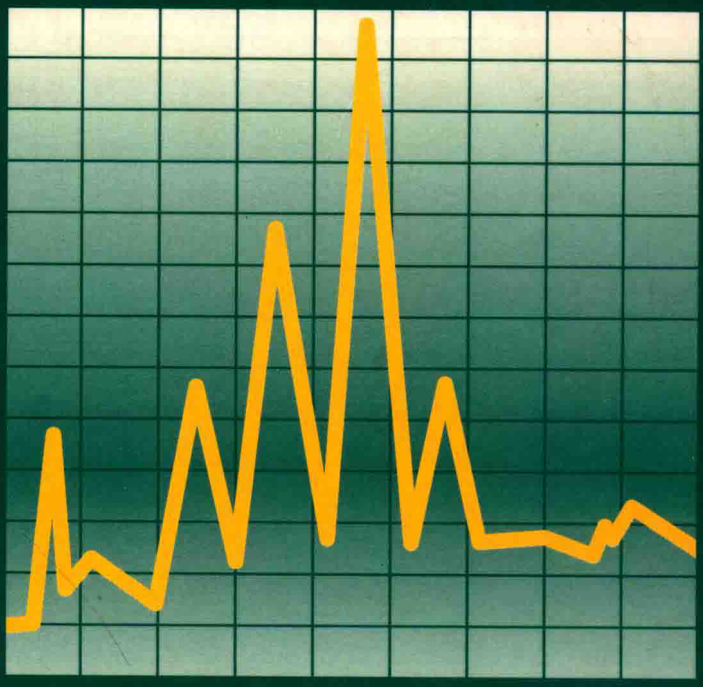
Phytochemical Methods

A GUIDE TO MODERN TECHNIQUES OF PLANT ANALYSIS

THIRD EDITION



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A guide to modern techniques of plant analysis

Third edition

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Preface to first edition

While there are many books available on methods of organic and biochemical analysis, the majority are either primarily concerned with the application of a particular technique (e.g. paper chromatography) or have been written for an audience of chemists or for biochemists working mainly with animal tissues. Thus, no simple guide to modern methods of plant analysis exists and the purpose of the present volume is to fill this gap. It is primarily intended for students in the plant sciences, who have a botanical or a general biological background. It should also be of value to students in biochemistry, pharmacognosy, food science and 'natural products' organic chemistry.

Most books on chromatography, while admirably covering the needs of research workers, tend to overwhelm the student with long lists of solvent systems and spray reagents that can be applied to each class of organic constituent. The intention here is to simplify the situation by listing only a few specially recommended techniques that have wide currency in phytochemical laboratories. Sufficient details are provided to allow the student to use the techniques for themselves and most sections contain some introductory practical experiments which can be used in classwork.

After a general introduction to phytochemical techniques, the book contains individual chapters describing methods of identifying phenolic compounds, terpenoids, fatty acids and related compounds, nitrogen compounds, sugars and their derivatives and macromolecules. The attempt has been made to cover practically every class of organic plant constituent, although in some cases, the account is necessarily brief because of space limitations. Special attention has, however, been given to detection of endogeneous plant growth regulators and to methods of screening plants for substances of pharmacological interest. Each chapter concludes with a general reference section, which is a bibliographic guide to more advanced texts.

While the enormous chemical variation of secondary metabolism in plants has long been appreciated, variation in primary metabolism (e.g. in

the enzymes of respiration and of photosynthetic pathways) has only become apparent quite recently. With the realization of such chemical variation in both the small and large molecules of the plant kingdom, systematists have become interested in phytochemistry for shedding new light on plant relationships. A new discipline of biochemical systematics has developed and, since the present book has been written with some emphasis on comparative aspects, it should be a useful implement to research workers in this field.

In preparing this book for publication, the author has been given advice and suggestions from many colleagues. He would particularly like to thank Dr E.C. Bate-Smith, Dr T. Swain, Dr T.A. Smith and Miss Christine Williams for their valuable assistance. He is also grateful to the staff of Chapman and Hall for expeditiously seeing the book through the press.

Reading,
June 1973

J.B.H.

Preface to second edition

Since the preparation of the first edition, there have been several major developments in phytochemical techniques. The introduction of carbon-13 NMR spectroscopy now provides much more detailed structural information on complex molecules, while HPLC adds a powerful and highly sensitive analytical tool to the armoury of the chromatographer. With HPLC, it is possible to achieve for involatile compounds the type of separations that GLC produces for volatile substances. Spectacular developments have also occurred in the techniques of mass spectrometry; for example, the availability of a fast-atom bombardment source makes it possible in FAB-MS to determine the molecular weight of both very labile and involatile plant compounds. These techniques are described briefly in the appropriate sections of this new edition.

During the last decade, the number of new structures reported from plant sources has increased enormously and, among some classes of natural constituent, the number of known substances has doubled within this short time-span. The problems of keeping up with the phytochemical literature are, as a result of all this activity, quite considerable, although computerized searches through *Chemical Abstracts* have eased the burden for those scientists able to afford these facilities. In order to aid at least the student reader, literature references in this second edition have been extensively updated to take into account the most recent developments. Additionally, some new practical experiments have been added to aid the student in developing expertise in studying, for example, phytoalexin induction in plants and allelopathic interactions between plants.

Since the first edition, phytochemical techniques have become of increasing value in ecological research, following the realization that secondary constituents have a significant role in determining the food choice of those animals that feed on plants. Much effort has been expended on analysing plant populations for their toxins or feeding deterrents. Most such compounds were included in the first edition, except for the plant

tannins. A new section has therefore been included on tannin analysis in Chapter 2.

With this new edition, the opportunity has been taken to add two appendices – a checklist of TLC procedures for all classes of plant substance and a list of useful addresses for phytochemists. Some errors in the first edition have been corrected, but others may remain and the author would welcome suggestions for further improvements.

As with the first edition, the author has benefited considerably from help and advice of many colleagues. He would particularly like to thank his co-workers in the phytochemical unit and his students, who have been willing guinea pigs in the development of new phytochemical procedures.

Reading,
December 1983

Jeffrey B. Harborne

Preface to the third edition

Since 1984, when the second edition was published, there have not been any spectacular discoveries in phytochemical methods. Nevertheless, it has been a period of consolidation, when improvements to existing methods have been introduced. The first journal specifically dealing with plant analysis, called *Phytochemical Analysis*, was launched in 1990. The use of HPLC as an analytical tool has now spread to almost every class of secondary metabolite and this has been taken into account during the revision of the second edition. New references to the primary literature have been added. Sections on alkaloids, amines, iridoids, lipophilic flavonoids, glucosinolates and sesquiterpene lactones have all been expanded. The application of phytochemical methods to plants of ethobotanical importance has increased significantly in recent years and a brief account of the application of phytochemistry to medicinal plants has been added to chapter 1.

1990 was an important landmark for phytochemists. It saw the publication of the *Dictionary of Natural Products*, edited by John Buckingham, under the imprint of Chapman and Hall. This dictionary provides the phytochemical worker for the first time with a complete list of all known plant (and animal) chemical constituents and moreover, it is being regularly updated. One can now swiftly answer the question: have I found a new plant substance or not? A wealth of books and reviews on methods of plant analysis have appeared in the last decade and these have been added to the appropriate reference lists at the end of each chapter.

In preparing this new edition, the author wishes to thank once again colleagues in the phytochemical research service at Reading and also his many research students, over the years, who have tried out many of the procedures described herein. He also thanks Miss Valerie Norris for her expert assistance in the preparation of this edition, and the publishers for their encouragement.

Reading
February 1998

Jeffrey B. Harborne

Glossary

GENERAL ABBREVIATIONS

TLC = thin layer chromatography
GLC = gas liquid chromatography
PC = paper chromatography
UV = ultraviolet
IR = infrared
NMR = nuclear magnetic resonance
HPLC = high performance liquid chromatography
MS = mass spectroscopy
R_F = mobility relative to front
RR_t = relative retention time
nm = nanometres
mol.wt. = molecular weight
M = molar

CHEMICALS

BSA = *N,O*-bis (trimethylsilyl) acetamide
PVP = Polyvinylpyrrolidone (for removing phenols)
BHT = butylated hydroxytoluene (anti-oxidant)
EDTA = ethylenediaminetetracetic acid (chelating agent)
Tris = tris(hydroxymethyl)methylamine (buffer)
PMSF = phenylmethane sulphonyl fluoride
SDS = sodium dodecyl sulphate

CHROMATOGRAPHIC SUPPORTS

Kieselguhr = diatomaceous earth
Decalso = sodium aluminium silicate
DEAE–cellulose = diethylaminoethyl-treated
PEI–cellulose = polyethyleneimine-treated
ECTEOLA–cellulose = epichlorohydrin-triethanolamine-alkali treated

PPE = polyphenyl ether
TXP = trixylenylphosphate
DEGS = diethyleneglycol succinate
Apiezon L = stop-cock grease
Carbowax = polyethylene glycol
Chromosorb = firebrick support
ODS = octadecylsilane
OV = methyl siloxane polymer
SE = silicone oil
XE = nitrile silicone
Embacel = acid-washed celite support
Poropak = styrene polymer support

CHROMATOGRAPHIC SOLVENTS

MeOH = methanol
EtOH = ethanol
iso-PrOH = iso-propanol
n-BuOH = *n*-butanol
iso-BuOH = iso-butanol
PhOH = phenol
$NHEt_2$ = diethylamine
Et_2O = diethyl ether
HCO_2H = formic acid
HOAc = acetic acid
$CHCl_3$ = chloroform
CH_2Cl_2 = methylene dichloride
EtOAc = ethyl acetate
Me_2CO = acetone
MeCOEt = methyl ethyl ketone
C_6H_6 = benzene

Contents

1

Methods of plant analysis

1.1 INTRODUCTION

The subject of phytochemistry, or plant chemistry, has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, their natural distribution and their biological function.

In all these operations, methods are needed for separation, purification and identification of the many different constituents present in plants. Thus, advances in our understanding of phytochemistry are directly related to the successful exploitation of known techniques, and the continuing development of new techniques to solve outstanding problems as they appear. One of the challenges of phytochemistry is to carry out all the above operations on vanishingly small amounts of material. Frequently, the solution of a biological problem in, say, plant growth regulation, in the biochemistry of plant–animal interactions, or in understanding the origin of fossil plants depends on identifying a range of complex chemical structures which may only be available for study in microgram amounts.

It is the purpose of this book to provide, for the first time, an introduction to present available methods for the analysis of plant substances and

to provide a key to the literature on the subject. No novelty is claimed for the methods described here. Indeed, the purpose is to outline those methods which have been most widely used; the student or research worker can then most rapidly develop his own techniques for solving his own problems.

Some training in simple chemistry laboratory techniques is assumed as a background. However, it is possible for botanists and other plant scientists, with very little chemistry, to do phytochemistry, since many of the techniques are simple and straightforward. As in other practical subjects, the student must develop his own expertise. No recipe, however precisely written down, can substitute in the laboratory for common-sense and the ability to think things out from first principles. Examples of practical experiments which can be worked through to gain experience are provided in most sections of the following chapters. These can readily be adapted for laboratory courses and many have already been used for this purpose.

The range and number of discrete molecular structures produced by plants is huge and such is the present rate of advance of our knowledge of them that a major problem in phytochemical research is the collation of existing data on each particular class of compound. It has been estimated, for example, that there are now over 10000 known plant alkaloids and such is the pharmacological interest in novel alkaloids that new ones are being discovered and described, possibly at the rate of one a day.

Because the number of known substances is so large, special introductions have been written in each chapter of the book, indicating the structural variation existing within each class of compound, outlining those compounds which are commonly occurring and illustrating the chemical variation with representative formulae. References are given, wherever possible, to the most recent listings of known compounds in each class. Tables are included, showing the R_F values, colour reactions and spectral properties of most of the more common plant constituents. These tables are given mainly for illustrative or comparative purposes and are not meant to be exhaustive.

Phytochemical progress has been aided enormously by the development of rapid and accurate methods of screening plants for particular chemicals and the emphasis in this book is inevitably on chromatographic techniques. These procedures have shown that many substances originally thought to be rather rare in occurrence are of almost universal distribution in the plant kingdom. The importance of continuing surveys of plants for biologically active substances needs no stressing. Certainly, methods of preliminary detection of particular classes of compound are discussed in some detail in the following chapters.

Although the term 'plant' is used here to refer to the plant kingdom as a whole, there is some emphasis on higher plants and methods of analysis

for micro-organisms are not dealt with in any special detail. As a general rule, methods used with higher plants for identifying alkaloids, amino acids, quinones and terpenoids can be applied directly to microbial systems. In many cases, isolation is much easier, since contaminating substances such as the tannins and the chlorophylls are usually absent. In a few cases, it may be more difficult, due to the resilience of the microbial cell wall and the need to use mechanical disruption to free some of the substances present.

There are a number of organic compounds, such as the penicillin and tetracycline antibiotics (Turner, 1971; Turner and Aldridge, 1983), which are specifically found in micro-organisms and their identification is not covered here, because of limitations on space. Lichens also make a range of special pigments, including the depsidones and depsides. These are analysed by special microchemical methods, based on colour reactions, chromatographic and spectral techniques. A comprehensive account of the chemistry of lichens is given by Culberson (1969). The analysis of lichen pigments is mentioned briefly here in Chapter 2 (p. 101).

The chemical constituents of plants can be classified in a number of different ways; in this book, classification is based on biosynthetic origin, solubility properties and the presence of certain key functional groups. Chapter 2 covers the phenolic compounds, substances which are readily recognized by their hydrophilic nature and by their common origin from the aromatic precursor shikimic acid. Chapter 3 deals with the terpenoids, which all share lipid properties and a biosynthetic origin from isopentenyl pyrophosphate. Chapter 4 is devoted to organic acids, lipids and other classes of compound derived biosynthetically from acetate. Chapter 5 is on the nitrogen compounds of plants, basic substances recognized by their positive responses to either ninhydrin or the Dragendorff reagent. Chapter 6 deals with the water-soluble carbohydrates and their derivatives. Finally, Chapter 7 briefly covers the macromolecules of plants, nucleic acids, proteins and polysaccharides, which are easily separated from other constituents by their high molecular weights.

In the remainder of this introductory chapter, it is proposed to discuss, in general terms, methods of extraction, separation and identification. A final section will include some examples of the application of phytochemical methods in different areas of plant science.

There are two major reference works available on methods of plant analysis. The first is entitled *Methods in Plant Biochemistry*, with P.M. Dey and J.B. Harborne as series editors; this has appeared in a 10 volume set (1989–1997). The second is *Modern Methods of Plant Analysis*, a new series edited by H.F. Linskens and J.F. Jackson, which began publication in 1985 and has reached about 20 volumes. Many other texts deal *inter alia* with phytochemical methods and these are listed in the references at the end of this and subsequent chapters. A current journal *Phytochemical Analysis* is