticide endrin [10] and carbon tetrachloride [1,11] were dissolved in corn oil and administered orally at single doses or 6.0 mg/kg and 75 mg/kg, respectively. Control animals received the corresponding vehicles.

Urine Collection. Rats were placed in metabolism cages (Nalgene Co., Rochester, NY) for urine collection between 21.75 hrs and 26.15 hrs after treatment. During urine collection, the animals were allowed free access to tap water but received no food. The urine collecting vessels were positioned over styrofoam containers filled with dry ice which permitted the collection of urine in the frozen state over the 4.5 hr period. The collected urine was also free from contamination of food particles since the animals received no food during the urine collection period.

Derivitization and Extraction of Lipid Metabolites. 2,4-Dinitrophenylhydrazine (DNPH) was used as the derivatizing agent in the identification and quantitation of urinary metabolites. DNPH (310 mg) was dissolved in 100 ml of 2N HCl to make the derivatizing reagent. In 50 ml screw-capped Teflon lined tubes, 1.0 ml aliquots of urine, 8.8 ml of H_2O, and 0.20 ml (3.13 μmoles) of DNPH reagent were mixed, followed by the addition of 20 ml pentane. The tubes were intermittently shaken for 30 min, and the organic phases were removed. The aqueous phases were extracted with additional 20 ml aliquots of pentane. The pentane extracts were combined, evaporated under a stream of nitrogen in a 37° C

water bath, and reconstituted in 0.40 ml of acetonitrile. A 20 μl aliquot of each sample was injected onto the high pressure liquid chromatographic (HPLC) column and the peaks isocratically eluted as described below.

HPLC. The HPLC system consisted of a model 510 Waters pump (Milford, MA), a Waters model U6K loop injector, a 30 cm reverse phase (C_{18}) Bondapack column fitted with a 5 μ RP OD-GU pre-column cartridge, a model 484 Waters tunable absorbance detector and a Fisher Recordall (series 5000) strip chart recorder. The acetonitrile/water (49/51) mobile phase was filtered (0.45 μ, Nylon-66), degassed using a Millipore filtration kit (Rannin, Woburn, MA) and pumped at a flow rate of 1 ml/min. The detector was set at a wavelength of 330 nm and 0.01 absorbance units full scale (AUFS). The chart recorder speed was 0.25 cm/min. Following injection of a sample, the isocratic elution was carried out for 40 min.

Hydrazone Standards. Synthetic hydrazone derivatives were prepared by reacting 30 ml of DNPH stock solution with an excess (1-3 mmoles) of formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) or acetone (ACON). The reaction proceeded rapidly at room temperature. The precipitated hydrazones were filtered, dried and recrystallized from methanol. Solutions containing 50 ng/μl of the four synthetic hydrazones were prepared and chromatographed as described above. Urine samples were spiked with known amounts of each of the synthetic hydrazones to identify and/or confirm the urinary hydrazones by HPLC co-elution.

Acetonitrile solutions (100 μg/ml) of the four synthetic hydrazones were prepared and a UV-visible scan between 500-300 nm was obtained in a Perkin-Elmer Lambda 6 spectrophotometer in order to determine the absorption maxima for the four hydrazones.

To calculate the percent extraction recoveries, 0, 0.5, 1, 2, 3 or 5 μg of each of the synthetic hydrazones of FA, ACT, MDA and ACON were added to 1.0 ml urine samples

obtained from control animals during a single collection. The same extraction procedure described above was applied and the percent recoveries were calculated.

Calibration curves for each of the synthetic hydrazones were generated by chromatographing 10-700 ng of each and measuring the peak heights. Peak heights were proportional to the amount of hydrazone injected. The data were expressed as pmoles/12 hrs/kg body weight.

Gas Chromatography - Mass (GC-MS) Spectroscopy. In order to determine the identity of lipid excretion products in the urine, GC-MS was applied. The GC-MS system consisted of a Hewlett-Packard GC model 5890 with a 15 m capillary column (Supelco SPB-5, Bellefonte, PA) of 0.32 mm internal diameter and 0.25 μ film thickness. The injector was operated in the splitless mode. The temperature program was set as follows: starting temperature was 75° C and increased to 175° C at increments of 25° C/min. Between 175° C and 200° C the temperature was increased at a rate of 5° C/min, and finally to 300° C at increments of 25° C/min. The carrier gas was helium at an average linear velocity of 65.8 cm/sec. The mass spectrometer was a Finnigan Incos 50 quadrupole instrument with an ionization energy of 70 eV, and was set on electron ionization mode. The system was coupled to a Data General computer and a Printronix model MVP printer.

For GC-mass analysis, the four hydrazone derivatives of FA, AC, MDA and ACON were dissolved in chloroform (50 ng/μl). Similarly, hydrazine-derivatized urine samples were reconstituted in chloroform. Samples (2 μl) of standards and extracts were injected onto the GC-MS.

Following the full spectrum identification of each of the hydrazones, a selected ion monitoring (SIM) program was prepared and additional spectra were obtained in the SIM mode.

Statistical Methods. Significance between pairs of mean values was determined by Student's *t* test. A $P < 0.05$ was considered significant for all analyses.

RESULTS

Identification of Urinary Lipid Metabolites

Utilizing HPLC and GC co-elution methods as well as MS techniques, malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT) and acetone (ACON) were identified as urinary lipid metabolites.

UV-visible scan studies indicated that the absorption maxima of the four synthetic hydrazones of MDA, FA, ACT and ACON were 307, 349, 359 and 362 nm, respectively. Therefore, 330 nm was routinely used to monitor these compounds by HPLC. The extraction recoveries of the MDA, FA, ACT and ACON hydrazones were 74, 78, 88 and 91%, respectively, based on studies involving the addition of known amounts of the hydrazone derivatives to control urine.

Figure 1A depicts typical HPLC chromatograms of the four hydrazone standards, while Figures 1B and 1C contain representative HPLC chromatograms from urine of control and TCDD-treated rats, respectively. Figure 2A is a typical GC-elution profile of the hydrazones of standard FA, ACT, MDA and ACON, while Table I contains a summary of the HPLC and GC retention times of the four hydrazones. The retention times for the standards exactly corresponded with the chromatographic peaks for extracts of urine, and co-elution occurred when the standards were added to the urine samples for both HPLC and GC.

The order of elution of the four hydrazones upon GC was similar but not identical to that of the HPLC. Figure 2B is a typical GC profile of the hydrazone derivatives extracted from a urine sample. There are two notable differences as compared to the HPLC profile. The formaldehyde (FA) derivative (5.4 min) eluted first upon GC, and the acetone (ACON) derivative (7.4 min) eluted last. The order of GC elution of MDA and

FA was reversed in contrast to the order observed on HPLC. In addition, the acetaldehyde (ACT) derivative exhibited two peaks (6.3 and 6.6 min), corresponding to its syn and anti isomers which were separated by GC but not by HPLC (Figures 1A and 2A).

The MS data for the four lipid metabolites are presented in Figures 3-6. The molecular ions 210, 234, 224 and 238 of the synthetic hydrazones of FA, MDA, ACT and ACON, respectively, were identified (Figures 3A, 4A, 5A, and 6A). The same molecular ions were demonstrated in extracts of urine samples following GC-MS (Figures 3B, 4B, 5B and 6B). It should be noted that the MS of urinary MDA (Fig. 4B) also contains peaks of FA since the GC separation of the MDA and FA was incomplete. However, the results clearly show that FA, MDA, ACT and ACON were excreted in the urine of rats.

Selective ion monitoring was also used to provide further confirmation of the identity of the four lipid metabolites in urine. Ion chromatograms for a mixture of the hydrazones of the four reference standards and an extract of urine are presented in Figures 7A and 7B, respectively. The ions which were selected in this display mode are 158 and 234 for MDA, 210 for FA, 224 for ACT, and 238 for ACON. A comparison of Figures 7A and 7B clearly demonstrates that the mass ions produced by the four standards were associated with the components of urine that were separated by GC. The results provide further confirmation of the identities of the four lipid metabolites in the urine of rats.

Quantitation of Urinary Lipid Metabolites. Previous studies by Ekstrom and associates [7] have quantitated the urinary excretion of MDA by HPLC following treatment with hydroquinone and chloroform. No other urinary lipid peroxidation products were examined or identified. However, several large peaks eluting downfield from the MDA peak are readily observable on the HPLC chromatogram published by Ekstrom and co-workers [7] but were not identified.

The results in Table II provide quantitative data for control, and TCDD-, paraquat-, endrin-, and carbon tetrachloride-treated animals 24 hrs post-treatment. The results are

presented as nmoles/kg body weight/4.5 hrs. As can be seen, these four toxicants produced varying increases in the excretion of MDA, FA, ACT and ACON as compared to the results for control animals. Of the four metabolites, FA was excreted in the greatest amount. The greatest increase (10.7-fold) in the excretion of the four metabolites occurred with ACON in response to paraquat. Paraquat administration also resulted in a 2.4-fold increase in ACT excretion, while paraquat and endrin produced 2.2- and 2.1-fold increases in FA excretion.

DISCUSSION

Formaldehyde (FA), acetaldehyde (ACT), malondialdehyde (MDA) and acetone (ACON) have been identified and quantitated in the urine of rats employing a single HPLC system. Furthermore, four toxicants with apparently different mechanisms of free radical-induced cell injury [1,8,10] all enhance the excretion of these four lipid metabolites [Table II]. Direct comparisons of urinary lipid metabolites in response to the four toxicants can not be made since the effects are dependent upon dose and the differing toxicokinetics of each xenobiotic. Furthermore, urine collections were made at only a single interval.

Previous studies have identified MDA in various biological matrices by means of diverse chromatographic techniques. Draper et al. [12] applied HPLC procedures to thiobarbituric acid derivatized urine samples, and were able to identify low levels of MDA after an acid hydrolysis procedure was applied. Alterations in free MDA levels in the urine of rats treated with the herbicide paraquat have been reported by Tomita et al. [9]. Paraquat is known for its redox cycling and ability to induce an oxidative stress [9].

Ekstrom et al. [6] reported the detection of urinary MDA after derivatizing with DNPH and separating the adducts by HPLC procedures. The identity of the MDA hydrazone was confirmed only by retention time. Later, Ekstrom et al. [7] confirmed the identity of the MDA hydrazone derivative standard by means of mass spectrometry. However, the identity of MDA from urine which had been derivatized with DNPH was not

confirmed by mass spectrometry. Furthermore, no attempt was made to identify any of the other peaks clearly present on the UV-visible trace of the chromatograph.

Serum MDA has been identified by Kawai et al. [13] using similar HPLC techniques to those of Ekstrom et al. [7], while Largilliere and Melancon [14] determined free MDA in plasma by HPLC. Lee and Csallany [15] have assessed free MDA in rat liver by HPLC. Tomita et al. [16] reported a method for the determination of urinary MDA by means of gas chromatography and electron-capture detection utilizing pentafluorophenylhydrazine as the derivatizing agent. Poli et al. [17] identified MDA, 4-hydroxynonenal, propanol, butanone and hexanal in liver utilizing a combination of thin-layer chromatography and HPLC techniques. However, FA, ACT and ACON were not identified.

Free ACT in blood has been determined as the DNPH derivative by HPLC [18]. Formaldehyde and ACT have been identified in urban air [19] and industrial surfactants [20] as their DNPH derivatives by HPLC. However, these procedures have not been applied to biological samples.

The sources of the four lipid metabolites which have been identified are not entirely clear. The increases in these products may be due to either lipid peroxidation or beta oxidation. Dhanakoti and Draper [11] demonstrated that urinary MDA excretion was enhanced following administration of the known liver toxin carbon tetrachloride and the free radical generating anthracycline antineoplastic antibiotic adriamycin. Furthermore, the fate of radiolabeled MDA administered to rats was examined. MDA appeared to be extensively metabolized to acetate and carbon dioxide. Based on these observations, the urinary acetaldehyde identified in this study may arise from the breakdown of MDA which is formed due to lipid peroxidation. Previous studies have shown that TCDD [20], paraquat [1], carbon tetrachloride [1], and endrin [10] produce marked increases in the formation of MDA and other thiobarbituric acid reactive substances in the liver.

The enhanced formation of ACON in response to disease states such as diabetes as a consequence of enhanced beta oxidation is also well known [21]. Winters et al. [22]

reported that rat liver microsomes metabolized glycerol to FA. Glycerol is a product of the metabolism of triglycerides by adipose tissue and other tissues that possess the enzyme that activates glycerol, namely, glycerol kinase. Liver and brown tissue are known to have high glycerol kinase levels [22]. Other possible sources of FA might include the breakdown of MDA to acetate or ACT and a one carbon fragment [11], and/or the cleavage of a one carbon fragment from acetoacetic acid with the formation of ACON.

The HPLC methodology used in conjunction with the application of GC-MS provides conclusive identification and quantitation of MDA, FA, ACT and ACON excretion in the urine of rats. Furthermore, an increased excretion of these lipid metabolites occurs in response to free radical-induced cell injury. The methodology is simple and requires relatively little work-up time. The preparation of a 1.0 ml urine aliquot can be completed in less than 2 hrs including the elution by HPLC. Furthermore, excellent reproducibility and sensitivity are achieved. The detection limit is approximately 50 picomoles for any of the four lipid metabolites in a 1.0 ml urine aliquot in a 20 μl injection volume. Numerous applications of this procedure exist for the study of exposure to environmental pollutants as well as altered lipid metabolism in various disease states.

ACKNOWLEDGEMENTS

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TABLE I

Retention times of urinary lipid metabolites upon high pressure liquid chromatography (HPLC) and gas chromatography (GC)

Hydrazone Derivative of	Retention time (minutes)	
	HPLC	GC
Malondialdehyde (MDA)	9.6	5.4
Formaldehyde (FA)	11.2	5.1
Acetaldehyde (ACT)	14.6	6.3, 6.6
Acetone (ACON)	19.5	7.4

TABLE II

Excretion of malondialdehyde, formaldehyde, acetaldehyde and acetone by rats treated with endrin, carbon tetrachloride, paraquat and TCDD[1]

Excretion Product	nmoles/kg body weight/4.5 hours				
	Control	Endrin Treated (4.5 mg/kg)	Carbon Tetrachloride Treated (0.25 ml/100 G)	Paraquat Treated (75 mg/kg)	TCDD Treated (100 mg/kg)
Malondialdehyde (MDA)	1.45 ± 0.23	3.67 ± 0.31*	1.94 ± 0.26*	3.69 ± 0.35*	1.77 ± 0.16
Formaldehyde (FA)	32.87 ± 2.11	70.60 ± 4.81*	43.56 ± 3.52*	73.61 ± 5.91*	49.31 ± 3.58*
Acetaldehyde (ACT)	1.77 ± 0.28	3.41 ± 0.29*	2.31 ± 0.32*	7.25 ± 0.54*	2.49 ± 0.20*
Acetone (ACON)	2.81 ± 0.31	7.92 ± 0.47*	5.71 ± 0.57*	30.01 ± 2.13*	5.29 ± 0.63*

[1]Each value is the mean of four animals. Urine was collected from 21.75 hrs to 26.15 hrs post-treatment (4.5 hrs).

*$P < 0.05$ with respect to the control group.

LEGENDS

FIG. 1. HPLC of DNPH derivatives of malondialdehyde (MDA), formaldehyde (FA), acetaldehyde (ACT), and acetone (ACON): A - standards; B - urine from control animals; C - urine from TCDD-treated animals.

FIG. 2. A - Full scanning GC of DNPH derivatives of formaldehyde (FA), malondialdehyde (MDA), acetaldehyde (ACT), and acetone (ACON) standards; B - Full scanning GC of rat urine.

FIG. 3. A - Mass spectrum of DNPH derivative of formaldehyde standard; B - mass spectrum of DNPH derivatized formaldehyde in rat urine.

FIG. 4. A - Mass spectrum of DNPH derivative of malondialdehyde standard; B - mass spectrum of DNPH derivatized malondialdehyde in rat urine.

FIG. 5. A - Mass spectrum of DNPH derivative of acetaldehyde standard; B - mass spectrum of DNPH derivatized acetaldehyde in rat urine.

FIG. 6. A - Mass spectrum of DNPH derivative of acetone standard; B - mass spectrum of DNPH derivatized acetone in rat urine.

FIG. 7. A - Selective ion monitoring for the DNPH derivatives of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224), and acetone (238) standards; B - Selective ion monitoring of the DNPH derivatives of malondialdehyde (158, 234), formaldehyde (210), acetaldehyde (224), and acetone (238) from rat urine.

Fig. 1

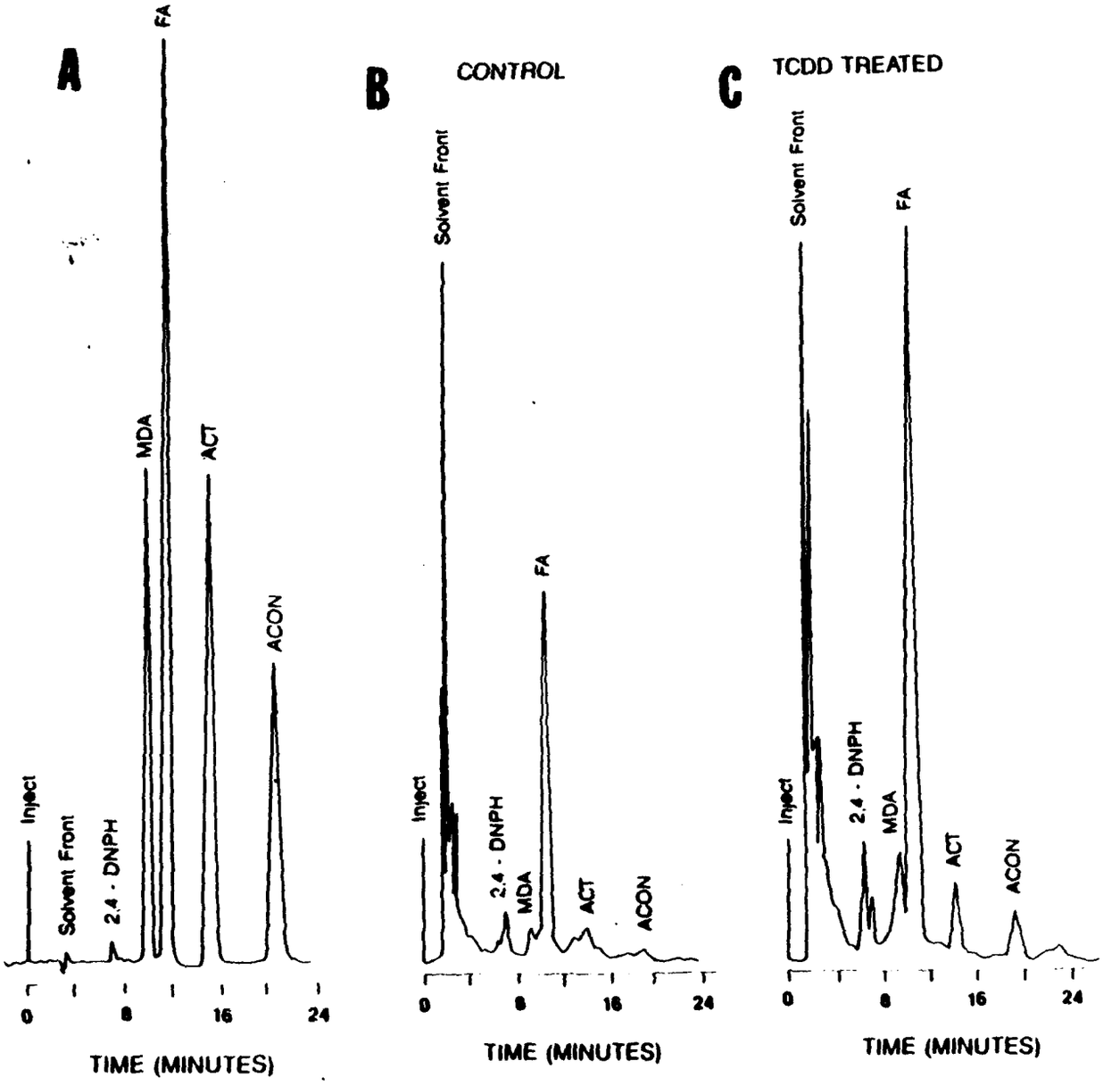
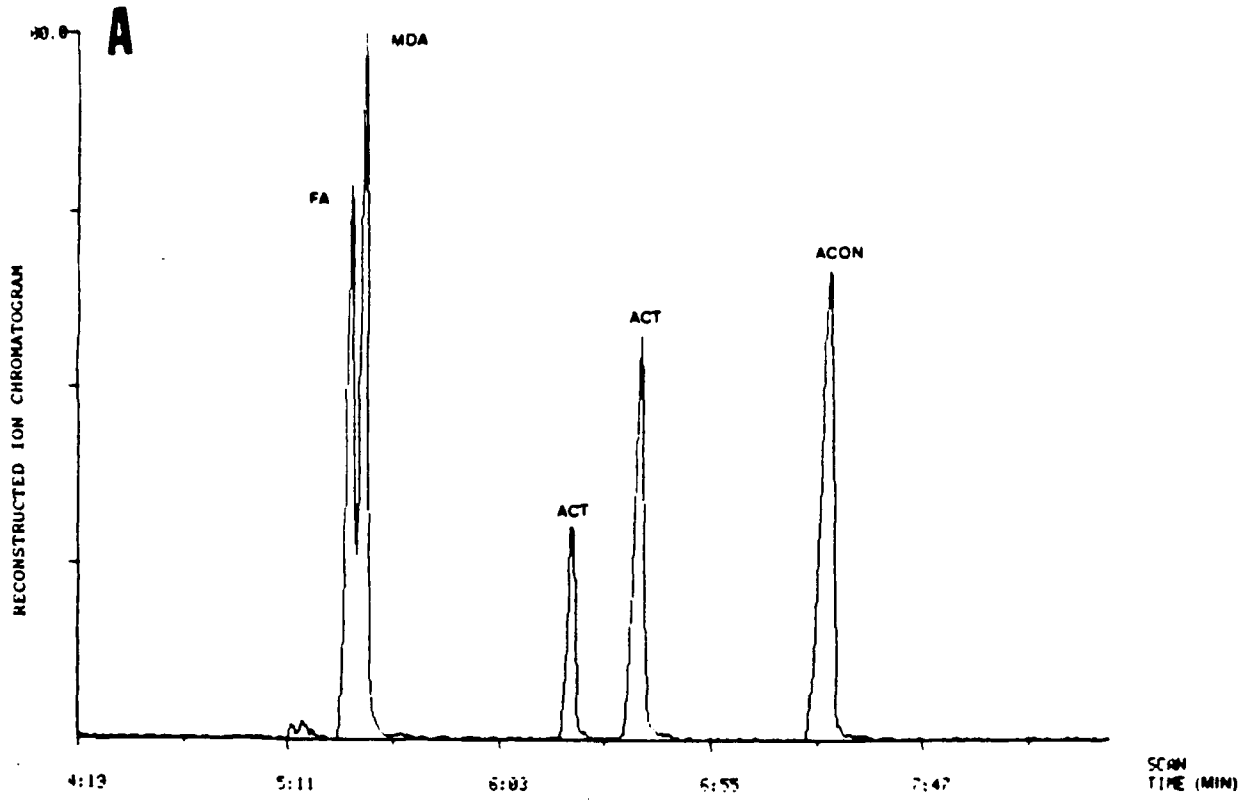

A
FA
MDA
ACT
ACON
Inject
Solvent Front
2,4 - DNPH
0
8
16
24
TIME (MINUTES)
B
CONTROL
Solvent Front
FA
Inject
2,4 - DNPH
MDA
ACT
ACON
0
8
16
24
TIME (MINUTES)
C
TCDD TREATED
Solvent Front
FA
Inject
2,4 - DNPH
MDA
ACT
ACON
0
8
16
24
TIME (MINUTES)

Fig. 2


A
MDA
FA
ACON
ACT
ACT
RECONSTRUCTED ION CHROMATOGRAM
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5:11
6:03
6:55
7:47
SCAN
TIME (MIN)

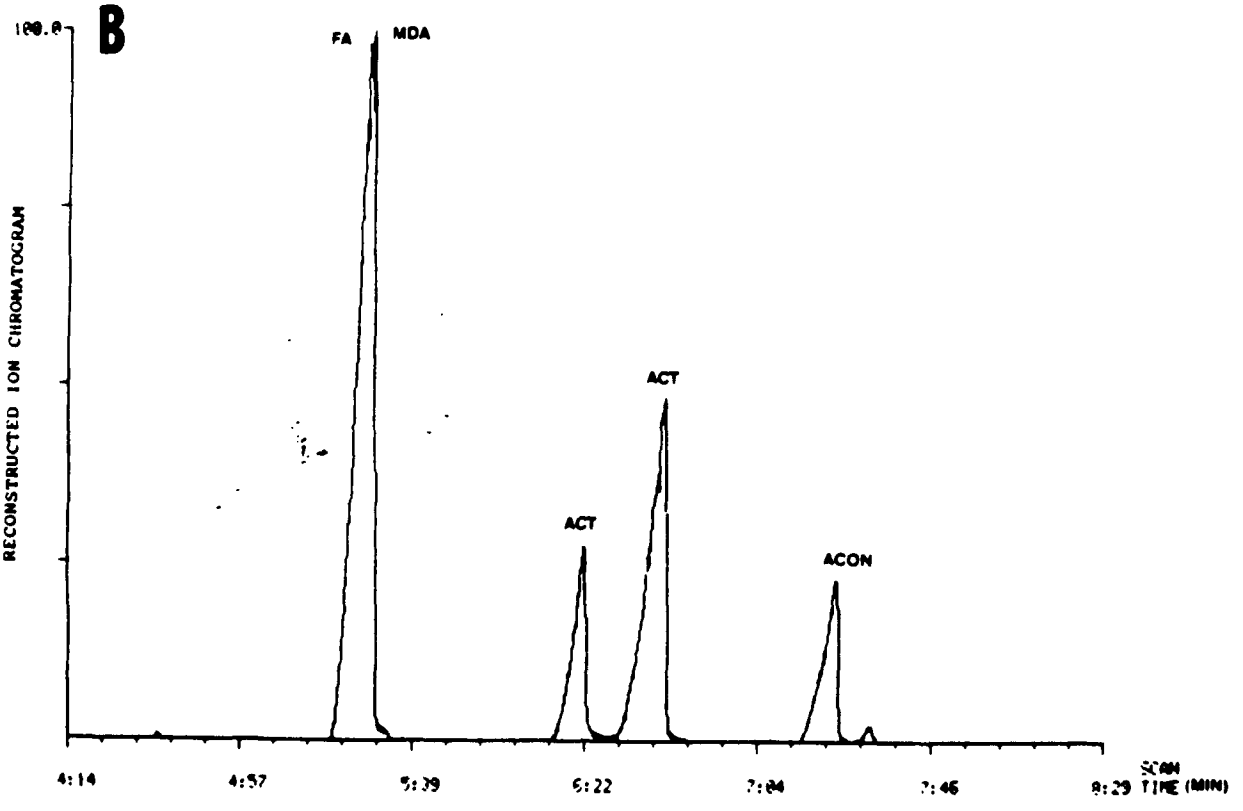
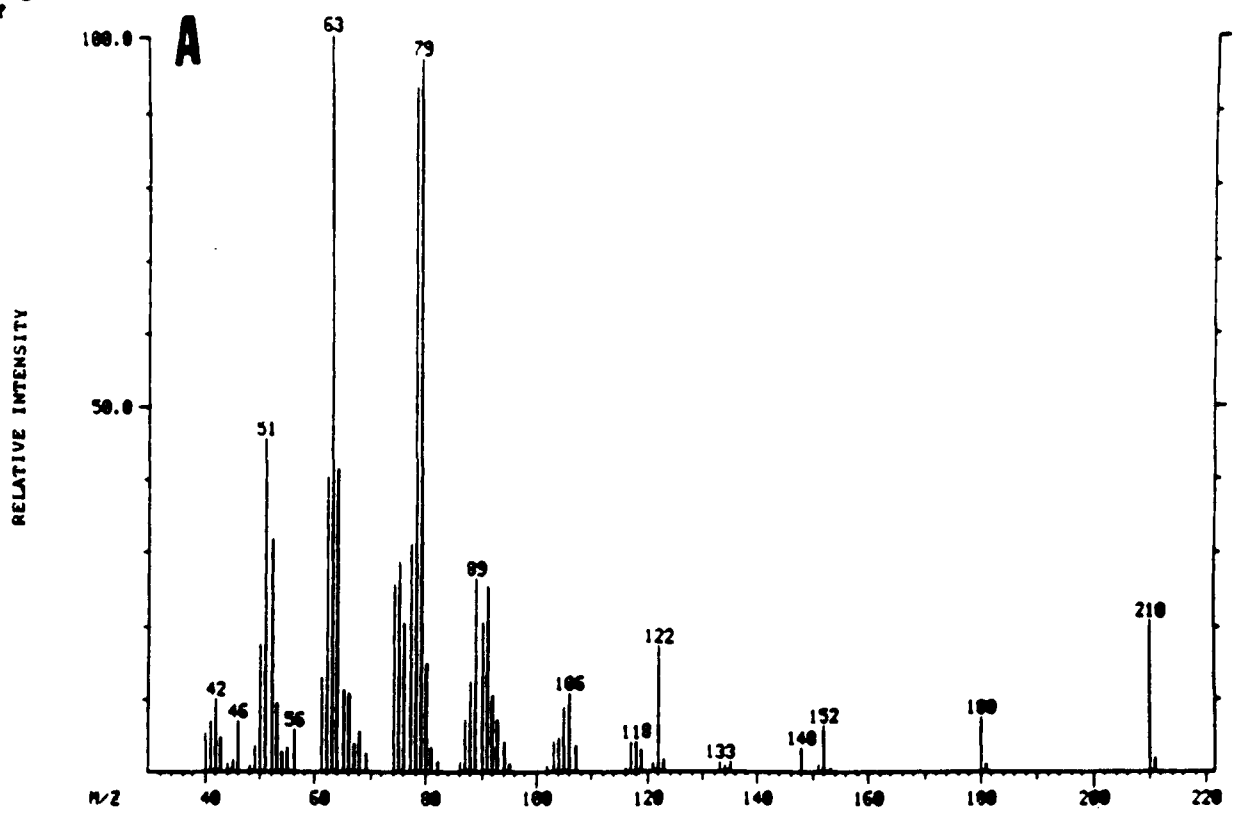

B
FA
MDA
ACT
ACT
ACON
RECONSTRUCTED ION CHROMATOGRAM
4:14
4:57
5:39
6:22
7:04
7:46
SCAN
TIME (MIN)

Fig. 3


A
100.0
50.0
RELATIVE INTENSITY
63
79
51
89
210
122
42
46
56
106
118
133
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152
180
M/Z
40
60
80
100
120
140
160
180
200
220

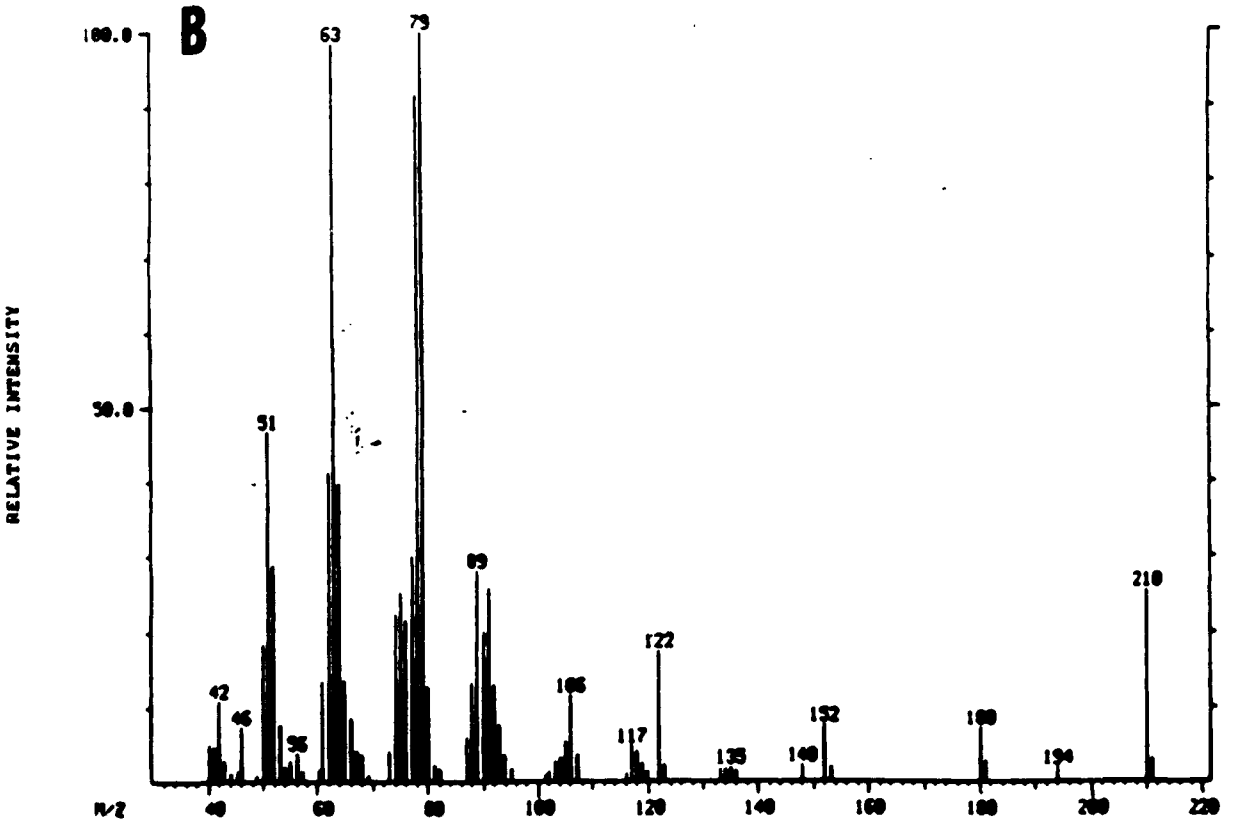
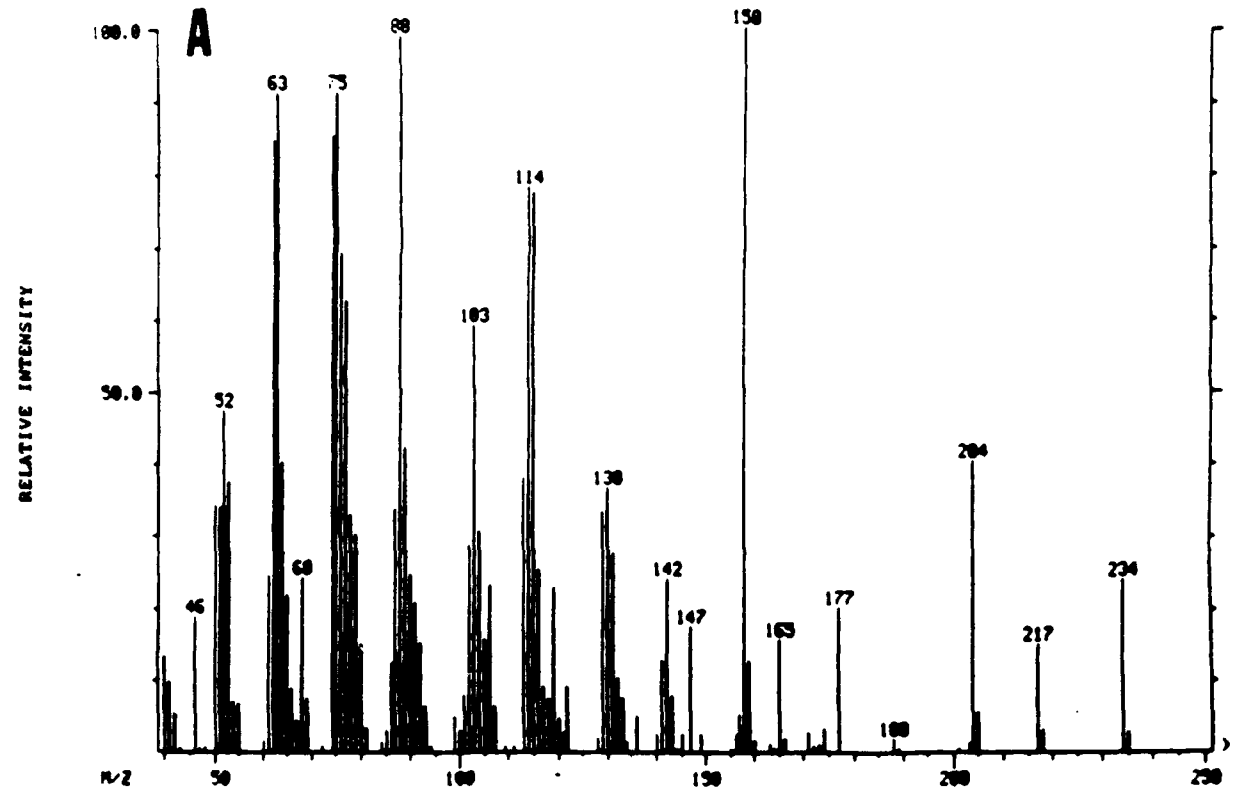

B
100.0
50.0
RELATIVE INTENSITY
63
79
51
89
210
122
42
46
56
106
117
135
140
152
180
194
M/Z
40
60
80
100
120
140
160
180
200
220

Fig. 4


A
100.0
50.0
RELATIVE INTENSITY
M/Z
50
100
150
200
250
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52
63
69
79
88
103
114
138
142
147
158
165
177
188
204
217
234

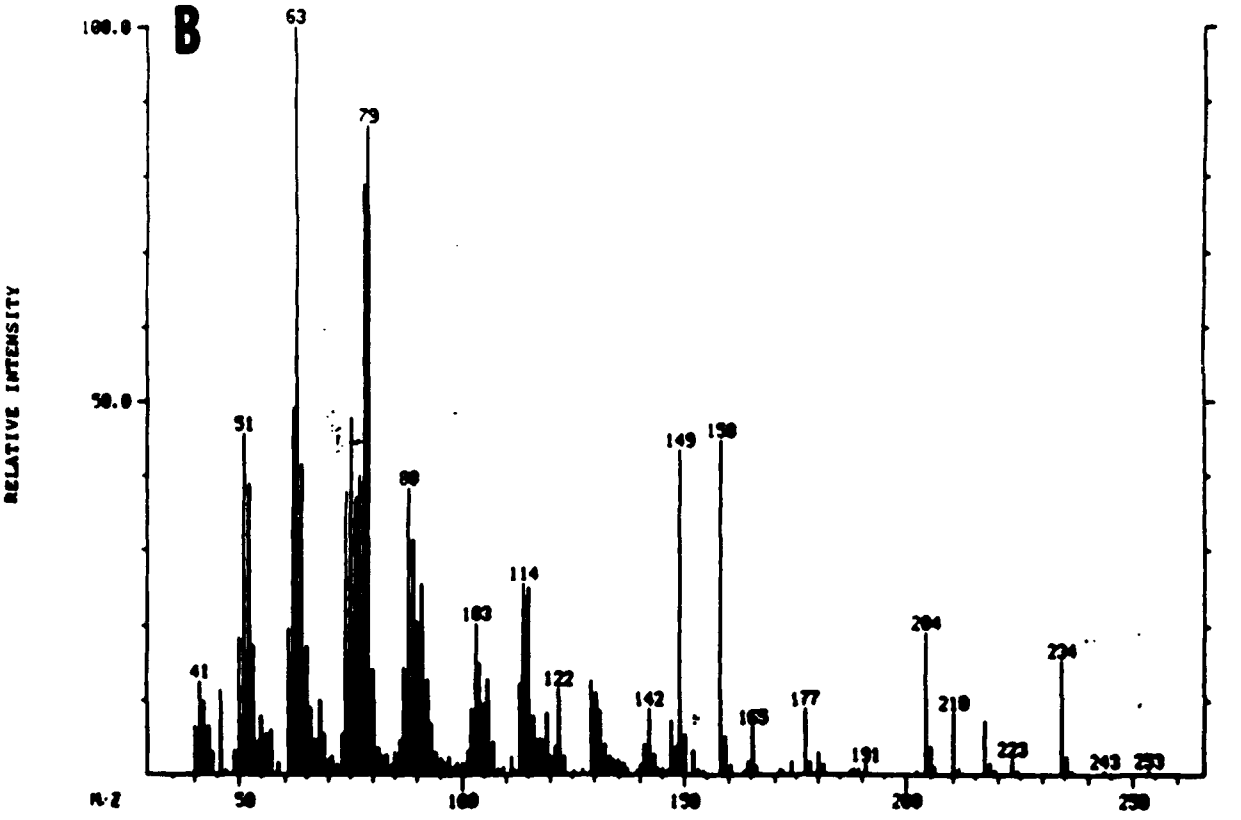
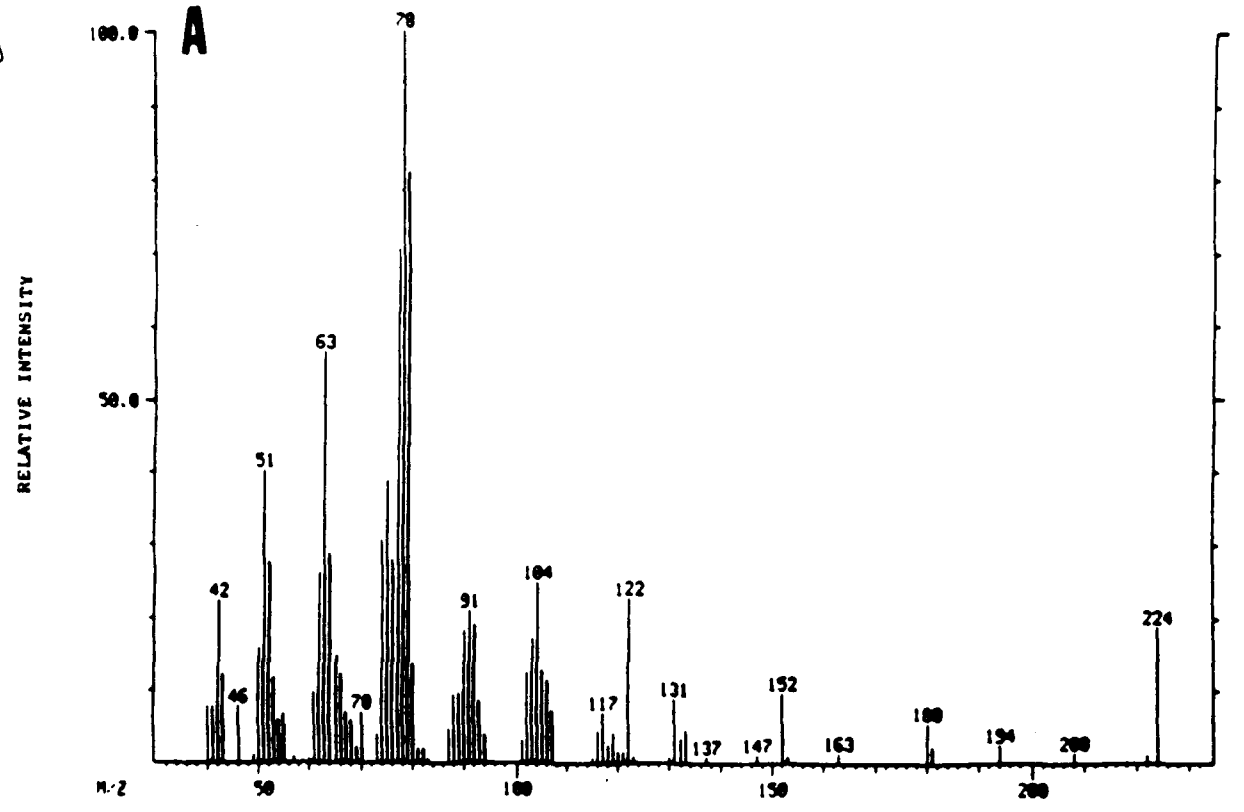

B
100.0
50.0
RELATIVE INTENSITY
M/Z
50
100
150
200
250
41
51
63
79
89
103
114
122
142
149
158
165
177
191
204
210
223
224
243
253

Fig. 5


A
RELATIVE INTENSITY
100.0
50.0
M/Z
50
100
150
200
79
63
51
42
46
70
91
104
122
117
131
137
147
152
163
180
194
200
224

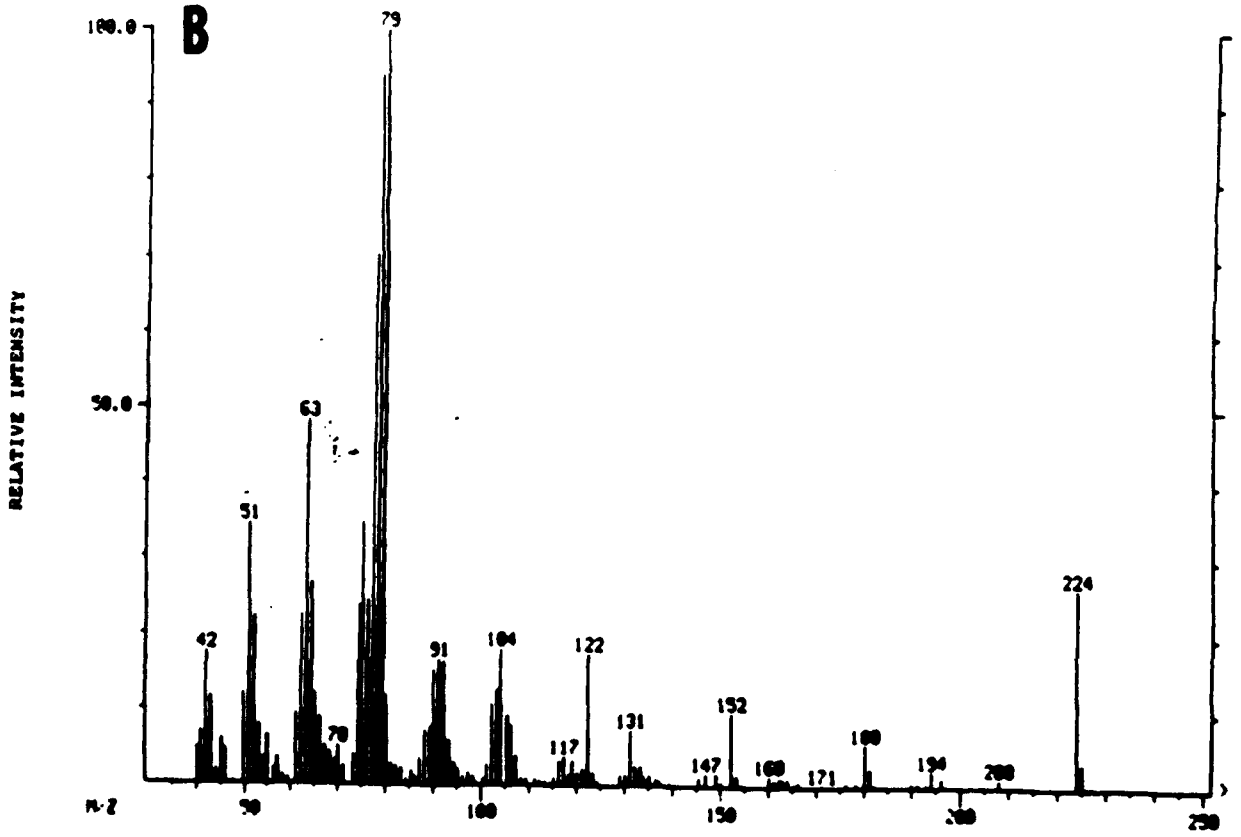
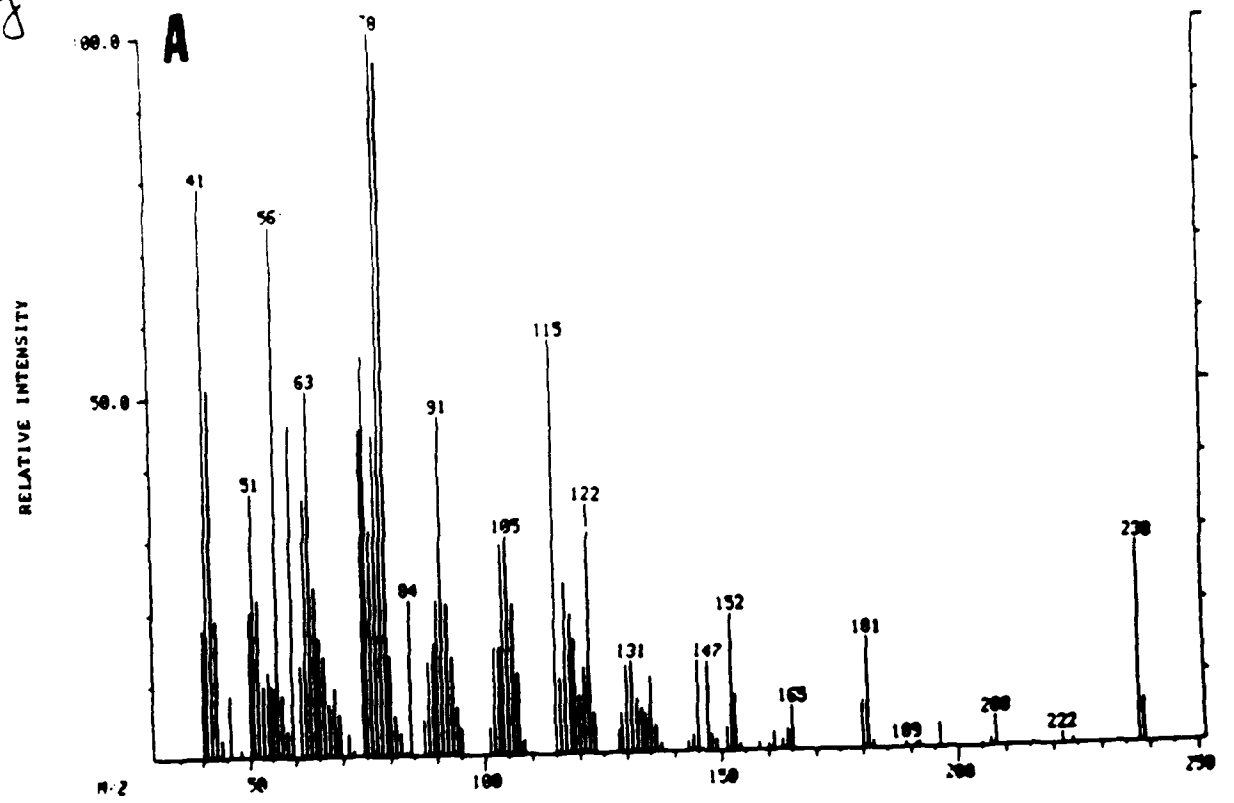

B
RELATIVE INTENSITY
100.0
50.0
M/Z
50
100
150
200
250
79
63
51
42
70
91
104
122
117
131
147
152
160
171
180
194
200
224

Fig. 6


A
RELATIVE INTENSITY
100.0
50.0
M/Z
50
100
150
200
250
41
56
63
51
78
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84
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122
131
147
152
165
181
189
200
222
238

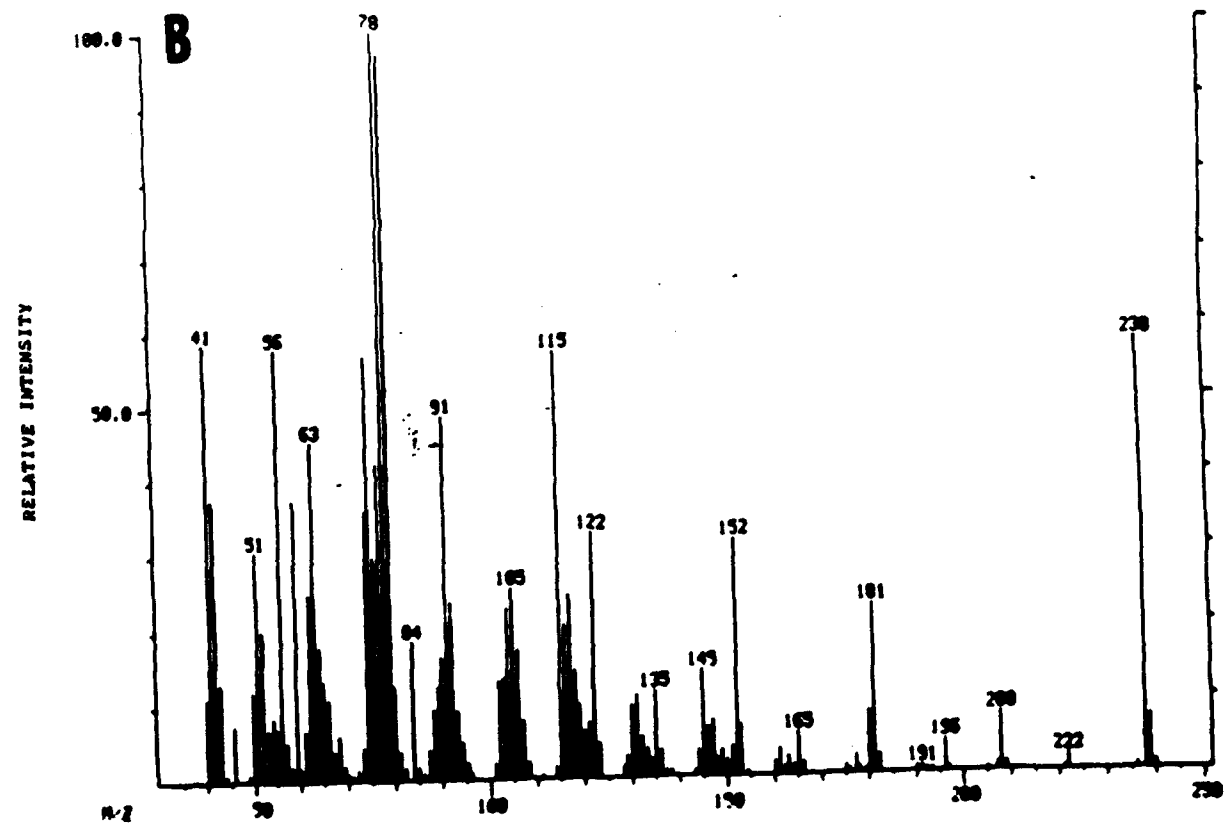
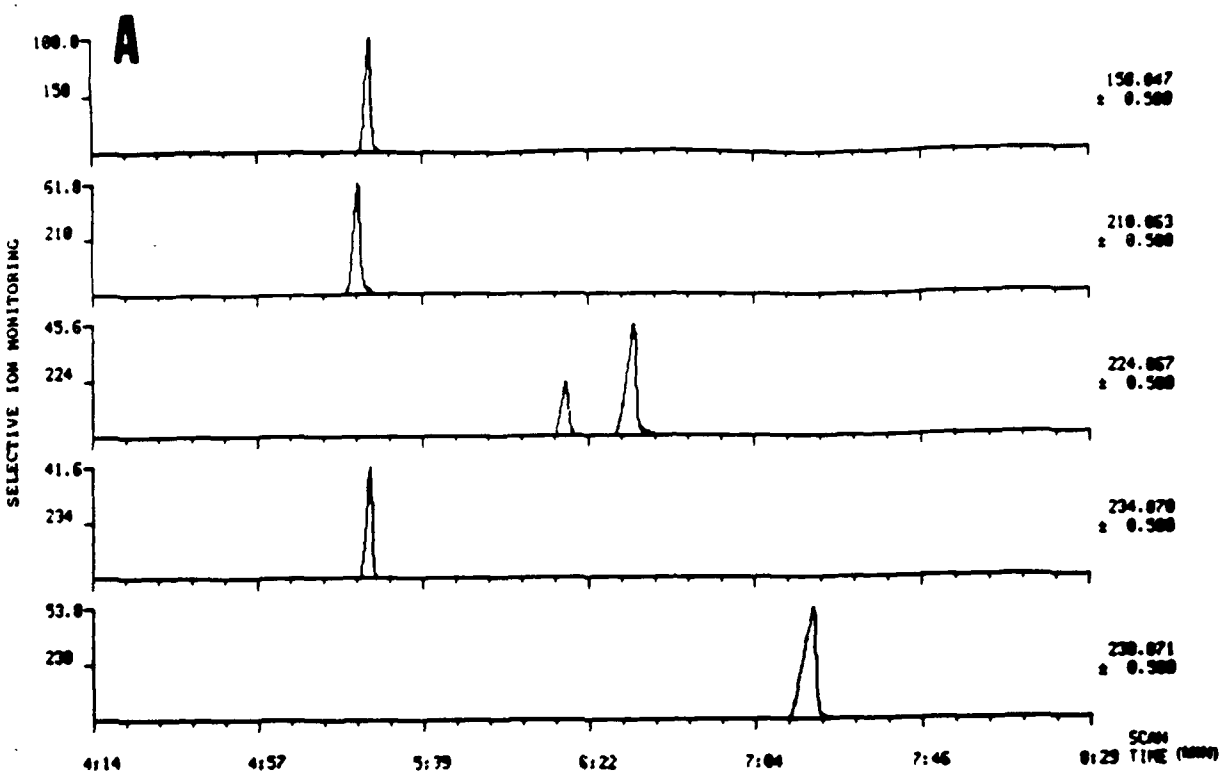
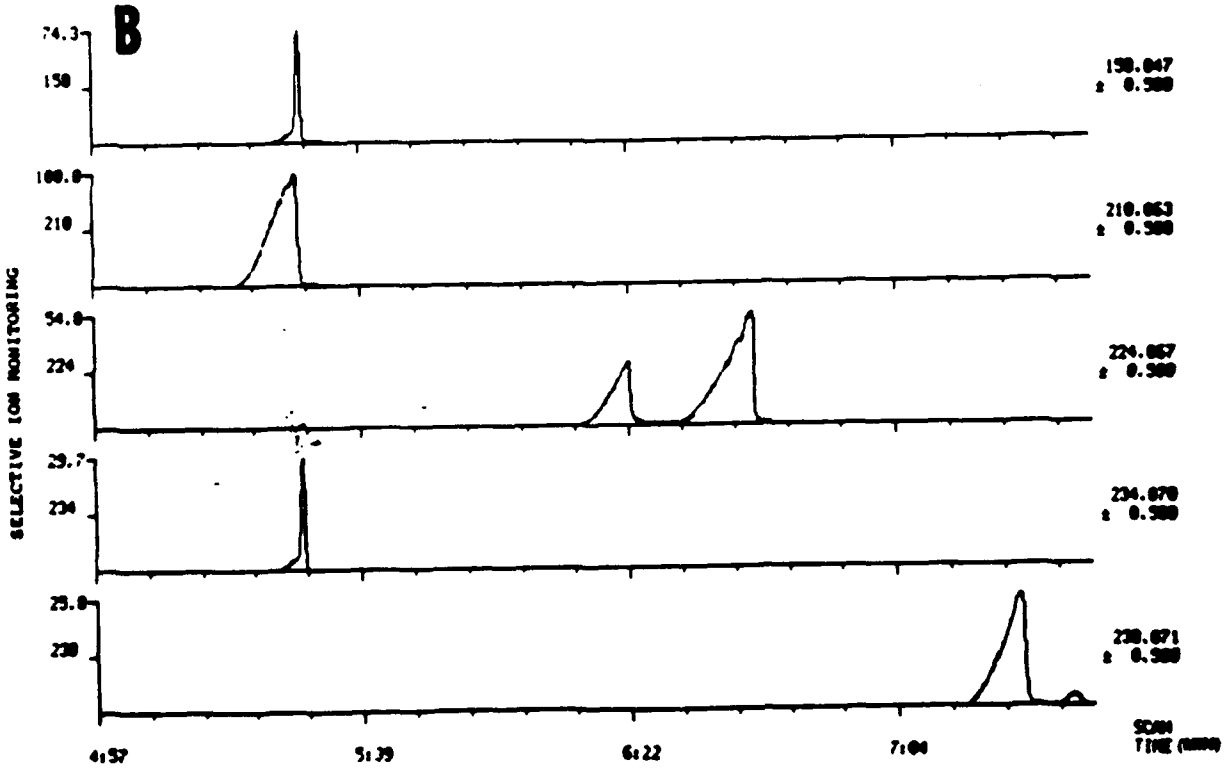

B
RELATIVE INTENSITY
100.0
50.0
M/Z
50
100
150
200
250
41
56
63
51
78
91
84
115
105
122
125
145
152
165
181
191
196
200
222
238

Fig. 7


A
SELECTIVE ION MONITORING
100.0
150
51.0
210
45.6
224
41.6
234
53.8
238
150.047
± 0.500
210.053
± 0.500
224.057
± 0.500
234.070
± 0.500
238.071
± 0.500
4:14
4:57
5:39
6:22
7:04
7:46
8:29
SCAN TIME (min)


B
SELECTIVE ION MONITORING
74.3
150
100.0
210
54.8
224
29.7
234
25.8
238
150.047
± 0.500
210.053
± 0.500
224.057
± 0.500
234.070
± 0.500
238.071
± 0.500
4:57
5:39
6:22
7:04
SCAN TIME (min)