HEYDEN INTERNATIONAL TOPICS IN
SCIENCE
Editor: L. C. THOMAS

Paper & Thin Layer Chromatographic Analysis of Environmental Toxicants

M. E. GETZ

HEYDEN

PAPER AND THIN LAYER CHROMATOGRAPHIC ANALYSIS OF ENVIRONMENTAL TOXICANTS

M. E. GETZ

Agricultural Research Service
United States Department of
Agriculture, USA

HEYDEN

LONDON · PHILADELPHIA · RHEINE

Heyden & Son Ltd., Spectrum House, Hillview Gardens,
London NW4 2JQ, UK
Heyden & Son Inc., 247 South 41st Street, Philadelphia, PA 19104, USA
Heyden & Son GmbH, Münsterstrasse 22, 4440 Rheine, Germany

Getz, M E
Paper and thin layer chromatographic analysis of environmental toxicants.—(Heyden international topics in science).
1. Pollution—Measurement
2. Pollution—Toxicology
3. Environmental chemistry
4. Paper chromatography
5. Thin layer chromatography
I. Title
614.7 TD177 79-41719

ISBN 0-85501-451-2

This is a private publication reflecting the opinions of the author and in no way implies US Government or Department of Agriculture policy.

Set by Eta Services (Typesetters) Ltd., Beccles, Suffolk
Printed litho and bound in Great Britain by W. & J. Mackay Ltd., Chatham

PAPER AND THIN LAYER CHROMATOGRAPHIC ANALYSIS OF ENVIRONMENTAL TOXICANTS

HEYDEN INTERNATIONAL TOPICS IN SCIENCE

Editor: L. C. Thomas

RTL/2 Design and Philosophy	J. G. P. BARNES
Introduction to PASCAL	C. A. G. WEBSTER
Computer Applications in Ship Technology	C. KUO
An Introduction to Computer-based Library Systems	L. A. TEDD
Computers and Instrumentation—A Practical Handbook of Measurement, Interfacing and Control Circuits	A. CARRICK
Analysis with Ion-selective Electrodes	P. L. BAILEY
Analysis of Drugs of Abuse	E. BERMAN
Toxic Metals and their Analysis	E. BERMAN
Environmental Pollution Analysis	P. D. GOULDEN
Paper and Thin Layer Chromatographic Analysis of Environmental Toxicants	M. E. GETZ
Biometeorology—The Impact of the Weather and Climate on Humans and their Environment (Animals and Plants)	S. W. TROMP
Colour: its Measurement, Computation and Applications	G. J. CHAMBERLIN and D. G. CHAMBERLIN

FOREWORD

The object of this series of monographs is the timely dissemination of information on topics of current interest in science. Techniques, ideas and applications are described in sufficient detail to enable those who are not specialists in a particular subject to appreciate the applicability of the subject matter to their own work. Bibliographies included in these monographs will enable the reader to pursue the subject to any desired depth. The volumes will thus form compact definitive texts for the specialist also.

One topic of continuing current interest in science is that of the environment and its pollution. The present volume deals with the application of the relatively simple and inexpensive techniques of paper and thin layer chromatography to the detection, and determination, of toxic or potentially toxic contaminants of the environment, with special emphasis on the air we breathe, the food we eat, and the water we drink.

Because of the relatively cheap and simple methods involved, this volume will be of especial interest to analysts without access to more sophisticated and expensive equipment and, in particular, to those in developing countries. The extensive collection of chromatographic reference data (for animal drugs and feed additives, air and water pollutants, pesticides, herbicides, food additives, cosmetics and natural toxicants), which are included in this volume, make it a valuable laboratory reference source for all scientists working with these materials.

L. C. Thomas

PREFACE

'They shall rule the fish of the seas, the birds of the sky, the cattle, the whole earth and all the creeping things that creep on earth'.

Genesis: 1.26–27

Many centuries have passed since the above words were written. During this time man has not only used what nature has provided but has also, unfortunately, abused her. Today, in the twentieth century we are acutely aware of our environment. Pollution, ecology and environment are everyday words. There is much emphasis on the interaction between man and his surroundings, and the beneficial and detrimental effects of his influence on the environment.

As science and technology grow at an ever increasing rate, man has realized that though it is highly improbable that he could destroy the planet earth, he can certainly destroy those elements which allow him to dominate it. The introduction into nature of artificial chemical factors makes it necessary to ascertain if the environment is capable of diluting or altering these artifacts to prevent permanent detrimental effects. This need has heralded a new era in analytical chemistry. New separation techniques coupled with the availability of highly sophisticated electronic instrumentation have enabled the analyst to measure chemicals in ever smaller quantities.

Chromatographic techniques have facilitated the task of environmental analysis. This monograph is concerned with the liquid–liquid and liquid–solid adsorption phenomena as manifested in paper and thin layer chromatography. The two techniques are reviewed as analytical methods for monitoring natural and man-made pollutants and toxicants in the environment. The author, having selected the more significant topics from this vast subject, has tried to include enough pertinent information to enable an experienced analyst to develop a methodology for solving his particular problem.

Today, it is possible to make paper and thin layer chromatography as quantitative as other chromatographic methods by *in situ* determination of spot density. This may be accomplished with the help of optical scanning instruments by applying spectrophotometric methods of assay to the desired compounds after they have been resolved on a paper or thin layer chromatogram. It is no longer necessary to extract material from a migrated spot before quantifying it spectrophotometrically or otherwise.

Many of the methods described can be extended to be quantitative by the application of *in situ* optical scanning, which means that there are now three quantitative chromatographic techniques available to the analyst; gas–liquid chromatography with its many detectors, high performance liquid chromatography, and thin layer or paper chromatography with its range of chromogenic reagents.

I would like to thank my wife Eve for her constructive criticism and editing which always improves the content of my writings.

M. E. Getz

November 1979

CONTENTS

CHAPTER 1

DRUGS AND ANIMAL FEED ADDITIVES

Man has been growing crops and raising animals for food for many thousands of years, but it has only been during the last two centuries that antibiotics and other drugs have been applied to the improvement of the many products of agronomy. One development of these applications has been the mixing of specific chemicals with animal feeds to increase resistance to disease and parasites in livestock.

Maintaining the health of the livestock population is important from two points of view. Firstly, it helps to ensure good quality meat, poultry, eggs and milk products for the consumer, at reasonable prices. Secondly, it greatly reduces the transmission of common diseases from animal to animal, and more importantly from animals to man.

Since the demand for animal protein has increased with the expanding world population, and agricultural practices are striving to keep production at a high level, antibiotics and other drugs have become increasingly important for maintaining efficient and competitive livestock industries. A number of drugs are used for the regulation of livestock growth and for the control of diseases such as coccidiosis, histomoniasis, worms and other epidemic diseases and parasites that can infect farm animals. Tables 1.1 and 1.2 list many of the chemicals that are used for these purposes in the United States.

The increasingly advanced technology of animal treatment has necessitated more complex regulation of these treatments by government. Any chemical used for the treatment of animals must now be shown to be both effective and safe for the animal, and to be free from harmful effects to the consumer. However, the possibility of drug residues in edible meat, milk, eggs, poultry and fish products always exists. This has meant that chemical analysis, which has always played an important role in governmental supervision of such chemical usage, has become increasingly necessary as the chemicals used in animal husbandry continually change.

TABLE 1.1
Antibiotics commonly used for animal treatment

Bacitracin	Neomycin
Chlortetracycline	Novobiocin
Erythromycin	Nystatin
Griseofulvin	Oleandomycin
Hygromycin B	Oxytetracycline
Lincomycin	Penicillin G
Monensin	Tylosin

Paper and thin layer chromatography can be useful tools for determining the identity and amount of chemicals in animal feed, and for monitoring the amounts of chemical residues in edible tissues and products.

1.1 EXTRACTION AND ISOLATION

The first step in any trace analytical procedure is the extraction of the desired chemical from the matrix in which it is contained. This can be done by various means such as:

(1) Stripping, used in the extraction of Novobiocin from feed.[1] A 1 g sample is placed in a stoppered graduated cylinder or flask, and extracted twice by vigorous shaking with ethyl acetate. The supernatant liquid is decanted and diluted to volume for microbiological assay or further purified for chromatographic separation.
(2) Soxhlet extraction, applied to extracting Griseofulvin from a feed sample.[2] A few grams of the finely-ground sample are placed in a fat-free thimble and extracted with chloroform for 2 h. The chloroform extract is evaporated down to a small volume and then diluted to 100 ml for further analytical treatment.
(3) High speed homogenization, whereby Clopidol residues are extracted from muscle, liver and kidney[3] with methanol and HYflo Super-Cel. The homogenate is filtered and the methanol filtrate is saved for further isolation steps.

After the relevant compound or compounds have been extracted, it is usually necessary to render them pure enough to be chromatographed with a minimum of interference from other co-extractives. This step is commonly referred to as the clean-up procedure, and experience has shown that a very efficient clean-up is usually necessary to prepare a sample for analysis by paper or thin layer chromatography. A widely applied bipartite clean-up method employs liquid–liquid partitioning between polar and nonpolar solvents,

TABLE 1.2
Drugs commonly used on animals in the United States

Common name[a]	Chemical name
Aklomide	2-Chloro-4-nitrobenzamide
Amprolium	1-(4-Amino-2-*n*-propyl-5-pyrimidinyl)-2-picolinium chloride
Arsanilic acid	*p*-Aminobenzenarsonic acid
Buquinolate	Ethyl-4-hydroxy-6,7-diisobutoxy-3-quinolinecarboxylate
Decoquinate	Ethyl-6-*n*-decyloxy-7-ethoxy-4-hydroxy-3-quinolinecarboxylate
DES	Diethylstilbesterol
Dimetridazole	1,2-Dimethyl-5-nitroimidazole
Enheptin	2-Amino-5-nitrothiazole
Ethopabate	Methyl-4-acetamido-2-ethoxybenzoate
Furazolidone	*N*-(5-Nitro-2-furfurylidene)-3-amino-2-oxazolidone
Glycarbylamide	4,5-Imidazoledicarboxamide
Ipronidazole	1-Methyl-2-isopropyl-5-nitroimidazole
Nequinate	Methyl-7-benzyloxy-6-butyl-1,4-dihydro-4-oxo-3-quinolinecarboxylate
Nicarbazin	4,4′-Dinitrocarbanilide-2-hydroxy-4,6-dimethylpyrimidine
Nifursol	3,5-Dinitrosalicylic acid
Nihydrazone	5-Nitro-2-furfuraldehyde acetylhydrazone
Nithiazide	1-Ethyl-3-(5-nitro-2-thiazolyl)urea
Nitrodan	3-Methyl-5-(*p*-nitrophenyl-azo-rhodanine)
Nitrofurazone	5-Nitro-2-furaldehyde semicarbazone
Nitromide	3,5-Dinitrobenzamide
Nitrophenide	*m,m*′-Dinitrodiphenyldisulfide
Nitarsone	4-Nitrophenylarsonic acid
Phenothiazine	
Piperazine	
Racephenicol	DL-Threo-2,2-dichloro-*N*-(β-hydroxy-α-hydroxymethyl)-*p*-(methylsulfonyl)phenethyl acetamide
Reserpine	
Roxarsone	4-Hydroxy-3-nitrobenzenearsonic acid
Sulfathiazole	
Sulfamerazine	
Sulfadimethoxine	2,5-Diamino-5-(4,5-dimethoxy)methylbenzyl pyrimidine
Sulfaguanidine	
Sulfaquinoxaline	
Sulfamethazine	*N*-(4,6-Dimethyl-2-pyrimidinyl)sulfanilamide
Sulfanitran	Acetyl-(*p*-nitrophenyl)sulfanilamide
Thiobendazole	2-(4-Thiazolyl)benzimidazole
Zoalene	3,5-Dinitro-*o*-toluamide

[a] Where the common and chemical names are identical the chemical name is not repeated.

followed by column chromatography on adsorbents such as alumina, silica gel or Florisil.

There are two major steps in preparing a sample for analysis. The compound must first be extracted from the containing matrix and then separated from unwanted co-extractives so that there is little or no interference when developing the thin layer or paper chromatogram.

1.2 ANALYSIS OF ANTIBIOTICS

Antibiotics are used for promoting the growth of livestock and poultry, and it is generally accepted that they are capable of three major types of biological action.[4]

(1) A metabolic effect, in which the antibiotic affects the metabolism of the animal favorably, so that it grows at a faster rate.
(2) The suppression of undesirable organisms, which compete with the animal for nutrients, and the stimulation of desirable organisms.
(3) The suppression of organisms that cause disease.

1.2.1 Bacitracin

The antibiotic bacitracin is usd as a growth promoter, as an inhibitor of liver abscesses, for increasing egg production and hatchability, for suppressing enteritis and chronic respiratory diseases, and for minimizing the effects of stress.

A successful thin layer separation (TLC) has been obtained by Ikekawa *et al.*[5]

Adsorbent: Silica Gel G
Developing solvents: (1) ethanol–ammonium hydroxide–water (8:1:1)
(2) ethanol–water (4:1)
R_F: (1) =0.58; (2) =0.13.

The compound can be made visible with ninhydrin, or UV light or by bio-autography.

Nussbaumer[6] also resolved bacitracins A and B by TLC.

Adsorbent: Silica Gel G
Developing solvent: *n*-butanol–acetic acid–water (4:1:2)
Bacitracin A, $R_F = 0.35$; bacitracin B, $R_F = 0.22$.

The spots were detected in UV light after heating the chromatogram to 110 °C, or with ninhydrin dissolved in a butanol–acetic acid solution.

1.2.2 Chlortetracycline and Oxytetracycline

Chlortetracycline is used as a growth promoter, as a preventive against anaplasmosis, bacterial pneumonia, foot rot, liver abscesses, respiratory infections, shipping fever, enteritis, coccidiosis, synovitis, atrophic rhinitis and cervical abscesses, and for increasing egg production and hatchability. Oxytetracycline is used to increase milk production, to produce better eggshell texture, and to prevent blue comb, cholera, hepatitis, bacterial diarrhea, enterotoxemia, leptospirosis, and hexamitiasis in animals and man.

Paper chromatography can be used to resolve these two antibiotics. One approach has been to develop the paper chromatogram with a solvent mixture of *n*-butanol, acetic acid and water (4:1:5).[7] The migrated spots were detected by bioautography and the R_F values for oxytetracycline and chlortetracycline were 0.60 and 0.69, respectively.

Another approach was to impregnate the paper with pH 3.5 McIlvaine's citrate–phosphate buffer,[8] and then to develop the chromatogram with a solvent system consisting of chloroform, nitromethane and pyridine (10:20:30), using bioautography to detect the spots. (R_F oxytetracycline = 0.13; R_F chlortetracycline = 0.50.)

Kelly and Buyska[9] impregnated the chromatographic paper with 0.1M EDTA solution and then developed with a mixture of *n*-butanol, acetic acid and water (4:1:5). This mixture forms two phases of which the upper phase is used for development. Bioautography is used for detection. This solvent system gave an R_F of 0.59 for oxytetracycline and an R_F of 0.76 for chlortetracycline. When the upper phase of the solvent mixture, *n*-butanol–ammonium hydroxide–water (4:1:5) was used to develop the chromatogram oxytetracycline and chlortetracycline had R_F values of 0.27 and 0.47, respectively.

Two other useful paper chromatographic systems were developed by Bohonas *et al.*,[10] and Hickey and Phillips.[11] The former soaked chromatographic paper with 0.3M pH 3 phosphate buffer, air-dried the paper and then developed in *n*-butanol saturated with water. Bioautography was used for detection; oxytetracycline gave an R_F of 0.40 and chlortetracycline an R_F of 0.65. The latter group chromatographed chlortetracycline without using a buffer but included acetic acid in the developing solvent.

Adsorbent: Schleicher and Schuell No. 507 chromatographic paper
Developing solvent: acetic acid–*n*-butanol–water (1:2:1)
R_F = 0.77.
Detection technique: bioautography

When TLC was applied to the resolution of the two tetracyclines some difficulties were apparent in the separations. However, Gyanchandani *et al.*[12] were able to develop a system for separating them.

The thin layer was prepared by mixing 50 g of celite with a 0.1M aqueous solution of EDTA plus a 20% polyethylene glycol 400 solution in glycerine (95:5). The plates were coated with a layer of 0.25 mm thickness, and dried overnight at room temperature. The following two developing solutions gave good resolution.

(1) Methyl ethyl ketone saturated with pH 4.7 McIlvaine's buffer.
R_F oxytetracycline = 0.60; R_F chlortetracycline = 0.76.
(2) Dichloromethane–ethyl formate–ethanol (9:9:2) saturated with pH 4.7 McIlvaine's buffer.
R_F oxytetracycline = 0.20; R_F chlortetracycline = 0.60.

Two chromogenic reagents gave good results: Fast Blue B (a diazonium salt), under alkaline conditions, gave a pink spot with chlortetracycline and a

yellow spot with oxytetracycline; a 50 mg per cent solution of diphenylpicrylhydrazyl sprayed under alkaline conditions gave a yellowish pink spot for chlortetracycline and a brownish yellow spot for oxytetracycline.

1.2.3 Erythromycin

This is used to promote growth, and to prevent respiratory diseases, coryza and stress. Majumdar and Majumdar[13] developed a paper chromatographic procedure for the resolution of erythromycin isomers, where the paper was developed with methanol, water and acetone (19:75:6), and the resulting spots were detected with bioautography using *Micrococcus pyogenes var. Aureus*, or *Bacillus subtilis* (erythromycin A, $R_F = 0.7$, and erythromycin B, $R_F = 0.6$).

A thin layer separation was accomplished[14] by coating a plate with a slurry of Silica Gel G in a 0.02N aqueous sodium acetate solution, developing the plate in methanol and 0.02N sodium acetate (120:30), then detecting the migrated compound with a solution of glucose, 85% phosphoric acid, water, ethanol and *n*-butanol (2 g + 10 ml + 40 ml + 30 ml + 30 ml). After spraying with the reagent solution the plate was heated at 150 °C for 5 min, erythromycin formed a blue grey color. ($R_F = 0.28$.)

1.2.4 Griseofulvin

This is a fungicide which is used for the systemic treatment of athletes foot in humans and also serves as a general fungicidal treatment for animals. It can be chromatographed using paper in conjunction with a number of solvent systems.[15] The paper may be:

(i) impregnated with chloroform and developed with a butanol-saturated water solution. ($R_F = 0.22$.)
(ii) impregnated with ethyl acetate and developed with butanol-saturated water. ($R_F = 0.22$.)
(iii) impregnated with ethyl acetate and developed with a butanol, ethanol and water mixture (5:1:4). ($R_F = 0.37$.)
(iv) impregnated with methyl ethyl ketone and developed with butanol-saturated water. (R_F value = 0.24.)

Detection of the migrated compound was carried out by UV irradiation. Thin layer techniques can also be used for the chromatography of Griseofulvin.[16] A plate is coated with a layer of Silica Gel HF254 and developed in two different solvent systems.

(1) Butyl acetate–acetone (4:1, $R_F = 0.49$)
(2) Chloroform–methanol (98:2, $R_F = 0.68$)

The migrated spot may be detected with a solution of 5% potassium carbonate and 0.2% potassium permanganate.

1.2.5 Lincomycin

This is used primarily for promoting growth in animals, and can also be separated by paper chromatography:[17] the chromatogram is developed with a solution of 1-butanol, acetic acid and water (2:1:1), and the antibiotic then detected by bioautography using *Sarcina lutea*. ($R_F = 0.70$).

Silica Gel G was used as adsorbent for the thin layer chromatography of lincomycin.[18] The chromatogram was developed in ethyl acetate, acetone and water (8:5:1), and the spot detected by bioautography using *Sarcina lutea*. ($R_F = 0.3$.)

1.2.6 Monensin

This antibiotic is used for the control of coccidiosis. The various types of monensin have been resolved by paper chromatography,[19] using Whatman No. 1 paper developed in a water, methanol, acetic acid benzene mixture (72:24:0.5:3.5). The migrated spots were detected by bioautography using *Bacillus subtilis ATCC 6633*. (Monensin A, $R_F = 0.50$; Monensin B, $R_F = 0.90$; Monensin C, $R_F = 0.35$.)

Thin layer chromatography of monensin was carried out with silica gel as adsorbent; the developing solvent was ethyl acetate, and the migrated compounds were detected by spraying with a 3% vanillin solution in 1.5% ethanolic sulfuric acid and heating at 100 °C until spots were a bright red color. (Monensin A, $R_F = 0.5$; Monensin B, $R_F = 0.5$; Monensin C, $R_F = 0.2$.)

1.2.7 Neomycin

This is used to treat bacterial enteritis, enterotoxaemia and dysentery. Paper chromatographic separation was obtained[19] by developing the chromatogram in a solvent mixture of *n*-propanol, glacial acetic acid and water (9:1:10). Neomycin B and Neomycin C gave R_F values of 0.26 and 0.30, respectively. They were both detected with ninhydrin. Another paper method[20] used methyl ethyl ketone, *t*-butanol, methanol and 6.5N ammonium hydroxide (16:3:1:6) as the developing solvent, and ninhydrin as the detecting agent. (Neomycin B, $R_F = 0.54$; Neomycin C, $R_F = 0.30$.) The R_F values were calculated relative to the R_F value for neamine, which was taken as 1.00.

An interesting approach to the thin layer separation of Neomycin was devised by Brodasky.[21] Plates were prepared from Nuchar activated carbon by making a slurry of the following composition: Nuchar C-190N + calcium sulfate ($CaSO4.\frac{1}{2}H_2O$) + water (30 g + 1.5 g + 220 ml). This was applied to glass plates immediately after mixing and allowed to air-dry. Coated plates were stored for 16 h before use. Chromatograms were then developed with a solution of 0.5N sulfuric acid, neutralized by exposure to ammonia fumes for