Copyright Warning & Restrictions

The copyright law of the United States (Title 17, United States Code) governs the making of photocopies or other reproductions of copyrighted material.

Under certain conditions specified in the law, libraries and archives are authorized to furnish a photocopy or other reproduction. One of these specified conditions is that the photocopy or reproduction is not to be "used for any purpose other than private study, scholarship, or research." If a, user makes a request for, or later uses, a photocopy or reproduction for purposes in excess of "fair use" that user may be liable for copyright infringement,

This institution reserves the right to refuse to accept a copying order if, in its judgment, fulfillment of the order would involve violation of copyright law.

Please Note: The author retains the copyright while the New Jersey Institute of Technology reserves the right to distribute this thesis or dissertation

Printing note: If you do not wish to print this page, then select "Pages from: first page # to: last page #" on the print dialog screen

Abstract

Title of Thesis: Biodegradation of Diflufenican using anaerobic microbial consortia.

Velukumar Nanjagowder: Master of Science in Environmental Science (toxicology option) 1991

Thesis directed by: Dr. David Kafkewitz, Professor Biological Sciences dept. Rutgers University, Newark

In this thesis I examine the feasibility of using anaerobic microbial consortia from digested anaerobic sludge to degrade Diflufenican, a fluorinated herbicide released into the environment as a result of agricultural usage. The compound is manufactured by Rhone Poulenc and it is an active ingredient of agricultural herbicides. The anaerobic digested sludge served both as a source of microorganisms and as a growth medium. Initial studies were conducted by making a stock solution of 1 ppm concentration of Diflufenican in acetone, the optimal solubility of the compound as suggested in the material safety data sheet is 0.05 ppm in water.

Anaerobic sewage sludge obtained from a neighboring treatment plant was filtered using laboratory filter paper then stored to be used for the preparation of samples and controls. The samples consist of a suspension of Diflufenican in filtered sludge without prior sterilization. The controls consist of a suspension of Diflufenican in sterilized sludge. Anaerobic conditions were obtained in the samples and controls

by bubbling nitrogen gas through the sludge for a few minutes and then sealing the bottles. The samples and controls were incubated at room temperature and analyzed every two weeks to check for Diflufenican degradation. Analytical difficulties in detecting Diflufenican were encountered because of its low solubility and its concentration in samples and controls were too low for detection. Preparation of standards for Diflufenican led to precipitation in solution of concentrations that were high enough for detection. Alternate methods of concentrating the target compound in the samples and controls using the Baker analyte column , a column used to concentrate compounds in solution, proved unsuccessful. It was then decided to detect metabolites of the target compound to demonstrate degradation. Chemical analysis of Diflufenican by the department of chemistry of Rutgers University, Newark, revealed that the main metabolites were Niacin, 2,4-Difluoroaniline and Trifluorotoluene. It was decided to look for the metabolite 2,4-Difluoroaniline in the samples based on the hypothesis that the microorganisms would act on Diflufenican and degrade it releasing 2,4-Difluoroaniline. Standards for 2,4-Difluoroaniline were prepared and run on HPLC to obtain chromatograms for comparison.

Samples and controls of 6,8 and 12 weeks were run and chromatograms obtained. Although detection peaks were obtained in all of the above chromatograms, no perfect match was obtained between the peaks of the samples and those of the standards for 2,4-Difluoroaniline. This led to the conclusion that Diflufenican may be degraded by microorganisms to some other compounds other than 2,4-Difluoroaniline as it was not possible to detect this compound in the samples. Also it may be possible that the degradation of diflufenican may take a longer period of time to release the expected metabolites.

BIODEGRADATION OF DIFLUFENICAN USING ANAEROBIC MICROBIAL CONSORTIA

by

Velukumar Nanjagowder

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fullfillment of the requirements for the degree of Master of Science in Environmental Science (Toxicology Option).

1991

APPROVAL SHEET

Title of Thesis : BIODEGRADATION OF DIFLUFENICAN USING ANAEROBIC MICROBIAL CONSORTIA

Name of Candidate: Velukumar T. Nanjagowder
Master of Science in Environmental Science
(toxicology option)

Thesis & Abstract Approved:

Dr. Richard Trattner Ph.D.
Professor & Program director,
Dept. of Envi. Sci. NJIT.

Date

Dr. David Kafkewitz Ph.D.
Professor, Dept. of
Biological Sci. Rutgers Univ.

Date

Dr. Piero Armenante Ph.D.
Professor, Dept. of Chem. Engg
and Chem. NJIT.

Date

VITA

Name: Velukumar Nanjagowder

Address: 261, Kearny Avenue, Kearny, NJ 07032

Degree and Date to be conferred: M.S. 1991

Secondary Education:

Collegiate Institution attended	Dates attended	Degree	Date Conferred
New Jersey Institute of Technology, NJ	Sept 1989 to Oct 1991	M. Sc	October 1991

Major: Environmental Science (Toxicology Option)

ACKNOWLEDGEMENTS

My sincere gratitude to Dr. David Kafkewitz my thesis advisor, for his guidance, understanding, friendship and moral support throughout the research, without whose help it may not have been possible to achieve as much.

My heartfelt gratitude to Dr. Richard Trattner who has been instrumental in guiding and encouraging me throughout my entire period of education at NJIT, an eminent professor and a very compassionate human being.

Special thanks to Dr. Piero Armenante for being a member of my Masters thesis committee.

Also special thanks to all faculty members of the Otto H. York research center for their cooperation during the project.

Finally my special appreciation to Ms. Gwen San Augustin, research associate and Mr. Clint Brockway, Lab manager at NJIT for their kindness, understanding and timely help.

TABLE OF CONTENTS.

LIST OF FIGURES

1. INTRODUCTION

1.1 Overview

Public concern over the possible effects of chemicals on humans and their environments are largely focussed on a few classes of compounds. Among these compounds are pesticides and herbicides that are widely used today. Special emphasis should be laid on halogenated compounds, which have a tendency to remain persistent in the environment after their release. Reineke and Knackmuss(17) report that since their introduction in 1929, about 750,000 tons of polychlorinated biphenyls have been produced and an estimated 300,000 tons of the total have already been released into the environment. The remaining is at a risk of environmental release.

Among halogenated compounds of concern is Diflufenican an active ingredient of herbicides. Diflufenican contains five fluorine atoms per molecule. Three of the atoms are present as a trifluoromethane side chain, with the possibility of removal by degradation as volatile compounds. The remaining two fluorine atoms are attached to an aromatic ring and are likely to be environmentally persistent. Everyday use of herbicides releases large quantities of this chemical into the environment, thereby causing significant quantities of this compound and its metabolites to persist in the environment.

Muller and Lingens (15) and Reineke and Knackmuss(17) have demonstrated in their studies that microorganisms are capable of degrading a number of halogenated organic compounds and that this degradation can occur in aerobic or anaerobic conditions. Anaerobic degradation of the compound is brought about by reductive dehalogenation without changing the aromatic structure of the parent molecule. Anaerobic degradation is most effective against multihalogenated compounds, like Diflufenican used in this study. Reductive dehalogenation by anaerobic

microorganisms requires a consortium of organisms forming a food and electron chain (9,19,20). The rate of biodegradation can be enhanced by using enrichment cultures of anaerobic microorganisms, a process called bioaugmentation (8). This approach was also used in this study.

1.2 Relevance.

Diflufenican a fluorinated compound is commonly used and is toxic to man. When this compound is used in weed control it can be washed off the foliage and enter the soil and groundwater. The compound can become localized in sediments and water logged soils. Most sediments and water logged soils lack oxygen, this lack of oxygen is important as it is ideal for the degradation of multihalogenated compounds like Diflufenican. However, the presence of an appropriate microbial consortium is a must.

1.3 Approach:

An important aspect of this study is to make use of the dehalogenating capabilities of anaerobic consortia to degrade compounds within a short period of time. The major difficulty is in producing a microbial consortium that can function as a complete food chain, and permit electron flow via interspecies hydrogen transfer and sequential fermentations (9,13,20). However the selection of an appropriate consortia by the process of acclimation of the sludge can reduce the time for degradation of most halogenated compounds. In studies such as the one being presented here, sewage sludge when filtered and sterilized can serve as a culture medium. The liquid phase of the sludge is rich in trace nutrients and carbon sources which is required by the organisms that are present in the sludge. The anaerobic

sludge used in this study is a waste product produced by treatment plants and can be exploited to a great extent in biodegradation studies. Boyd et al (4,8) have demonstrated that sludge can yield dehalogenating organisms and that the pattern of compounds attacked and products formed can be used as a guide in predicting the outcome of new studies as this one. Sample cultures were analyzed every two weeks by HPLC, using filtered aliquots of supernatant fluid in the culture bottles to detect degradation products.

1.4 Expected Results

The results expected from this study is to detect the degradation of the target compound Diflufenican by demonstrating the appearance of 2,4- Difluoroaniline an important metabolite of the target compound in the samples. This is based on the assumption that the anaerobic consortia would act in a predictable way on the target compound and break it down releasing the expected metabolite. Also the usefulness of using anaerobic sludge in this and similar experiments can be justified.

2. LITERATURE SURVEY:

The following few paragraphs are a survey of some specific and successful experiments conducted to study the degradation of halogenated compounds using anaerobic sludge as a source of microorganisms.

2.1 Ann-Sofie Allard, Per-Ake Hynning, Carin Lindgren, Mikael Remberger and Alasdair H. Neilson studied the dechlorination of chlorocatechols by stable enrichment cultures of anaerobic bacteria by using metabolically stable anaerobic cultures obtained by enrichment with 5- bromovanillin, 5-chlorovanillin, catechin, and phloroglucinol to study dechlorination of chlorocatechols. A high degree of specificity in dechlorination was observed by them, and some chlorocatechols were found to be more resistant to dechlorination than others, only 3,5-dichlorocatechol, 4,5-dichlorocatechol, 3,4,5-trichlorocatechol, and tetrachlorocatechol were dechlorinated and not all of them were dechlorinated by the same consortium. 3,5-dichlorocatechol produced 3-chlorocatechol, 4,5-dichlorocatechol produced 4-chlorocatechol, and 3,4,5-trichlorocatechol produced either 3,5-dichlorocatechol or 3,4-dichlorocatechol; tetrachlorocatechol produced only 3,4,6-trichlorocatechol. They found that incubation of uncontaminated sediments without additional carbon sources brought about dechlorination of 3,4,5-trichlorocatechol to 3,5-dichlorocatechol. O-demethylation of chloroguaiacols was done by enrichment cultures, but catechin enrichment was unable to O-demethylate tetrachloroguaiacol. They found that none of the enrichments dechlorinated any of the polychlorinated phenols examined. Their results suggested that dechlorination was not dependent on enrichment with or growth at

the expense of chlorinated compounds and that it would be premature to formulate general rules for the structural dependence of the dechlorination reaction.

2.2 Claire Gouling, C.J. Gillen & E. Bolton worked on the biodegradation of substituted benzenes, they tested the ability of micro-organisms to degrade a range of substituted aromatic compounds. Their studies used the compounds as sources of carbon and energy, and degradation rates were monitored. They also investigated the effect of the presence of other aromatic compounds and the effects of rapidly metabolized substrates. The significance of using such inoculum for waste-treatment processes to hasten the biological processes was stressed by them.

2.3 Alasdair H. Neilson, Ann-Sofie Allard, Per-Ake Hynning and Mikael Remberger worked on the transformations of halogenated aromatic aldehydes by metabolically stable anaerobic enrichment cultures. They obtained metabolically stable enrichment cultures of anaerobic bacteria by elective enrichment of sediment samples from the Baltic Sea and Gulf of Bothnia. These sediment samles have been previously used to study the oxidation and reduction of the adelhyde group of various halogenated aromatic aldehydes. During the transformation of 5- and 6-chlorovanillin, 6-bromovanillin, 3-chloro-4-hydroxybenzaldehyde, 3,5-dichloro-4-hydroxybenzaldehyde, and 3,5-dibromo-4-hydroxybenzaldehyde, their studies demonstrated that the synthesis of the corresponding carboxylic acids, which were the principal metabolites, was invariably accompanied by partial reduction of the aldehyde to a hydroxymethyl group in yields of between 3 and 30%. They also observed the complete reduction to a methyl group with some of the halogenated

vanillins, but to an extremely limited extent with the halogenated 4-hydroxybenzaldehydes. One of their test consortium produced both the hydroxymethyl and methyl compounds from both 5- and 6-chlorovanillin: this led them to make the assumption that that the methyl compound was the ultimate reduction product. On the basis of the kinetics of formation of the metabolites, they were able to conclude that the oxidation and reduction reactions were related and they were also able to observe dehalogenation in one of their consortia. They also noted that in contrast to the transformations of 5- and 6-chlorovanillin, which produced chlorinated methylcatechols, the corresponding compounds were not observed with 5- and 6-bromovanillin: but the former was debrominated, forming 4-methylcatechol, whereas the latter produced 6-bromovanillin alcohol without demethylation. Similarly they noted that 3-chloro-4-hydroxybenzaldehyde formed chlorinated carboxylic acid and benzyl alcohol and the 3-bromo compound was debrominated with formation of 4-hydroxybenzoic acid, and, ultimately, phenol. On prolonged incubation, they found that the halogenated carboxylic acids were generally decarboxylated, so that the final products from these substrates were halogenated catechols or phenols. The study indicates that reductive processes that were detected during this experiment might occur in the environment during anaerobic transformation of halogenated aromatic aldehydes containing hydroxyl and/or methoxyl groups.

2.4 Mark D. Mikesell and Stephen A. Boyd worked on the reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms the inoculum they used was anaerobically digested municipal sewage sludge which had been acclimated to monochlorophenol degradation for more than 2 years. This sludge was shown to degrade pentachlorophenol (PCP).

They found that Di-, tri-, and tetrachlorophenols accumulated when PCP was added to each of the individual acclimated sludges. They also found that when the 2-chlorophenol- (2-CP), 3-CP-, and 4-CP-acclimated sludges were mixed in equal volumes, PCP was completely dechlorinated. The same results were obtained by them in sludge acclimated to the three monochlorophenol isomers simultaneously and with repeated PCP additions, 3,4,5,-trichlorophenol, 3,5-dichlorophenol, and 3-CP accumulated in less than stoichiometric amounts. They found that all chlorinated compounds disappeared after PCP additions were stopped. Also incubations with [^{14}C]PCP resulted in 66% of the added ^{14}C being mineralized to $^{14}CO_2$ and $^{14}CH_4$. Their intial studies showed that Technical-grade PCP was degraded at a rate very similar to that of reagent-grade PCP, but after repeated additions, the technical PCP was degraded more slowly. More experiments revealed that Pentabromophenol was also rapidly degraded by the mixture of acclimated sludges. The results they obtained shows that it is possible to demonstrate the complete reductive dechlorination of PCP by the combined activities of three chlorophenol-degrading populations.

2.5 Stephen A. Boyd, Daniel R. Shelton, Duane Berry and James M. Tiedje, experimented with the anaerobic biodegradation of phenolic compounds in digested sludge and examined the anaerobic degradation of phenol and the *ortho, meta,* and *para* isomers of chlorophenol, methoxyphenol, methylphenol (cresol), and nitrophenol in anaerobic sewage sludge diluted to 10% in a mineral salts medium. They studied 12 monosubstituted phenols of which only *p*-chlorophenol and *o*-cresol were not significantly degraded and the time required for complete substrate disappearance (in weeks) were: phenol (2), *o*-chlorophenol (3), *m*-chlorophenol (7), *o*-methoxyphenol (2), *m*- and *p*-methoxyphenol (1), *m*-cresol (7), *p*-cresol (3), and *o*-

, *m*-, and *p*-nitrophenol (1). However they observed complete mineralization of phenol, *o*- chlorophenol, *m*-cresol, *p*-cresol, *o*-nitrophenol, *p*-nitrophenol, and *o*-, *m*-, and *p*-methoxyphenol. They discovered that in general, the presence of Cl and NO_2 groups on phenols inhibited methane production and elimination or transformation of these substituents was accompanied by increased methane production. Their results indicated that *o*- Chlorophenol was metabolized to phenol, hence dechlorination was the initial degradation step. Also they found that the methoxyphenols were transformed to the corresponding dihydroxybenzene compounds, which were subsequently mineralized.

2.6 Amikam Horowitz, Joseph M. Suflita and James M. Tiedje worked on the reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms and found that Methane-producing freshwater lake sediment was found to dehalogenate chloro-, bromo-, and iodobenzoates by a reductive reaction in which the halogen was replaced by a hydrogen atom. They identified and confirmed the dehalogenated products by mass spectrometry, nuclear magnetic resonance, or chromatography. They also found that removal of the halogens to produce benzoate was necessary before mineralization to CH_4 + CO_2 could occur. In their experiments dehalogenation occurred after a lag period which lasted from 1 week to more than 6 months, depending on the chemical. They also observed that dehalogenation did not take place in the absence of CH_4 production, and it was inhibited by the addition of 20% O_2 and once the sediment was acclimated to halobenzoate dehalogenation, new additions of the halobenzoate were degraded without lag. They found that acclimation occured regardless of whether the parent substrates were eventually mineralized to CH_4 + CO_2. Their test sediments acclimated to bromo- and chlorobenzoate degradation generally metabolized

bromo- and chlorobenzoates, but sediments acclimated to iodobenzoate degradation only metabolized iodobenzoate. Also they discovered that previous acclimation of sediment to benzoate decomposition did not alter the pattern of dehalogenation, and sediment acclimated to dehalogenation was not concurrently acclimated to benzoate degradation. The presence of this specific behaviour, the lag period and subsequent acclimation of the microorganisms along with the absence of dehalogenation in sterile sediments and by sediments previously incubated at _ 39^{O}, suggests that this reaction was biologically catalyzed. Hence it can be concluded that a pathway for the reductive dehalogenation of aryl halides is present in anaerobic microorganisms of this methanogenic sediment.

2.7 Joseph M. Suflita, Joseph A. Robinson and James Tiedje studied the Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. They measured the kinetic parameters associated with the microbial dehalogenation of 3- chlorobenzoate, 3,5-dichlorobenzoate, and 4-amino-3, 5-dichlorobenzoate in anoxic sediment slurries and in an enriched methanogenic culture grown on 3-chlorobenzoate. They found that the initial dehalogenation of the substrates exhibited Michaelis-Menten kinetics and the apparent K_m values for the above substrates ranged from 30 to 67 uM. The pattern of degradation, however, was found to be unusual. The enrichment culture was found to accumulate partially dehalogenated intermediates to 72 and 98% of that possible when incubated with either 3,5dichloro- or 4-amino-3,5-dichlorobenzoate, respectively, but did not accumulate significant amounts of benzoate when 3-chlorobenzoate was the sole carbon and energy source. The accumulated intermediates were rapidly metabolized only after the parent substrate concentrations were nearly depleted (<5 uM). They developed a sequential Michaelis-Menten model to account for the

observed pattern of biodegradation. Using the new model they found , that relative differences in the K_m and V_{max} parameters for substrate and intermediate dehalogenations alone were insufficient to explain the transitory accumulation of intermediates. However, by inserting a competitive inhibition term, with the primary substrate as the inhibitor, the observed pattern of degradation was simulated. This led them to conclude that the dichlorinated substrates competitively inhibit the dehalogenation of the monochlorinated substrates. They also observed similar kinetic patterns for sediments, although the rates were slower than in the enrichment culture.

2.8 Walter Reineke, Hans-Joachim Knackmuss studied the microbial degradation of haloaromatics. According to their study the mechanisms of microbial degradation are as follows.

Biodegradation Mechanisms

The biodegradation of a halogenated compound can be considered complete only when its carbon skeleton is converted into intermediary metabolites and its organic halogen is returned to the mineral state. The crucial point is the removal of halogen substituents from the organic compound. This may occur at an early stage of the degradative pathway with reductive, hydrolytic, or oxygenolytic elimination of the halosubstituent. Alternatively, nonaromatic structures may be generated, which spontaneously lose halide by hydrolysis or hydrogen halide by beta elimination.

Displacement of Halogen Through Hydrogen

Molecular oxygen is required not only as the terminal electron acceptor during respiration, but also for insertion into the aromatic compounds during ring-activating hydroxylation and ring cleavage. Microorganisms have of necessity evolved different mechanisms for degradation in the absence of oxygen, including

ring fission of aromatic compounds. Although details of the pathways and the enzymes involved are still missing, metabolism of the aromatic ring in the absence of molecular oxygen is now known to proceed in at least five different situations (90): (*a*) through anaerobic photometabolism; (*b*) under nitrate-reducing conditions in mixed cultures and by single strains of *Bacillus* sp., *Pseudomonas* sp., and *Moraxella* sp.; (*c*) with sulfate as electron acceptor; (*d*) in consortia through fermentation coupled to methanogenesis, and (*e*) through fermentation.

Isolation of Haloaromatic-Degrading Strains

In four billion years microorganisms have evolved an extensive range of enzymes, pathways, and control mechanisms in order to be able to degrade a wide array of naturally obscuring aromatic compounds. In contrast, the list of pure, biochemically well characterized cultures able to grow at the expense of haloaromatics is short. Haloaromatic-assimilating microbial strains have been obtained by (a) enrichment from nature, (b) in vivo genetic manipulation, and (c) in vitro genetic engineering.

The theory of enrichment culture is simple. The haloaromatic compound to be degraded is supplied as the growth-limiting and usually sole source of an essential nutrient in a culture medium. Of the many organisms added at the start of the experiment, only those with the necessary degradative ability will grow significantly under these conditions. Enrichment cultures grown in chloroaromatics often require several months for isolation. This indicates that besides selection genetic events might be involved. For the degradation of some chlorinated aromatics natural gene exchange has to occur. An important prerequisite for the construction of bacterial strains capable of degrading novel chlorinated aromatic compounds is the recognition and acquisition of genes coding an enzyme sequence able to convert chlorinated aromatics to the respective chlorocatechols. Genes allowing total degradation of chlorocatechols are borne on some transmissible plasmids. The first

report of in vivo construction of a catabolic pathway for the mineralization of chlorinated aromatics using external genetic information for the acquisition of a novel phenotype described work with *Pseudomonas* strain B13 and *Pseudomonas putida* mt-2 and the novel growth substrate 4- chlorobenzoate. Strain B13 was isolated by enrichment culture with 3- chlorobenzoate. It oxidizes 3-chlorobenzoate to 3- and 4-chlorocatechol (see above) and uses the maleylacetate pathway for further breakdown. Strain B13 is unable to utilize 3-chlorobenzoate, since the benzoate 1,2-dioxygenase has a very narrow specificity and will not accept 4-chlorobenzoate as a substrate. However, strain B13 can oxidize 4-chlorocatechol, the expected metabolite in the degradation of 4-chlorobenzoate. The benzoate 1,2-dioxygenase in *Pseudomonas putida* mt-2 determined by the TOL plasmid has a broader specificity than the B13 enzyme and can accept 4-chlorobenzoate as a substrate.

Application of Degradative Strains

Certain xenobiotics, particularly those with polychlorinated aromatic rings, are not known to be growth substrates but are nevertheless subject to cometabolism. Recent work demonstrated that degradation of PCB in soil was not enhanced by addition of a PCB-cometabolizing, biphenyl-utilizing *Acinetobacter* strain. Enhancement of both substrate disappearance and mineralization was only brought about by the addition of the substrate analog, biphenyl. This substrate-analog enrichment selectively increased the number of biphenyl degraders and thereby the cometabolic activity within the indigenous microflora. Upon depletion of the (co)substrate the number of biphenyl oxidizers declined exponentially. Xenobiotics present in soil at rather high concentrations may be mineralized by laboratory strains that grow at the expense of the contaminant.

3. TARGET COMPOUND DIFLUFENICAN

The following is a brief description of the target compound.

CAS NO. 83164-33-4

USE An active ingredient of agricultural herbicides.

PHYSICAL AND CHEMICAL PROPERTIES

Colourless crystals; melting point 161 - 162C; vapour pressure 5.3×10^{-7} mm Hg @ 30C; stability - stable in air up to melting point; at 22C very stable in aqueous solution at pH 5, 7 and 9; fairly stable to photolysis; solubility in water at 25C, 0.05 mg/l. Soluble in most organic solvents, e.g. acetone 100, dimethyl formamide 100, acetophenone 50, isophorone 35, xylene 20, cyclohexane < 10, kerosene < 10 (all in g/l @ 20C); thermal stability - good, temperature of detection of first exotherm 246C; dust explosibility - minimum energy required to ignite dust cloud 68.4 mJ (air jet milled material).

4. MATERIALS & METHODS.

4.1 Inoculum & Growth Substrates:

Municipal Sewage sludge from Livingston sewage treatment plant Livingston New Jersey, was collected fresh from primary anaerobic digesters in two liter jars, tightly capped and stored at room temperature until ready for use. The sludge serves both as a source anaerobic microorganisms, as well as a growth media for the microorganisms. Room temperatures was selected to best simulate the external environmental conditions. The sludge was divided into two portions. One portion was double filtered with laboratory filter paper and stored to be used as sample inoculum the other was double filtered and autoclaved at 121 degrees for 30 minutes and was used to prepare controls. The samples consist of a suspension of Diflufenican in filtered sewage sludge not autoclaved and the controls consists of a suspension of Diflufenican, in filtered sewage sludge autoclaved twice at 121 degrees centigrade for 30 minutes each time to kill all the organisms.

4.2 Test Substrates:

The test substrate used is Diflufenican a halogenated compound and an active ingredient of agricultural herbicides. The compound has been described in detail in the MSDS. Metabolites of the compound as determined by GC-MS by the chemistry department of Rutgers University Newark, are 2,4 Difluoroaniline, Trifluorotoluene and Niacin.

It must be noted that the test compound is patented and available literature on the compound is minimal.

4.3 Experimental Procedure for the preparation of samples and controls.

Stock solution of the test compound was made with a concentration of 1 ppm in acetone. This was determined as the optimal solubility of the compound. At higher concentrations Diflufenican precipitated. It was also assumed that this low concentration would be ideal as it would have least toxicity and maximum microbial activity. 5ml aliquots of the Diflufenican stock solution were taken in 100ml bottles and left overnight for the acetone to vaporize. The following day 100ml of filtered sludge was added to each of the bottles, thus making the concentration of the test substrate Diflufenican 0.05 ppm. Autoclaved sludge was added to bottles which were then labelled as controls, and filtered sludge not autoclaved was added to other bottles which were then labelled as samples. One percent resauzurin was added to each bottle as a redox indicator to ensure that the samples and controls remained anaerobic. Nitrogen gas was bubbled through the contents of each of the bottles and the bottles were capped and sealed under strict anaerobic conditions. The samples and controls were allowed to incubate at room temperature.

4.4 Sampling and Analysis.

Sampling of the compound for biodegradation was done every two weeks. The HPLC was chosen as a quick and efficient method of detection for the compound. Difficulties in the analysis were experienced because at concentrations higher than 0.05 ppm Diflufenican would precipitate in solution and in the HPLC column. After further speculation it was decided that if biodegradation could not be demonstrated in terms of decrease in concentration of the target compound, the best solution would be to use analytical techniques to detect the appearance of the metabolite of the compound, in the samples. The metabolite chosen was 2,4 Difluoroaniline which could be broken off from Diflufenican by the action of the microorganisms on a simple amide bond, as per our hypothesis. Standards of 2,4 Difluoroaniline were prepared in concentrations that would be expected from the complete breakdown of the target compound on a mole for mole basis. Samples and controls were centrifuged using an IEC Centra- M centrifuge for five minutes at 10,000 RPM. The samples and controls were then filtered using a Millex GV filter of pore size 0.22micrometers. The filtered samples and controls were run every two weeks using HPLC to detect compounds that would yield chromatograms similar to the one for the 2,4 Difluoroaniline standards.

4.5 Setup & Calibration of HPLC for Analysis.

The mobile phase used was 50:50, water and Acetonitrile.

Column Packing Material Nova-Pak C18

Measurement conditions:

Measurement Time	10 min
Display Time	10 min
Wave length Range	210 --- 310 nm
Wavelength	227 254 260 280 nm
Band width	3 3 3 3

Chromatogram conditions:

CH 1: AUFS 0.500	Wavelength 227
CH 2: AUFS 0.500	Wavelength Max

Integrator Conditions:

Wavelength	M 1
Time Range	1 to 10 minutes
Column	3.9 mm ID 15.0 cm
Flow Rate	1.00 ml/min
Temperature	20.0 C
Flow Cell	10.00 mm
Pressure	1800 to 2000 psi
Injection volume	40 ul

5. RESULTS

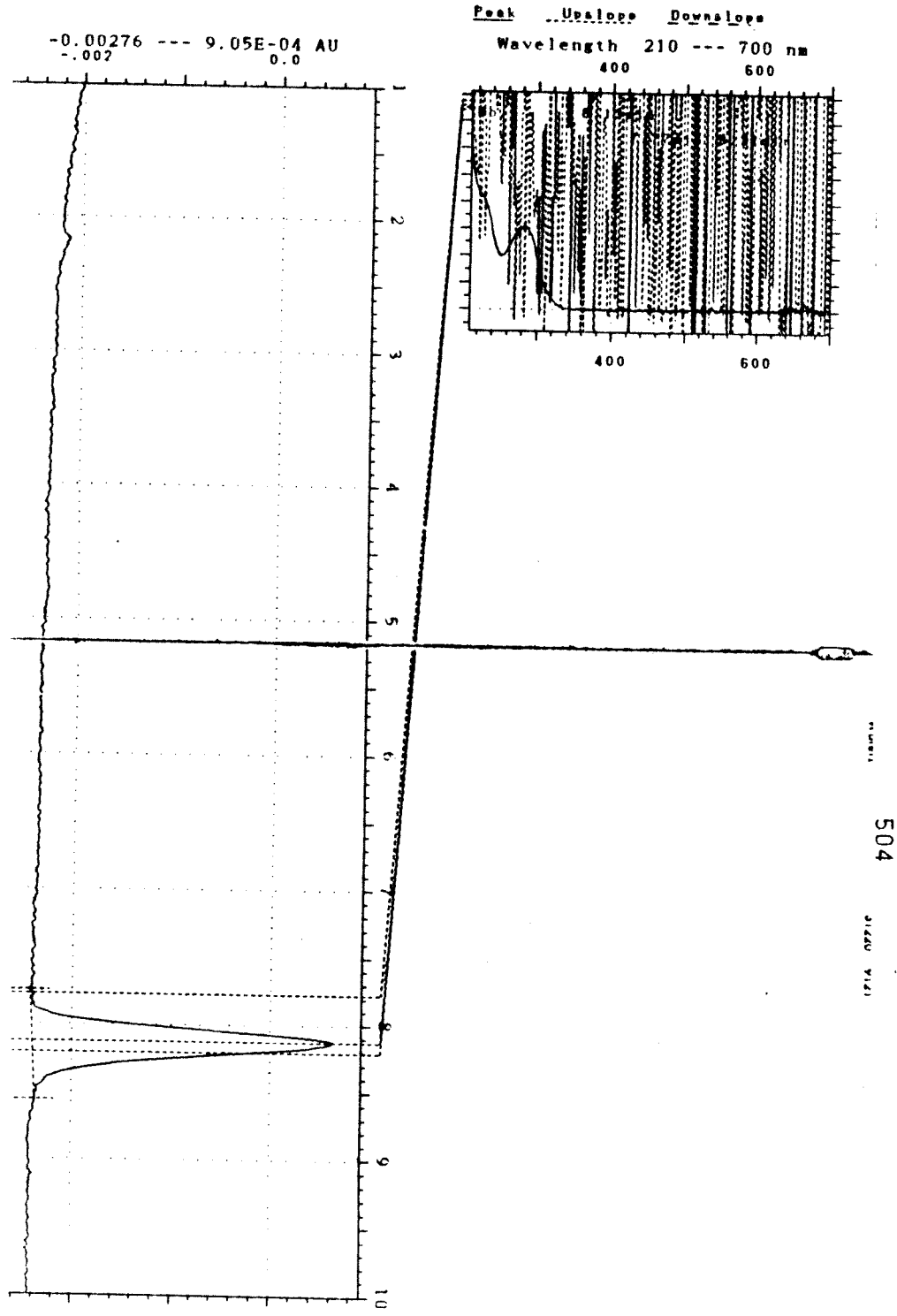
Diflufenican standards in a concentrations ranging from 0.1 ppm to 0.5 ppm in acetonitrile were run on HPLC, with the mobile phase of acetonitrile and water at 55:45, an injection quantity of 40 microliters and a flow rate of 1.5 ml per minute. The chromatogram obtained shows a peak with a retention time of eight minutes and maximum absorbance between 210 to 250 nm. (as shown in figure 1)

Due to analytical difficulties in measuring the disappearance of the target compound from anaerobic sewage sludge, it was decided that the best alternative was to look for one of the main metabolites of the target compound. The metabolites on chemical analysis were Niacin, Trifluorotoluene and 2,4 Difluoroanaline. 2,4 Difluoroaniline was chosen as Niacin would most probably be used up in microbial metabolism and we were interested in a halogenated compound that would be easily broken off from the target compound. It was calculated that if the microbial consortium acted on the entire concentration of the target compound, then the resulting concentration of the metabolites as described above would be in sufficient quantities in solution as to be detectable using high performance liquid chromatography. The above assumptions were made on the basis that the anaerobic microbial consortium would behave in a predictable way favoring the breakdown of the target compound as proposed. Standards of the metabolite 2,4 Difluoroaniline were prepared in low concentrations comparable to what might be expected if the target compound biodegraded in a predictable way. Standards for 2,4- Difluoroaniline were made at concentrations of 0.0001214 mg/ml, 0.001214 mg/ml and 0.002428 mg/ml. Chromatograms of these concentrations revealed peaks and a standard calibration curve was plotted (as shown in figure 2 & 3). Analysis of the samples and controls was done every two weeks using high performance liquid chromatography. Samples and control of the first six weeks did

not yield any significant or meaningful results. Samples analyzed after six weeks showed the appearance of a peak with a retention time of two minutes and maximum absorbance at between 200 and 220 nm (as shown in figure 4). 2,4-Difluoroaniline controls analyzed at 6 weeks revealed a peak at 2.65 minutes with maximum absorbance at 220nm and another smaller peak at 3.56 (as shown in figure 5). 2,4-Difluoroaniline samples analyzed at eight weeks revealed a compound with a retention time of 1.54 minutes and maximum absorbance at 270nm, and another smaller peak with a retention time of 2minutes and maximum absorbance at 210 nm (as shown in figure 6). 2,4-Difluoroaniline controls analyzed at 8 weeks revealed two peaks one with a retention time of 2.72 minutes and maximum absorbance at 210 nm and the other with a retention time of 3.69 minutes and maximum absorbance at 200nm (as shown in figure 7). 2,4-Difluoroaniline samples analyzed at 12 weeks revealed two peaks one with a retention time of 1.53 minutes and maximum absorbance at 260 nm and another with retention time of 2 minutes and maximum absorbance at 210 nm (as shown in figure 8). 2, 4-Difluoroaniline controls analyzed at 12weeks revealed two peaks, one with a retention time of 1.96 minutes and maximum absorbance at 200nm and another with a retention time of 2.66 minutes and maximum absorbance at 210 nm (as shown in figure 9). Samples analyzed at six, eight and twelve weeks all showed results as discussed above.

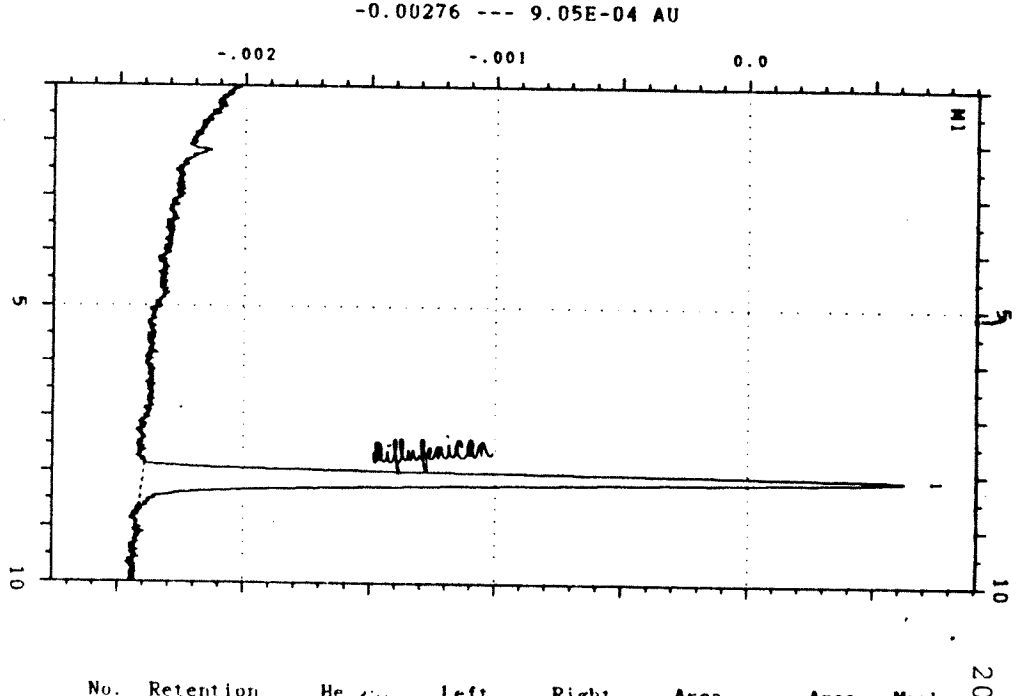
(N.B. Retention time and maximum absorbance for 2,4 Difluoroaniline standards are 2.30 minutes and 220 and 230 nm). Although some peaks from the sample chromatograms of 6,8,12 weeks are in close proximity to the peak in the chromatogram for the 2,4-Difluoroaniline standard, in terms of analytical detection, they is no perfect match. Hence it can be concluded that Diflufenican was not biodegraded releasing 2,4 Difluoroaniline, but it may be possible that the target compound was degraded releasing other unknown compounds.

FIGURE 1



$CH_3CN : H_2O$ (55 : 45) 40 µl 1.5 ml/min

Waters 994	< I N T E G R A T O R >				
Date	: Apr/25/91 12:11:14		FILE NAME	: DIFLUFENICAN	
Wavelength	: M1				
Time range	: 1.00 --- 10.00	min	Smoothing	: 5	points
Interval	: NORMAL		Minimum area	: 1.0E-06	AU*min
Time double	: 100	min	Slope	: 1.0E-05	AU/min
Minus peak	: OFF		Drift	: 1.0E-05	AU/min
Paper speed	: 10.0	mm/min	Height	: 1.0E-04	AU
Baseline correct	: OFF		Width	: 0.1	min
Sample name	: DIFLUFENICAN		Packing material	: NOVA-PAK C18	
Column	: 3.9mmID* 15.0cm		Mobile phase	: CH3CN,H2O 50,50	
Flow rate	: 1.00	ml/min	Pressure	: 2190.0	PSI
Temperature	: 20.0	°C	Injection volume	: 30	µl
Flow cell	: 10.00	mm			

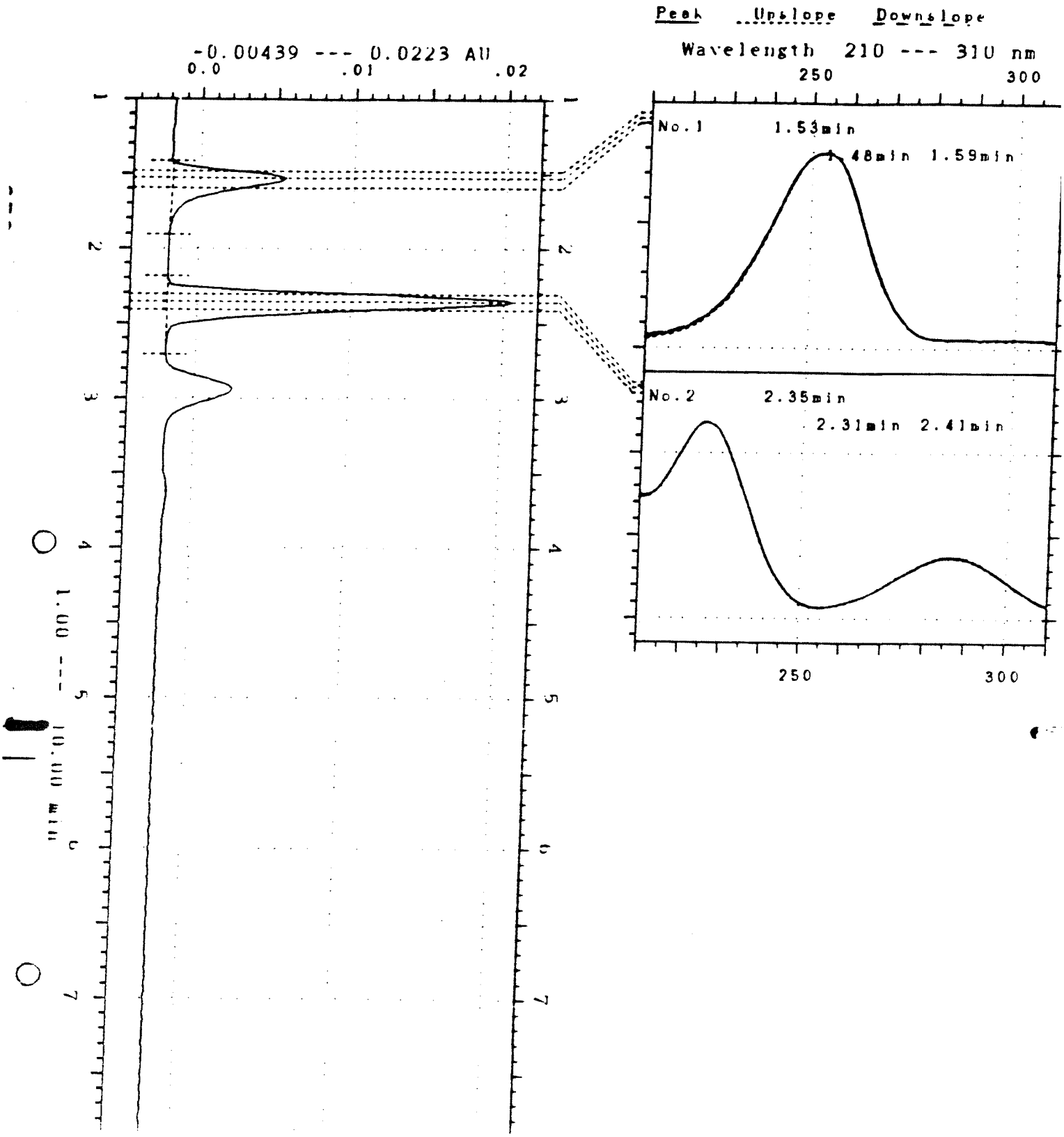


No.	Retention [Min]	He [ ]	Left [Min]	Right [Min]	Area [AU*Min]	Area [%]	Mark
1	8.13	0.6[illegible]02	7.87	9.98	6.964E-04	100.0	

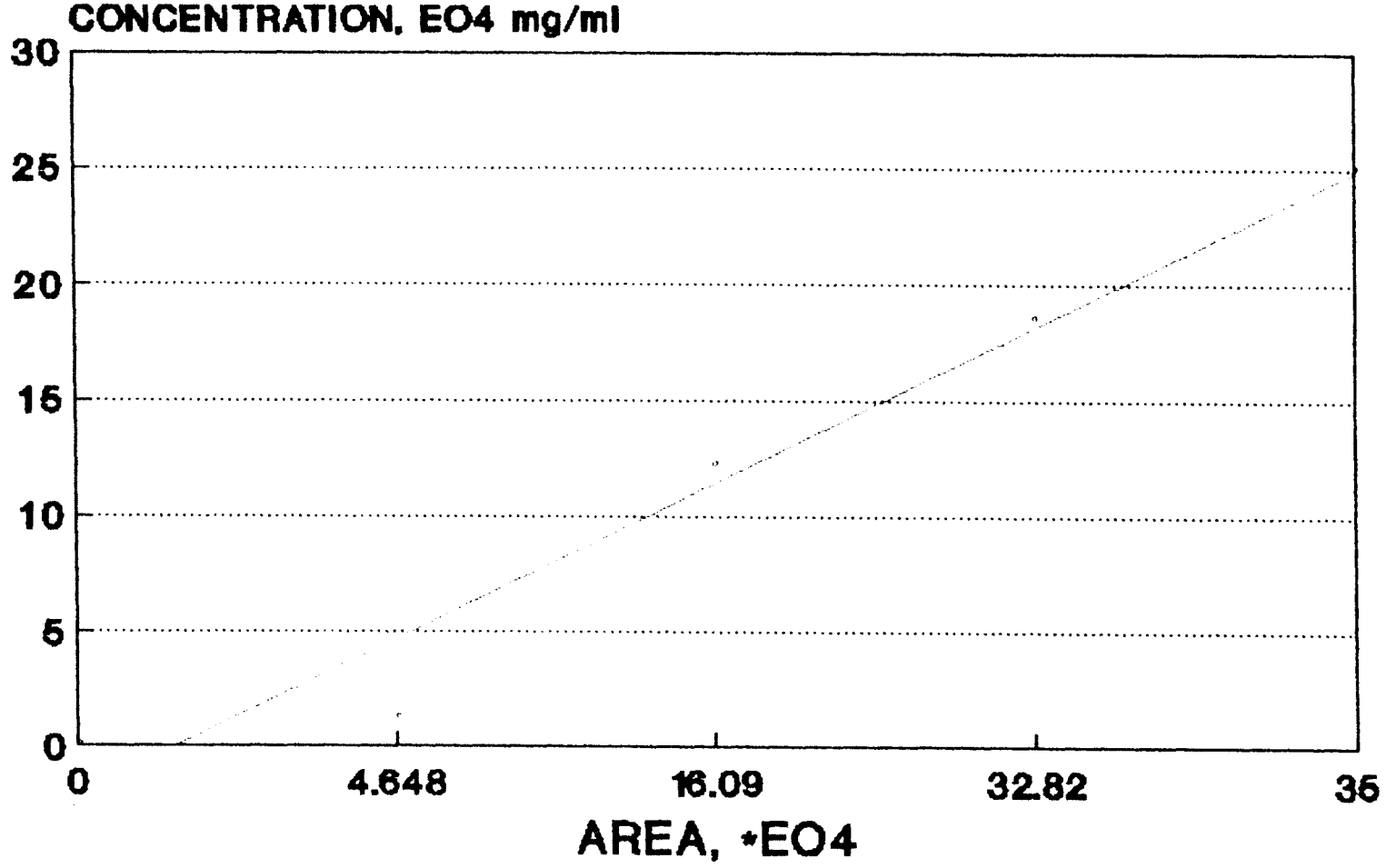
FIGURE 2

CHROMATOGRAM FOR 2,4 DIFLUOROANILINE STANDARDS

Peak_no.	Time	page	Peak_no.	Time	page
1	1.53 min	1			
2	2.35 min	1			



2, 4 DIFLUOROANILINE STANDARDS METABOLITE OF DIFLUFENICAN



Lowest detectable conc. using HPLC

FIGURE 3

FIGURE 4

CHROMATOGRAM FOR 2,4 DIFLUOROANALINE SAMPLES 6 WEEKS

Peak_no.	Time	page	Peak_no.	Time	page
2	2.00 min	1			

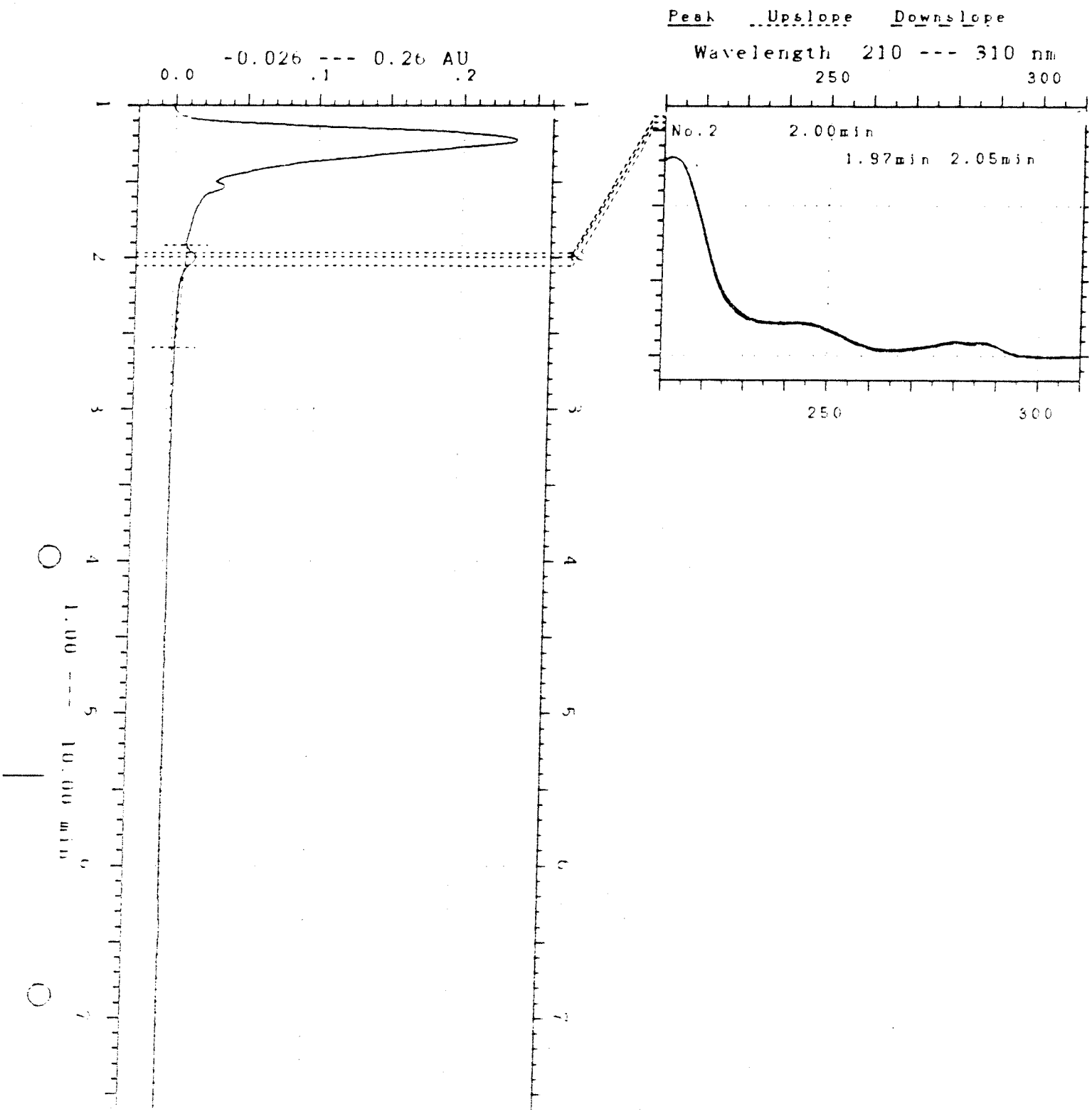


FIGURE 5 **CHROMATOGRAM FOR 2,4 DIFLUOROANALINE CONTROLS 6 WEEKS**

Peak_no.	Time	page	Peak_no.	Time	page
2	2.65 min	1			
3	3.56 min	1			

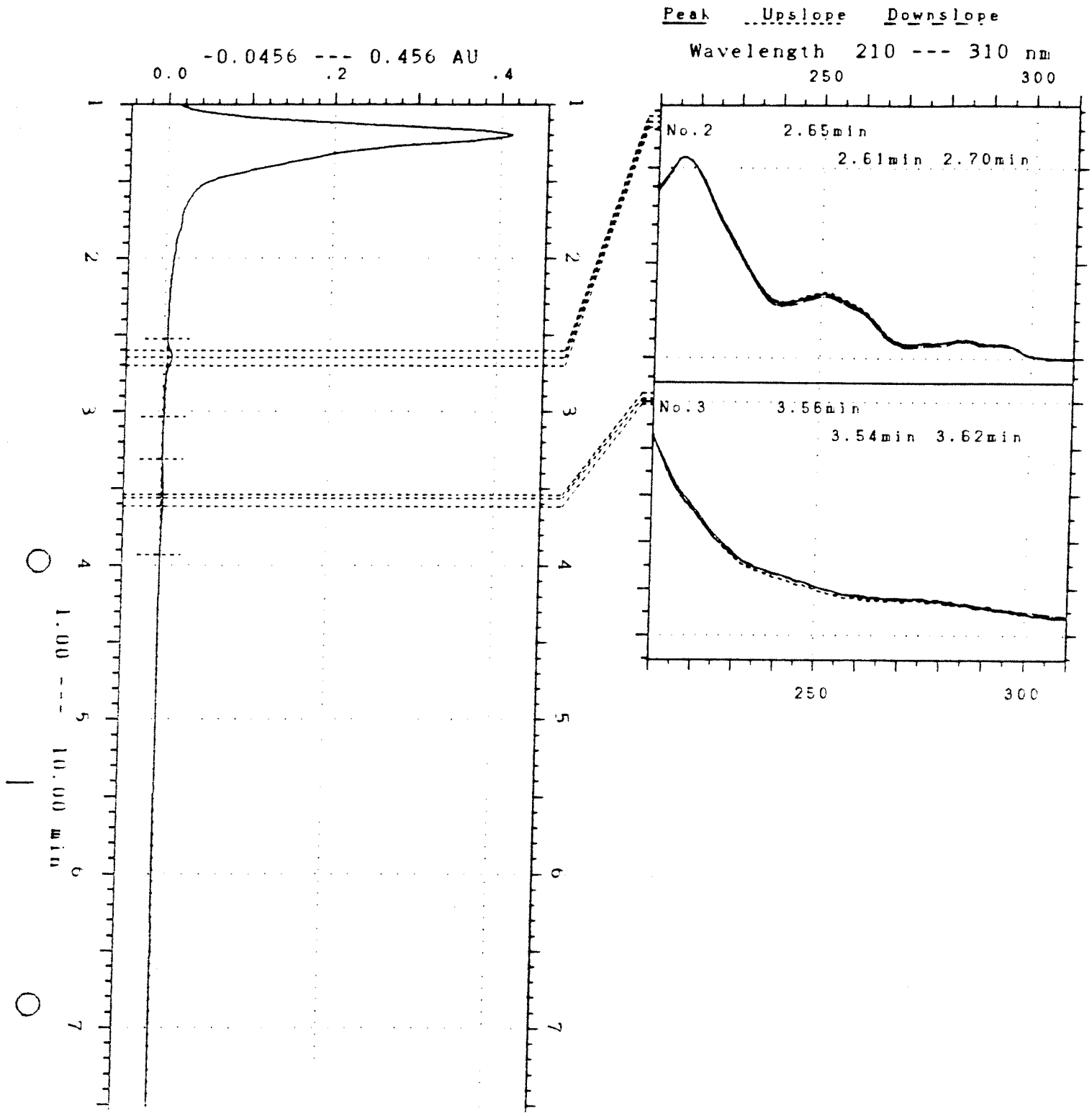


FIGURE 6 **CHROMATOGRAM FOR 2,4 DIFLUOROANALINE SAMPLES 8 WEEKS**

Peak_no.	Time	page	Peak_no.	Time	page
2	1.54 min	1			
3	2.00 min	1			

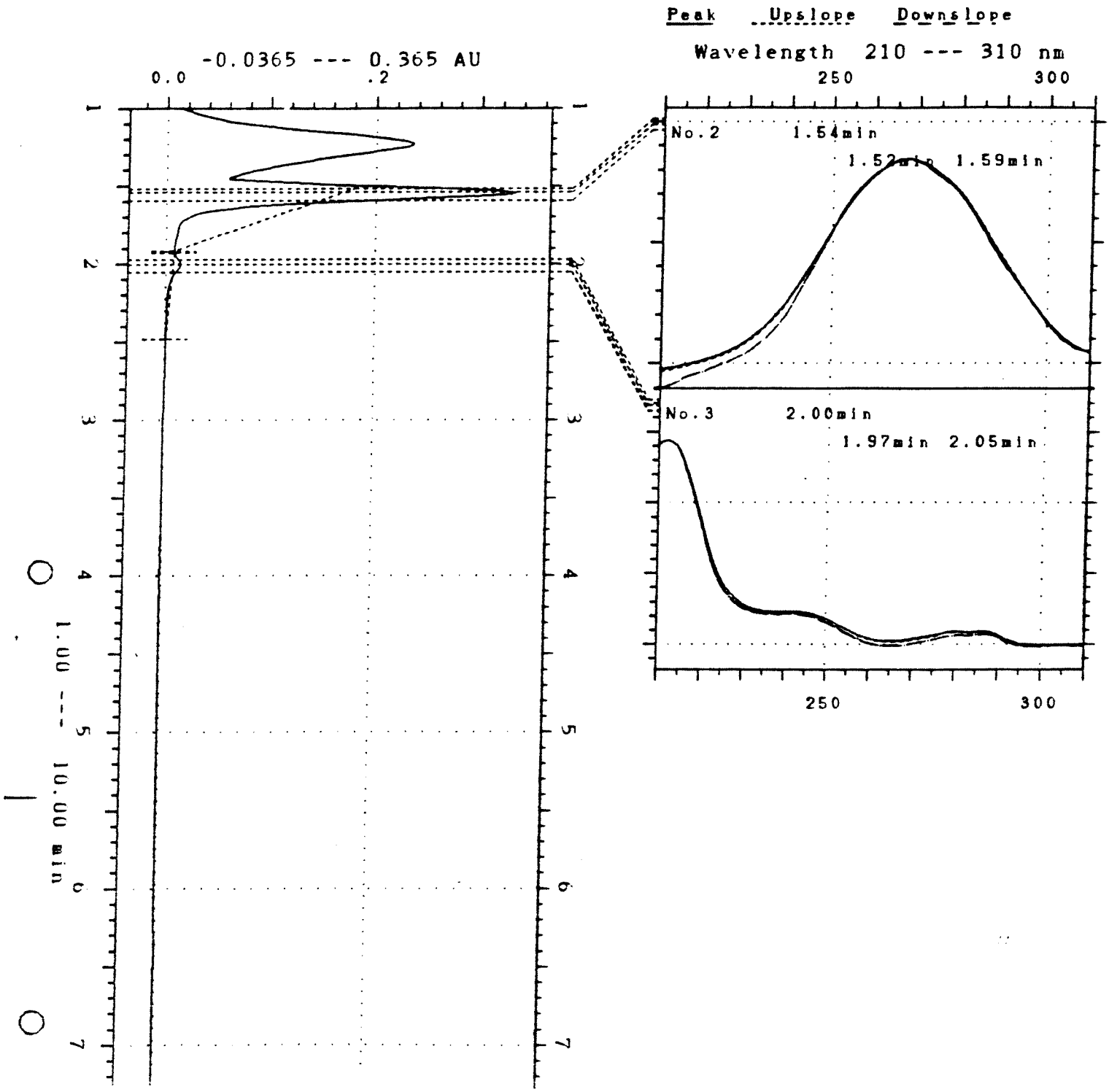


FIGURE 7

CHROMATOGRAM FOR 2,4 DIFLUOROANALINE CONTROLS 8 WEEKS

Peak_no.	Time	page	Peak_no.	Time	page
2	2.72 min	1			
3	3.69 min	1			

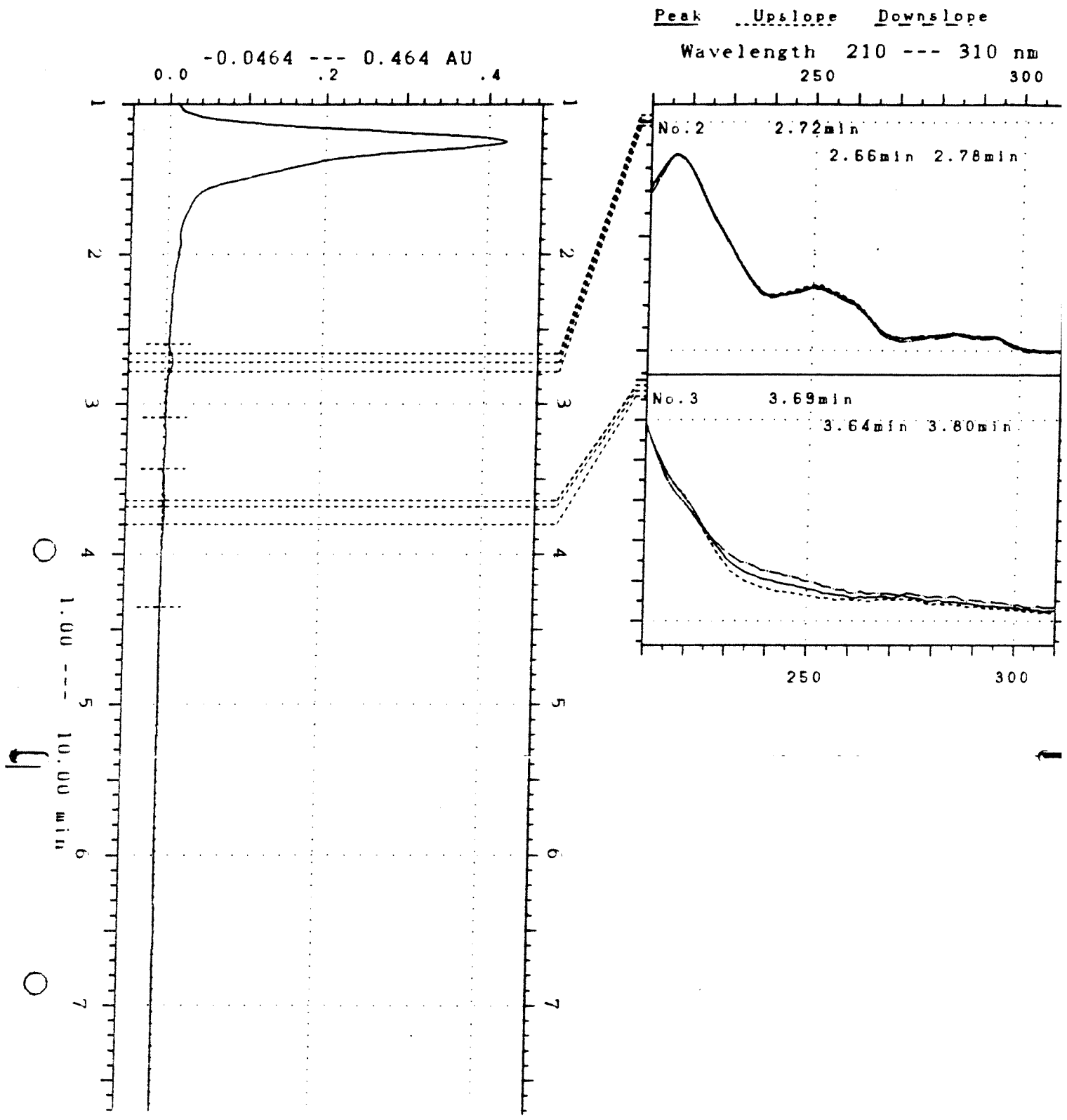


FIGURE 8 **CHROMATOGRAM FOR 2,4 DIFLUOROANALINE SAMPLES 12 WEEKS**

Peak_no.	Time	page	Peak_no.	Time	page
2	1.53 min	1			
3	2.00 min	1			

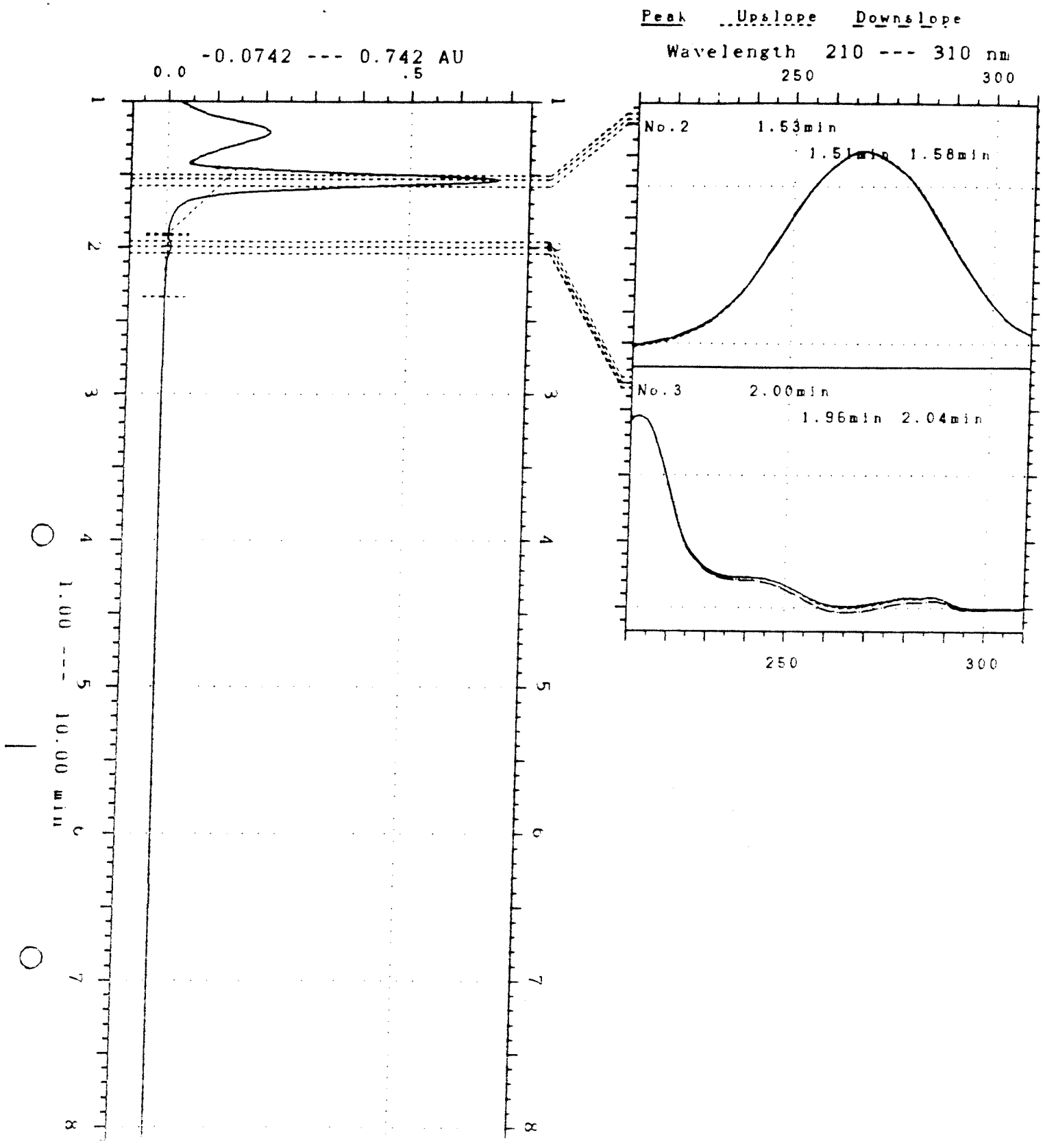
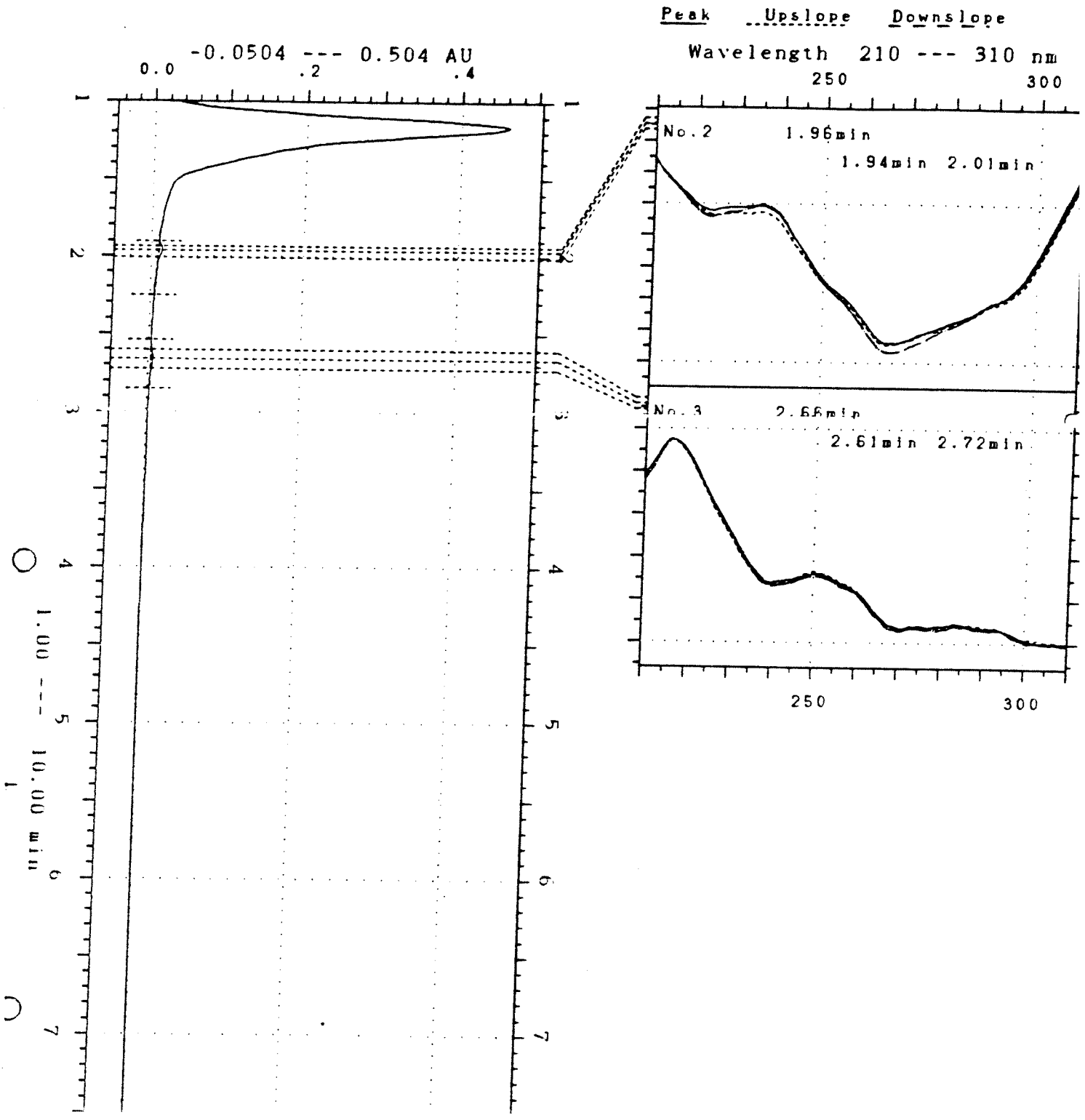


FIGURE 9

CHROMATOGRAM FOR 2,4 DIFLUOROANALINE CONTROLS 12 WEEKS

Peak_no.	Time	page	Peak_no.	Time	page
2	1.96 min	1			
3	2.66 min	1			



6. DISCUSSION

The study was directed towards finding ideal anaerobic conditions and microbial consortia that would degrade Diflufenican. Analytical difficulties in detecting the degradation of Diflufenican was due to the relative insolubility of the compound (0.05 ppm only in water). As a result it was decided to try and detect one of the main metabolites, 2,4-Difluoroaniline which would be expected to appear in the cultures if the target compound was acted upon by the organisms in a predictable way. HPLC chromatograms of samples of 6, 8, and 12 weeks showed the appearance of a compound with a retention time of two minutes and weak absorbance between 200 and 220 nm. Although it was confirmed that this chromatogram did not match the one from the 2,4-Difluoroaniline standards, and hence not the metabolite we expected to appear, it is not unrealistic to assume that it could be some other compound produced by the degrading action of the microorganisms. The variations in the results from those which we expected may be attributed to the following reasons (a) The microorganisms in the sludge were not acclimated to the target compound and hence the degrading action is much slower than expected due to a lag period. (b) The physical appearance of the samples of 6,8, 12 weeks were definitely more turbid than the controls of the same periods this leads us to conclude that the microbial activity may have been optimal but the concentration of the target compound was so low that degradation of the compound may have released metabolites in such low quantities making it difficult for detection.

(C) Using higher concentrations of the target compound may prove toxic to the microbial consortia, but there is a possibility that the organisms may undergo genetic variations to adapt and may produce degradation, thus making it possible to

isolate pure cultures that act on the target compound. (d) As the target compound has been patented and previous studies on fluorinated compounds cannot be applied in their entirety to this particular compound, it is not possible for us to determine whether better results are to be expected over a period of weeks or months. (e) As an important backup experiment we have decided to do similar studies using the main metabolite 2,4-Difluoroaniline as the target compound and look for degradation. 2,4-Difluoroaniline is more water soluble than the target compound Diflufenican used in this study and contains two fluorine atoms, it will be interesting to note the action of the anaerobic microbial consortia on this compound.

7. References.

1. Alasdair H. Neilson, Ann-Sofie Allard, Per-Ake Hynning, and Mikael Remberger. Applied and Environmental Microbiology, Sept. 1988, p. 2226-2236.

2. Ann-Sofie Allard, Per-Ake Hynning, Carin Lindgren, Mikael Remberger, and Alasdair H. Neilson. Applied and Environmental Microbiology, 1/91, p. 77-84.

3. Amikam Horowitz, Joseph M. Suflita and James M. Tiedje. Applied and Environmental Microbiology, May 1983, p. 1459-1465.

4. Boyd, S.A., Shelton, D.R., Berry, D., & J.T. Tiedje. 1983 Appl. Environ. Microbiol. 45:50.

5. Claire Gouling, C.J. Gillen & E. Bolton. Journal of Applied Bacteriology, 1988, 65, 1-5.

6. Cord-Ruwisch, R., Seitz, H.J., & R. Conrad. 1988 Arch. Microbiol. 149:350.

7. Dolfing, J. & J.M. Teidje. 1986 FEMS Microbiol. Ecol. 38:293.

8. Goulding, C. Gillen, C.J., and E. Bolton. 1988 J. Appl. Bacteriol 65:1.

9. Horowitz, A.J., J.M. Sulfita, & J.M. Teidje. 1983 Appl. Environ. Mircobiol., 45:1459.

10. Iannotti, E.L., D. Kafkewitz, M.J. Wolin & M.P. Bryant. 1973 J. Bacteriol. 114:1240.

11. Joseph M. Suflita, Joseph A. Robinson and James M. Tiedje. Applied and Environmental Microbiology, May 1983, p. 1466-1473.

12. Mark D. Mikesell and Stephen A. Boyd. Applied and Environmental Microbiology, Oct. 1986, p. 861-865.

13. Mohn, W.W. & Teidje, J.M. 1990 Arch. Microbiol. 153:267.

14. Mikesell, M.D., & S.A. Boyd. 1986 Appl. Environ. Microbiol. 52:861.

15. Muller, R. & F. Lingens. 1986 Angew. Chem. Int. Ed. Eng. 23:779-789.

16. Neilson, A.H., Allard, A.S., Hynning, P.A. & M. Remberger. 1988 Appl. Environ. Microbiol. 54: 2226.

17. Reineke, W. & H.J. Knackmuss. 1988 Ann. Rev. Microbiol. 42:263-287.

18. Stephen A. Boyd, Daniel R. Shelton, Duane Berry,and James M. Tiedje. Applied and Environmental Microbiology, July 1983, p. 50-54.

19. Sulfita, A.J., A. Horowitz, D.R. Shelton & J.M. Teidje. 1982 Science, 218, 1115.

20. Sulfita, J.M., J.A. Robinson & J.M. Teidje. 1983 Appl. Environ. Microbiol., 45:1466.

21. Walter Reineke, Hans-Joachim Knackmuss. Annual Review of Microbiology, 1988, 42:263-87.

MATERIAL SAFETY DATA SHEET FOR DIFLUFENICAN

CAS NO. 83164-33-4

USE An active ingredient of agricultural herbicides.

PHYSICAL AND CHEMICAL PROPERTIES Colourless crystals; melting point 161 - 162C; vapour pressure 5.3 x 10^{-7} mm Hg @ 30C; stability - stable in air up to melting point; at 22C very stable in aqueous solution at pH 5, 7 and 9; fairly stable to photolysis; solubility in water at 25C, 0.05 mg/l. Soluble in most organic solvents, e.g. acetone 100, dimethyl formamide 100, acetophenone 50, isophorone 35, xylene 20, cyclohexane < 10, kerosene < 10 (all in g/l @ 20C); thermal stability - good, temperature of detection of first exotherm 246C; dust explosibility - minimum energy required to ignite dust cloud 68.4 mJ (air jet milled material).

TRANSPORT INFORMATION Not restricted.

CLASSIFICATION FOR SUPPLY (CLASSIFICATION, PACKAGING AND LABELLING OF DANGEROUS SUBSTANCES REGULATIONS 1984)

CLASSIFICATION Harmful.

RISK PHRASES Harmful by inhalation.

HANDLING Work with chemicals should be carried out to high standards of housekeeping and personal hygiene.

Avoid breathing dust.

For handling operations involving a risk of exposure to dust, goggles (BS 2092/D) and approved respiratory protection should be worn.

The dust is **EXPLOSIVE** when mixed with air in critical proportions and in the presence of a source of ignition.

EXPOSURE LIMITS Rhone Poulenc Control Limit 10 mg/m^3 (8 hour TWA Value).

PERSONAL PROTECTION Workers should wear rubber footwear, overalls and PVC gloves.

HEALTH HAZARDS Acute toxicity of this product is low.

Acute oral LD50 for rats is >2,000 mg/kg.

Acute dermal LD50 for rats is >2,000 mg/kg.

Acute inhalation of LC50 for rats is >2.34 mg/l.

Oral LD50 for dog and rabbit were greater than 5,000 mg/kg.

No deaths or significant signs were at the maximum achievable atmospheric concentration of 2.34 mg/l.

No dermal sensitization properties.

Long term administration of Diflufenican at very high dose levels illicited an adaptive response by the liver of experimental animals. Characterized mainly be hepatocellular hypertrophy. The response was readily reversible on withdrawal of the herbicide.

Carcinogenicity studies in the mouse and rat showed no evidence of increased tumour frequency and it is concluded that Diflufenican is not a carcinogen.

Mutagenicity studies suggest that Diflufenican is unlikely to present any genetic risk.

Diflufenican is not considered to be a teratogen.

EMERGENCY ACTION
FIRST AID
CONTAMINATION - Always seek medical advice in cases of serious personal contamination.

EYES - Irrigate with clean water for at least 15 minutes. Get medical help.

SKIN - Flood exposed skin with water. Remove contaminated clothing then wash skin with soap and water.

IF SWALLOWED - If patient is conscious was out mouth, if breathing stops start artificial respiration. Keep patient warm, comfortable at rest. Get medical help.

IF INHALED - Move patient to fresh air. If breathing stops, start artificial respiration. Get help.

- NEVER attempt to give anything to a semi-conscious or unconscious person.

IMMEDIATE

MEDICAL ADVICE To be administered by qualified medical personnel. No specific antidote. Symptomatic measures. Supportive measures.

EMPTYING THE STOMACH

In the event of a large amount being swallowed, stomach emptying is recommended, provided this can be performed within 4 hours of ingestion.

1. The method of choice of emptying the stomach is by gastric lavage with adequate control to prevent aspiration of any fluid into the lungs.

2. The administration of ipecacuanha* - under medical or nursing supervision - is the preferred method for inducing vomiting but its use in first-aid should be restricted to occupational health centres. It should not be recommended to first-aiders.

*Emetic dose for adults + 10-20 ml of Ipecacuanha Tincture (BP) followed, if necessary, by a similar dose after 20 minutes.

3. Pharyngeal stimulation is not the preferred method since it is not often effective in causing vomiting in adults.

4. Administration of salt solution is definitely contraindicated.

5. No attempt should be made to induce vomiting in unconscious or semi-conscious people.

FURTHER MEDICAL ADVICE Telephone Rhone Poulenc Ltd., 01-592-3060. During office hours ask for Medical Information Department. At other times the Duty Security Officer will find a respondent.

Doctors may obtain information and advice from the nearest Poisons Information Centre.

SPILLAGE for small amounts - Avoid breathing dust and other personal contact, wear gloves, preferably vacuum up the material, or cover with inert absorbent materials (e.g. sand, earth, vermiculite, etc.), carefully sweep up and place into a labelled receptacle for safe disposal.

for large amounts - Deal with all spillages immediately.

Keep people away and upwind.

Turn off all sources of ignition. No smoking.

Avoid contact with eyes, skin and clothing, wear overalls, rubber boots, gloves and goggles or face shield.

If contamination of drains, streams, watercourses, etc., is unavoidable, warn the Local Water Authority immediately.

Contain by surrounding and covering with inert absorbent material (sand, earth, vermiculite, etc.).

Sweep up carefully and shovel sweepings into marked open-head container.

Spread more inert absorbent materials over the entire spill area, sweep up and shovel sweepings into the marked container ("dry sweeping" process).

Repeat the "dry sweeping" process at least twice more.

Finally wash contaminated area with detergent and water. Absorb as much as possible into inert materials sweep up and add sweepings into the marked container.

Place marked container in a safe open area to await disposal.

DISPOSAL

a. Local authority may accept the sweepings, or

b. arrange disposal by a licensed contractor, or

c. dispose of safely, and in accordance with the Control of Pollution Act 1974, and regulations made thereunder.

FIRE

Combustible and may give rise to dangerous fumes.

Use waterspray, fog or foam.

Firefighter should wear protective clothing and breathing apparatus for fires involving large amounts of this product.

If contamination of drains or watercourses is unavoidable, warn the local Water Authority immediately.

STORAGE

Store in a cool, dry, well ventilated area away from sources of heat, food, drink and animal feedstuffs.

ADDITIONAL INFORMATION

ECOLOGICAL HAZARDS

Diflufenican has very low toxicity to avian and aquatic animals.

LC50 96 hr rainbow trout 75 mg/L
LC50 96 hr carp 105 mg/L

LD50 bobwhite quail over 2150 mg/kg
LD50 mallard duck over 4000 mg/kg