STUDIES ON THE PHENOLIC COMPOUNDS OF APPLE LEAF TISSUE

(*Malus pumila* Mill.)

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for the degree of Master of Horticultural Science
in Plant Science

by

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Chapter 1

INTRODUCTION - THE PHENOLIC COMPOUNDS IN HIGHER PLANTS

1.1 The Natural Phenolic Compounds in Higher Plants

A number of books dealing wholly or partly with the natural phenolic compounds have been published in recent years. A comprehensive treatment of phenolic compounds is presented in one of them (1). Specific discussion of the flavonoid group of phenolic compounds is also found elsewhere (2,3). In these references much of the literature on phenolic compounds is cited and reviewed.

1.1.1 The nature of phenolic compounds

Substances which possess at least one aromatic ring bearing an hydroxyl substituent constitute the phenolic compounds. Several hundred such compounds are known in plants. They represent a number of groups of compounds including the simple monocyclic phenols, the flavonoids and their relatives, the xanthones, phenolic quinones, alkaloids and sterols, besides polymeric materials such as the tannins and lignins (4).

Further discussion of the natural phenolic compounds in this thesis will be largely confined to the two groups of monocyclic phenols (benzoic and cinnamic acid derivatives) and the flavonoid group of compounds. This recognises the relative importance of these

three groups of phenolic compounds in plants in general and in the genus Malus in particular. While the flavonoid compounds constitute the largest group of natural phenols (4), the phenolic acids of the benzoic and cinnamic acid groups are present in variety in many plants (5). The following outline of each of these three groups will illustrate their nature.

1.1.2 Benzoic acid derivatives (C6 - C1 compounds)

Most of the simple phenolic acids (see Table 1) are apparently widely distributed in Angiosperms. For example, an examination of the leaves of 122 plants representing 86 families showed that p-hydroxybenzoic and gentisic acids occurred in 97% of the plants (6).

Three acids, p-hydroxybenzoic, vanillic and syringic acids, occur as ester groups in lignin and, as a rule, plants which do not contain lignin lack these acids (6). Other benzoic acid derivatives occur in plants (5), some of which are rich sources of derivatives with unusual hydroxylation patterns (6).

The interrelationships of all these hydroxybenzoic acids are not clear at present since the origin of the carbon skeleton of some is unknown (7).

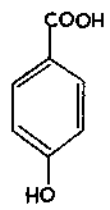
1.1.3 Cinnamic acid derivatives and related compounds (C6 - C3 compounds)

The common cinnamic acid derivatives are shown in Table 1 and are all derivatives of trans-cinnamic acid. These acids are represented in combined form in practically every higher plant. In a sample of the leaves of Angiosperms the frequencies of occurrence of

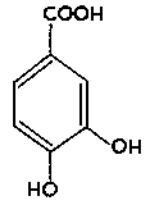
Table 1

STRUCTURE OF SOME BENZOIC AND CINNAMIC ACID DERIVATIVES

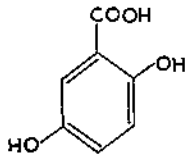
Benzoic Acid Derivatives



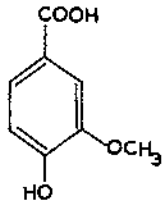
p-hydroxybenzoic acid



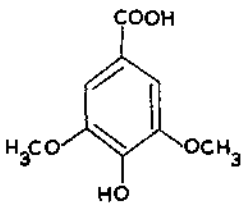
protocatechuic acid



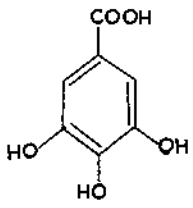
gentisic acid



vanillic acid

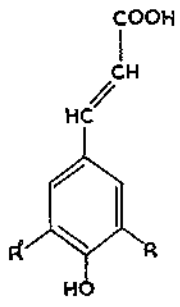


syringic acid



gallic acid

Cinnamic Acid Derivatives



General structure

R = R' = H	p-coumaric acid
R = OH; R' = H	caffeic acid
R = OCH_3; R' = H	ferulic acid
R = R' = OCH_3	sinapic acid

p-coumaric, caffeic, ferulic and sinapic acids were 49, 63, 48 and 32% respectively (6).

Other cinnamic acid derivatives are rare, for example, 3,4,5-trimethoxycinnamic acid. The o-hydroxycinnamic acids are readily cyclized to coumarins during isolation and tend to be recorded as such. So far the only o-hydroxycinnamic acid isolated is o-coumaric acid (6).

The alcohols corresponding to p-coumaric, ferulic and sinapic acids (p-coumaryl, coniferyl and sinapyl alcohol respectively) are thought to be precursors of lignin, a polymer containing such C6 - C3 units (6).

Other C6 - C3 compounds include the coumarins, the isocoumarins and the chromones, while the lignans which occur in heartwoods are dimers of C6 - C3 units (6).

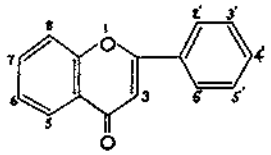
1.1.4 The flavonoid compounds (C6 - C3 - C6 compounds)

The flavonoid nucleus is represented by the structure of flavone, 2-phenylchromone, in Table 2 (8). The molecule consists of two benzene rings (A and B) joined by a three-carbon bridge which is formed into a γ-pyrone ring. In nature, various classes of flavonoid compounds occur and they are distinguished only by the state of oxidation of the three-carbon bridge (8). Details of these classes are presented in Table 2 and a few examples of specific compounds are given. The numbering of the flavone nucleus applies to all other classes except the dihydrochalkones and chalkones, where the C5 and C7 (flavone) become C2' and C4' and the aurones, where C5 and C7

Table 2

CLASSES OF THE FLAVONOID COMPOUNDS

The Flavonoid Nucleus



flavone

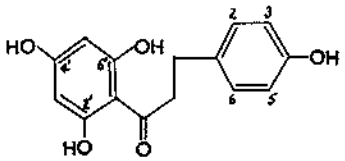
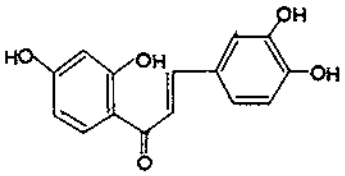
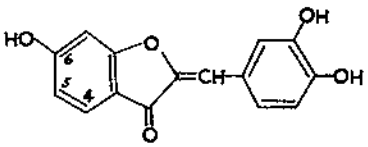
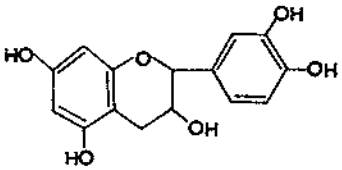
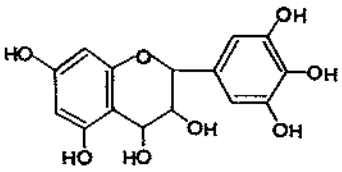
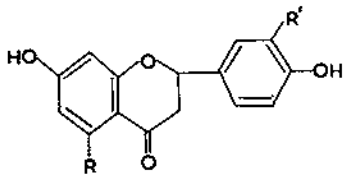
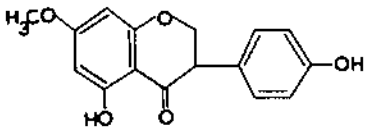
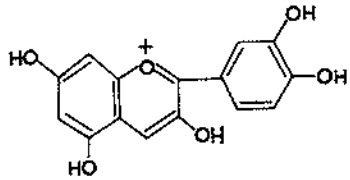
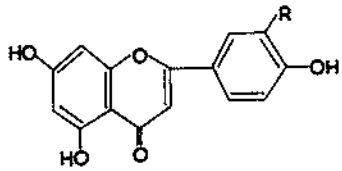
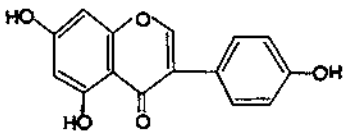
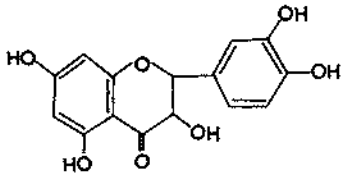
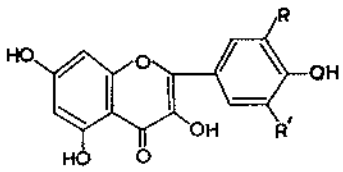
Class	Structure	Name of Compound
Dihydrochalkones		Phloretin
Chalkones		Butein
Aurones		Sulphuretin
Flavan-3-ols		Catechin
Flavan-3,4-diols		Leucodelphinidin

Table 2 (continued)

Flavanones		R = H;R' = OH Butin R = OH;R' = H Naringenin R = R' = OH Eriodictyol
Isoflavanones		Padmakastein
Anthocyanidins		Cyanidin
Flavones		R = H Apigenin R = OH Luteolin
Isoflavones		Genistein
Dihydroflavonols		Taxifolin
Flavonols		R = R' = H Kaempferol R = OH;R' = H Quercetin R = R' = OH Myricetin R=OCH_3;R'=H Isorhamnetin

become C4 and C6. While the dihydrochalkones, chalkones, aurones, isoflavanones and isoflavones lack the basic 2-phenylchromone skeleton they are nevertheless classed as flavonoids on the grounds of their close chemical and biosynthetic relationship with the group (8).

A number of compounds are known within each class of the flavonoids as a consequence of the substitution of largely hydroxyl and methoxyl groups in both the A and B rings. All these compounds may be referred to as flavonoid aglycones. There are certain common patterns of substitution in the A and B rings which may be explained on the basis of their biosynthetic origin. Usually hydroxyl groups in the A ring are at both the C5 and C7 positions or only at C7 and they normally remain unmethylated. From one to three hydroxyl or methoxyl groups may be present in the B ring. It is usual to find a free hydroxyl group in the C4' position with further orthohydroxyl or methoxyl groups at the C3' and C5' positions. These substitution patterns in the A and B rings are consistent with the derivation of the former ring from acetate units and the latter from cinnamic acid type precursors (8).

Compounds with less usual substitution patterns are the result of the introduction of hydroxyl or methoxyl groups into other positions in the rings or the removal of such groups from normal positions, or both (8). The extent of this variation in substitution differs with the particular class of flavonoid and where it is most marked and varied the greater is the variety of aglycones in the class.

With almost 100 flavonol and flavone aglycones known, the former

class accounting for nearly two-thirds of the total, variation in the substitution pattern is wide - from flavone itself to digicitrin, 3',5-dihydroxy-3,4',5',6,7,8-hexamethoxyflavone (8). An outline of the substitution pattern in flavones and flavonols is presented in Table 3.

Table 3

SUBSTITUTION IN FLAVONES AND FLAVONOLS (8)

Location of hydroxyl or methoxyl substituents	Frequency of occurrence
C5 and C7	over 90%
C4'	under 80%
C3'	about 50%
C6	over 33%
C8	about 25%
C2'	about 10%
C5'	about 10%

The hydroxyl substituent at C6 and C8 is methylated in 70-80% of cases but the C5 substituent is methylated in about only 15% of cases (8). A 4'-methoxyl group is rather common in flavones but rare in other flavonoids (6).

In the flavones only apigenin and luteolin are considered of frequent occurrence in Angiosperms (6). Kaempferol, quercetin and myricetin are the three most commonly occurring flavonols and in some groups of plants they can almost be regarded as common metabolites (8).

For example, these compounds are represented in the leaves of 90% of a sample of woody dicotyledons (8). In a sample of 1000 Angiosperms a leaf survey showed the frequency of occurrence of kaempferol, quercetin and myricetin to be 48%, 56% and 10% respectively. Probably every higher plant contains a flavone or flavonol (9). However, many of the reported flavones and flavonols (6) have been found so far only in a single species (8). The dihydroflavonols often co-occur with the related flavonols and those corresponding to kaempferol, quercetin and myricetin occur widely in heartwoods (6).

Of the twelve known anthocyanidins, six are common. They are flavylium cations structurally related to the flavonols and are unstable in the free state; salt formation or glycosylation stabilises them. The common ones are widely distributed in higher plants, in leaves, flowers and fruits, where they may make a considerable contribution to plant colour (6).

Flavanones are of fairly general distribution and naringenin and eriodictyol are relatively common (6). The isoflavanones are rare (8). Isoflavones are largely confined to one sub-family on the Leguminosae, the Papilionatae. Twenty-six isoflavones are known and 20 of them have been isolated from the Leguminosae (6).

The 20 naturally occurring chalkones are probably very limited in their distribution. Chalkones undergo partial or complete ring closure to yield the corresponding flavanones when treated with dilute alkali, or usually dilute acid. This conversion is a ready one in the case of 2',4',6'-trihydroxychalkones and is mediated enzymatically by flavanone synthetase in several plants (8). Only six aurones are

known (8). With the chalkones they constitute the anthochlor pigments which are abundant only in the Compositae, despite their occurrence in several other families (9).

Four dihydrochalkone compounds are known to occur naturally. Phloretin, the most common one, is present in Malus and in the Ericaceae, where asebogenin, the 4'-methylether of phloretin, is also present. Smilax glycyphylla (Liliaceae) contains phloretin too. Four species of Malus contain a 3-hydroxyphloretin compound. The fourth dihydrochalkone occurs in the essential oil of Populus balsamifera (6).

The flavan-3-ols may be collectively called catechins (8,10) and several natural ones are recorded (10). In substitution patterns they correspond to known aglycones in other classes of flavonoid compounds. The name catechin is given to a specific flavan-3-ol. In nature it occurs as (+)-catechin, often called d-catechin, and its laevorotatory diastereoisomer (-)-epicatechin, or l-epicatechin, is also a natural product. Both isomers are of wide distribution and frequently occur together (10).

Leucoanthocyanins, or leucoanthocyanidins, are compounds which yield anthocyanidins when boiled with aqueous or alcoholic hydrochloric acid. All leucoanthocyanidins for which structures are definitely established are flavan-3,4-diol derivatives, but their widespread occurrence in nature may mean that other structures await discovery (11). Leucoanthocyanidins are especially widespread in the woody dicotyledons, 60% having these compounds in their leaves (8). They undergo oxidation and polymerisation readily in vitro (11). The main mode of occurrence is in the polymeric form, as flavolans, which are

especially abundant in heartwoods where they are usually responsible for the dark colour (8). Structural determinations have been restricted to the flavan-3,4-diol derivatives (10). With three asymmetric centres in the molecule, a variety of optically active forms is possible (11).

The flavan-3-ols and the flavan-3,4-diols are oxidised in plants to brown pigments. Flavolans, themselves coloured, are also oxidised (8). The condensed tannins are polymeric forms of the flavan-3-ols and flavan-3,4-diols (8,11). The hydrolysable tannins, based on gallic acid (3,4,5-trihydroxybenzoic acid) is the other group of commercial tanning agents (6).

1.1.5 Biosynthetic interrelationships

A brief outline of the current understanding of the biosynthesis of the benzoic and cinnamic acid derivatives and the flavonoid compounds is presented here to underline the interrelationships of these groups of natural phenols in higher plants. (See Figure 1 (12,13).)

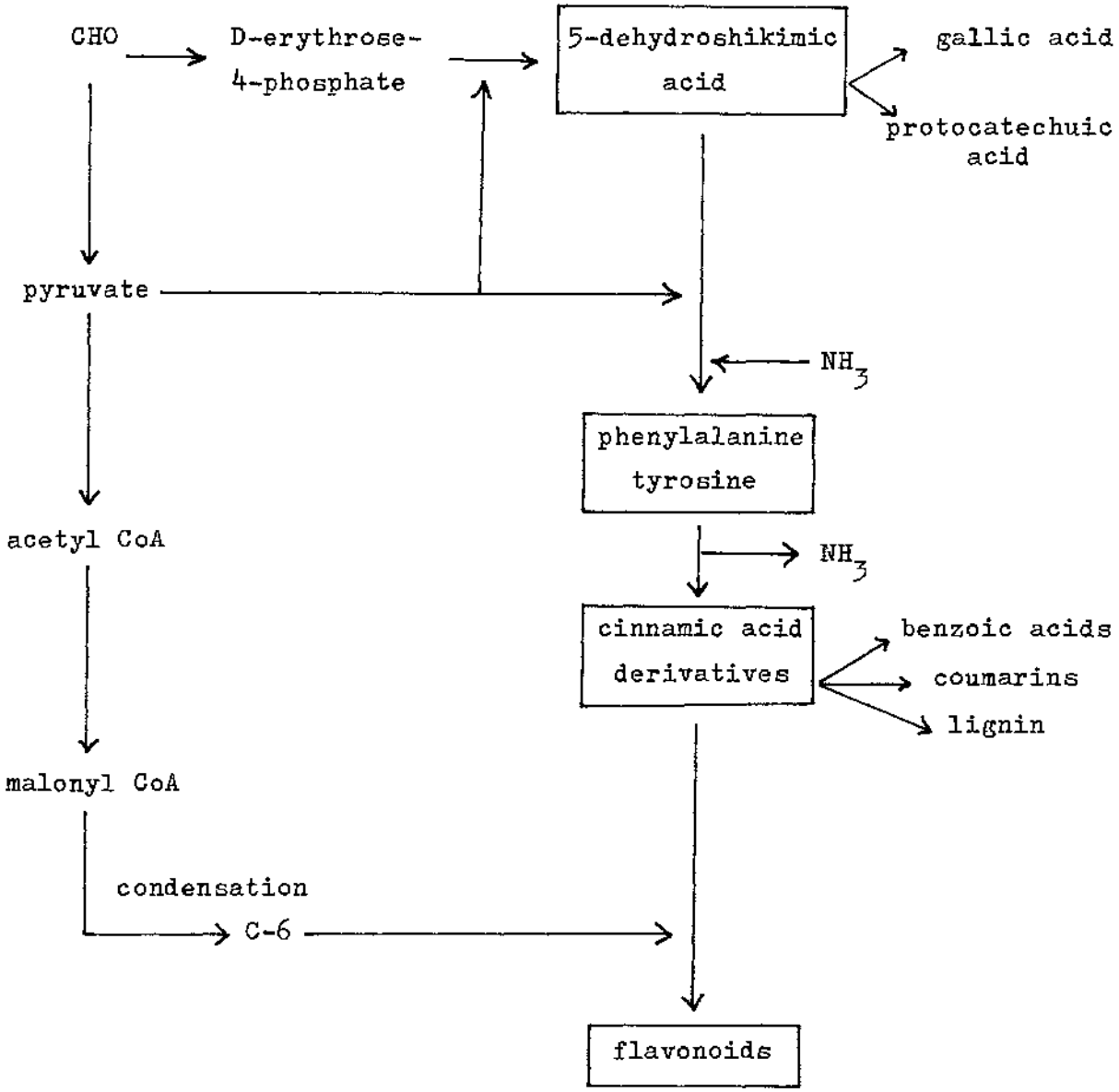
Cinnamic acid derivatives are thought to be derived from the amino acids phenylalanine and tyrosine, themselves synthesised in plants via the shikimic acid pathway.

Another general pathway which is well established for the synthesis of the aromatic ring in plants is the one involving the head to tail condensation and subsequent cyclisation of acetate units. This pathway may give rise to benzoic acid derivatives which may also arise by β-oxidation of appropriate cinnamic acid derivatives.

The carbon skeleton of the flavonoid compounds (C-15) comprises a C-6 unit (acetate derived) and a C6 - C3 unit (cinnamate derived);

Figure 1

SCHEME FOR BIOSYNTHESIS OF PHENOLIC COMPOUNDS IN HIGHER PLANTS



thus the biosynthesis involves the condensation of precursors derived from the two major pathways of aromatic ring biosynthesis in plants.

1.2 The Occurrence of Phenolic Compounds

In this section the main forms in which the phenolic compounds introduced above are found in higher plants will be discussed briefly.

1.2.1 Occurrence of the three groups of phenolics in the plant kingdom

Many organisms, from bacteria to higher plants, synthesise benzoic and cinnamic acid derivatives, but the combination of C-6 and C6 - C3 precursors in C-15 flavonoid compounds is restricted almost entirely to the flowering plants and ferns (7,14). Microorganisms can synthesise the C6 - C3 (phenylpropane) unit while fungi can synthesise both the C-6 and C6 - C3 precursors but cannot combine them into flavonoid compounds (14). All parts of higher plants contain flavonoid compounds but certain compounds tend to be restricted to particular tissues (8,14).

1.2.2 Natural forms of phenolic compounds

In the living cells of higher plants phenolic compounds normally occur in combined forms, as glycosides and esters (15,12,7). Storage tissues (seeds, berries) and dead or dying tissues (heartwood of trees) may contain free phenols (15). The main combined form of phenolic compounds is the glycosides and, with several hundred sugar-aglycone combinations recorded, the phenolic-O-glycosides is the most extensive

known group of plant glycosides. In the glycosides an hydroxyl group of the phenol is linked in β-configuration (with a few exceptions) to the sugar molecule (15). Most plants contain more than one glycoside of any aglycone (8).

Some general discussion on the sugars of the phenolic glycosides is presented by Harborne (15). The five common plant monosaccharides found attached to phenols are, in order of decreasing frequency of occurrence, D-glucose, D-galactose, D-xylose, L-rhamnose and L-arabinose. D-glucuronic acid may also occur in these glycosides.

Many phenolic diglycosides occur in plants, but the structures of only ten of their disaccharide units have been elucidated (15). Trisaccharide, but not tetrasaccharide, units occur in phenolic glycosides, and while structures for six trisaccharides have been proposed, they have not been rigorously established. More trisaccharide units have still to be characterised (15). The oligosaccharides of phenolic glycosides contain only 1$\longrightarrow$2 or 1$\longrightarrow$6 linkages, with the sugar at the reducing end nearly always glucose. Many are specific to the phenolic glycosides, being absent elsewhere in plants in the free or combined state (15).

The phenolic compounds which occur as esters are the phenolic acids chiefly of the benzoic and cinnamic acid types (7,12).

1.2.3 Flavonoid glycosides

In plants, flavonoid compounds occur typically as glycosides, in which an hydroxyl group is joined in a semi-acetal link to the sugar molecule. In some cases more than one hydroxyl group in a

molecule may be linked in this manner to an equal number of sugar molecules. The sugars found include mono-, di- and trisaccharides, combined through the C-1 of the sugar to form monosides, biosides and triosides. The frequency of occurrence of simple sugars in the glycosides, in decreasing order, is D-glucose, D-galactose, L-rhamnose, L-arabinose and D-xylose. D-glucuronic acid also occurs, at low frequency (8).

Flavonol glycosides constitute by far the largest and most varied class of phenolic glycosides and they have been isolated from many sources (15). Glycosylation is usually through the 3-position (about 70% of cases) while the 7-position is less commonly glycosylated (about 20% of cases) (8). C-4'-glycosides are known (8) as are 3,4'-diglycosides, but no 5-glycosides are recorded (15). In some more complex flavonol glycosides, p-coumaric, ferulic and caffeic acids are found as acyl groups attached to the sugar residues (15).

Glycosylation in flavones is most commonly at the C-7 hydroxyl group (80%), followed by the C-5 and C-4' groups (8). Flavone 8-C-glycosides are formed in plants, and nearly all 8-C-glycosides isolated are of the flavone class (15). The glycosidic pattern in flavanones is akin to that of the flavones, for example, 7-glucosides are common and 5-glycosides are known (15). Ten isoflavone glycosides are known and five are 7-glucosides. In the heartwood of trees many isoflavones occur in the free state (15).

Glycosylated anthocyanidins are called anthocyanins, and here the pattern of glycosylation is similar to that in flavonols (15). However, if a second position in the anthocyanin molecule is glycosyl-

ated, it is normally at the 5- rather than at the 7-position as with flavonols. Glycosylation in the 3-position appears essential for the stability of the anthocyanin pigments as they are unknown without a sugar in the 3-position. Some anthocyanins with hydroxycinnamoyl residues attached to their sugars are known (15).

Catechins and leucoanthocyanidins occur as the aglycones in heartwood and even in leaf tissue (7). In the leaf of the tea plant, Camellia sinensis, the common catechins occur esterified in the 3-position with gallic acid (16).

Aurones are present only as glucosides (8), and mainly as 6-glucosides (15). Glucose is the only sugar found in chalkone glycosides, which include monosides and biosides, so far (8). The sugar is usually in the 4'-position. Sometimes chalkone-2'-glucosides co-occur with the corresponding flavanone-5-glucosides (15).

Six dihydrochalkone glycosides occur. In Malus, phloretin occurs as the 2'- and 4'-O-glucosides and also as the arabinosyl-glucoside and xylosylglucoside. The same genus yields 3-hydroxy-phloretin-4'-O-glucoside. Phloretin-2'-O-rhamnoside occurs in Smilax glycyphylla (15).

1.2.4 Natural forms of cinnamic acid derivatives

The cinnamic acid derivatives occur in plants in more combined forms than any other group of plant phenols (15). They are usually found as esters of a variety of compounds, including cyclohexane carboxylic acids like quinic acid, sugars, organic acids such as tartaric, phenolic alcohols, flavonoids and alkaloids. Glucosides

are also known (7,15).

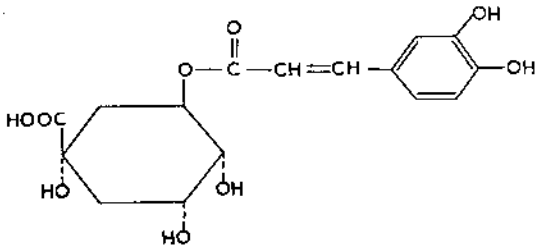
Conjugates of p-coumaric, caffeic and ferulic acids with quinic acid and with glucose occur widely in plant tissues. Many are found as trace components in very complex mixtures of phenolic compounds (17). Esters with quinic acid are common, especially in the case of caffeic acid; the 3-O-caffeoylquinic acid ester being known as chlorogenic acid (see Table 4). Other isomers are known (15,18). Many plants contain 3-O-p-coumaroylquinic acid, while the occurrence of 3-O-feruloylquinic acid is known (15). Glucose esters of p-coumaric, caffeic and ferulic acids are of common occurrence. These are monoesters with the structure shown in Table 4 (12). p-Coumaroyl, caffeoyl and feruloyl esters of shikimic acid have also been isolated (15).

Feeding the leaves of a variety of plants with L-phenylalanine, cinnamic, o-coumaric, m-coumaric, p-coumaric, caffeic, ferulic, sinapic and 3,4,5-trimethoxycinnamic acids, for periods of 1-3 days, yields mainly the glucose esters of the phenolic acids. The structures of the esters with p-coumaric, caffeic, ferulic and sinapic acids are identical with the natural esters of the same composition (7) (see Table 4). Plants may form some glycosides as well as the predominant esters when fed cinnamic acid derivatives, for example, the 3- and 4-β-glucosides of caffeic acid are formed in addition to large amounts of caffeoyl-D-glucose, in tomato plants fed caffeic acid (7). (See Table 4 for the structure of the glucoside.)

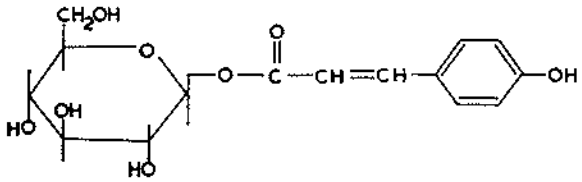
Shakuchirin (Table 4) is an example of a more complex compound containing a sugar and hydroxycinnamic acids. Many derivatives of this type exist but the complete structures of only a few have been

Table 4

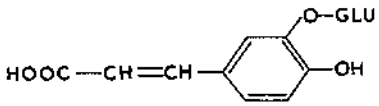
STRUCTURES OF SOME NATURAL HYDROXYCINNAMIC ACID DERIVATIVES



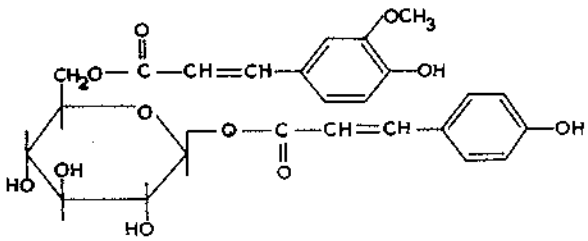
3-O-caffeoylquinic acid
(chlorogenic acid)



p-coumaroyl-D-glucose



caffeic acid-3-glucoside



1-p-coumaroyl-6-feruloylglucose
(chakuchirin)

determined (15).

1.2.5 Benzoic acid derivatives

Either esters or glycosides, or both, of a variety of benzoic acid derivatives are found in all higher plants (7). Apparently the composition and structures of many of these esters and glycosides are not known (5,7).

1.2.6 The significance of ester and glycoside formation

The reasons for the occurrence of phenolic compounds as esters and glycosides in actively metabolising plant tissues are not clearly understood.

Since plants convert administered phenolic compounds to glycosides and esters, these compounds may be looked upon as the products of detoxication reactions (7) which may prevent the interference of free phenols with some vital cellular mechanisms (15). The greater sap solubility and mobility in the cell vacuole of the glycosides over the parent phenols (8,15) may be significant. Glycosylation may protect the phenol from some enzymatic attack; potato phenolase does not oxidise the 3-glycosides of quercetin and myricetin, presumably because of steric reasons, for it will oxidise the corresponding aglycones (8).

1.2.7 The function of phenolic compounds in plants

Several functions have been suggested for phenolic compounds found in higher plants, but any vital function has yet to be uncovered.

The classification of phenolic compounds as 'secondary meta-

bolites' (12) suggests functions which are more specific to particular plants than to plants in general. The function of some flavonoid compounds, particularly the anthocyanins, as plant pigments in leaves, flowers and fruits is forwarded (8,9). It is also recognised that phenolic compounds may play a part in determining the resistance of certain plants to attack by pathogenic organisms (19).

Utilisation of phenolic glycosides and esters as respiratory or reserve materials seems not to occur, the compounds remaining as inert metabolic by-products once synthesised. In the light of this knowledge it is difficult to understand the not uncommon occurrence of from 1-5% of the fresh weight of some plant tissues as a particular flavonoid compound (7).

For some phenolics, labelling studies have indicated the compounds to be in a state of metabolic flux in the living plant (20) and other studies have demonstrated the effect of light duration (21) and light quality (22) on the levels of them. Taken together with the well known effects of phenolics on the activity of the indolylacetic acid oxidase enzyme system from plants (23), these results provide some indication of a possible physiological function for certain phenolics.

Chapter 2

INTRODUCTION - REVIEW OF LITERATURE

2.1 Isolation and Characterisation of Phenolic Compounds

The phenolic compounds, particularly the flavonoids, have attracted the attention of organic chemists for a considerable time and the earlier classical methods (24,25,26) have, in more recent times, been largely superceded by micro- or semi-micro methods in which chromatographic or spectroscopic techniques are mainly employed. Employment of these superior techniques has resulted in the identification of many new phenolic compounds, especially as combined forms, from a variety of plant sources. Further, some substances isolated by classical procedures have been shown to be mixtures (27). Specific examples of the application of micro-methods are to be found in many publications and are well illustrated in the work of Nordstrom and Swain (28) and of Harborne (29).

Valuable reviews of methods for the extraction, isolation and identification of phenolic compounds are found in sections of the books previously cited (1,2,3). The occurrence and structures of natural phenolic compounds in plant tissues are commonly determined by a suitable combination of extraction, isolation and identification procedures. The detailed procedures applied to the study of phenolic

compounds in a particular plant tissue are influenced by the nature of the plant tissue and the phenolic compounds present (26). Some main procedures which may be used are reviewed here, to give perspective to the methods chosen for the present investigation.

2.1.1 Extraction procedures

Extraction procedures are related to the solubility properties of the phenolic compounds and other unwanted compounds in the plant tissue. In general phenolic compounds are soluble in polar solvents. Phenolic glycosides are mostly water soluble while the corresponding aglycones are generally insoluble (26). Alcohols are widely used for extractions, while ether and ethyl acetate may be used for compounds with only a few hydroxyl groups or for flavonoid aglycones (26,27). Water, or very aqueous solvents are not favoured as extractants for various reasons. Many unwanted and potentially troublesome molecules are extracted, enzymes, including phenolases and glycosidases which act on phenolic compounds to alter their natural forms, are not inactivated readily unless the aqueous extract is boiled and finally, the aqueous solvent is removed only with some difficulty. In some cases a series of solvents may be used to extract a tissue (27).

Plant tissues may be extracted in the fresh or dried form. With fresh tissue, enzymes which act on phenolic compounds are released when tissues are disrupted and alteration of these compounds is avoided by prompt inactivation of the enzymes with organic extracting solvents and rapid heating of the extraction mixture (27,30). The tissue may be plunged directly into hot or boiling solvent (29). Plant material may

be dried and stored prior to extraction. Care must be exercised in the drying process to prevent the phenolic compounds being altered. Freeze-drying is an accepted procedure (20). More aqueous solvents may be required in the extraction of dried as compared with corresponding fresh material (27).

If lipids or photosynthetic pigments are extracted with the phenolic compounds, the crude extract is usually washed with light petroleum ether, ether, or hexane, to remove the bulk of most of these unwanted materials. Sometimes these materials are removed prior to the extraction of the phenolic compounds from fresh or dried material (26,27).

The crude extract is usually concentrated by controlled removal of the solvent under mild conditions (low temperatures, reduced pressure, nitrogen atmosphere) (27) before isolation of the individual phenolic compounds is attempted. In certain cases where the desired compounds have appropriate properties, techniques of solvent extraction, precipitation with neutral or basic lead acetate (26), complex formation with aqueous borate (24) and column chromatography (30) are used to advantage to remove unwanted material before the extract is finally concentrated. Certain of these steps are undertaken at times prior to the isolation of compounds by column chromatography (30).

2.1.2 Isolation procedures

The classical methods of solvent extractions, lead acetate precipitation and fractional crystallisation, while suited to the isolation of macro-amounts of certain phenolic compounds (24), are

poorly suited for the detailed examination of plant extracts (27). However, paper or column chromatographic techniques are of greater value for this purpose and are widely used in the isolation of phenolic compounds from plant extracts (26,27,30).

Paper chromatography has been extensively applied to the isolation of phenolic compounds from natural mixtures (26,30) and, when solvent systems are carefully chosen, considerable, and at times superior, resolution is achieved (5,30,31). The technique is simple and inexpensive (30) and enables individual compounds to be isolated in micro-amounts from complex natural mixtures (27,30,31). Paper chromatography has been a most valuable tool in the study of flavonoid compounds in plant materials following its first application to the separation of these compounds by Bate-Smith in 1948 (31) and the introduction of successful methods for the identification of flavonoid compounds using milligram quantities by Nordstrom and Swain (28). The same micro-techniques have been successfully applied to other groups of phenolic compounds (26). In preparative work the greatest limitation of paper chromatography is the small amount of crude extract which can be handled efficiently (30). Up to 100 mg of individual compounds can be isolated by large scale preparative paper chromatography (30,32) in which the solution for separation is evenly banded on a heavy grade of paper, for example, Whatman No. 3. Plant extracts in all stages of purification may be applied to the chromatogram (30).

The chromatogram is developed with a solvent normally selected from the wide range in use (26,30). (See Table 5.)

Table 5

SOLVENT SYSTEMS USED IN PAPER CHROMATOGRAPHY

Solvent	Proportions*	Uses	Reference
Acetic acid (HA)	2 - 60%	All types	(26)
Benzene-HA-water	125:72:3	Phenolic acids	(5)
		Flavonoid aglycs.	(38)
n-Butanol-HA-water	4:1:5 etc	All types	(30)
n-Butanol-ethanol-water	5:1:2	Phenolic acids	(26)
Ethyl acetate-HA-water	3:1:3	Many types	(26)
Isopropanol-NH_3-water	8:1:1	Phenolic acids	(26)
Phenol-water	saturated	All flavonoids	(30)
Water		Many types	(26)

* Where two layers form the organic one is used.

The bands of phenolic compounds on the dried chromatogram are detected by either their fluorescence in ultraviolet light or by visualisation on a test strip treated with a suitable spray reagent (26,30). (See Table 6.)

Compounds are eluted from the excised bands in aqueous ethanol or methanol (70-95%) and the concentrated eluates are re-banded on further sheets of paper for chromatography in selected solvents (27,30). Each band is eluted and rechromatographed in the selected solvents until a chromatographically pure (30) compound is obtained (27). Final eluates are most conveniently handled further in alcohol solution,

particularly where small quantities are isolated (30). However, compounds may be isolated crystalline from their eluates (32) though at times only with difficulty (30).

The solvent systems employed in a particular order in preparative chromatography are conveniently selected and ordered on the results of preliminary trials in which one- and two-dimensional paper chromatography are applied to the mixture to be separated. A standard grade of filter paper like Whatman No. 1 is commonly used in this preliminary work (30).

Table 6

REAGENTS USED TO LOCATE PHENOLICS ON PAPER CHROMATOGRAMS (26)

Composition	Concentration	Use
Aluminium chloride	1-5% in alcohol	All flavonoids
Ammonia vapour		Most compounds
BASES (Sodium hydroxide or carbonate)	Dilute aqueous	Most compounds
DIAZOTISED AMINES (p-nitroaniline and sulphanilic acid)	0.02 - 0.2%	All compounds
Ferric chloride plus potassium ferricyanide	Each 1%, equal volumes	All compounds
p-Toluenesulphonic acid	3% alcoholic	Flavans

While column chromatography has not been used so widely and successfully as paper chromatography, it has been employed to advantage

by some workers in the isolation of certain compounds from plant extracts, often in amounts in considerable excess of those conveniently isolated by paper chromatography (27,30). A somewhat greater degree of skill is required for the successful application of column as compared with paper chromatographic techniques and the application of the technique is further restricted by the lack of an ideal adsorbent for flavonoid compounds and the relatively long time needed for a run (30).

Column chromatography has been applied to all types of flavonoid compounds (30). Wender and his associates have developed methods for the large-scale isolation of flavonoid compounds, including flavonoid glycosides, from plant extracts (27,30). Hanson and Zucker (17) have demonstrated the high resolving power of a system of column chromatography on silica gel by the isolation, from the potato tuber, of at least eight conjugates of caffeic and quinic acids, along with other hydroxycinnamic conjugates. Adsorbents which have been used in the column chromatography of phenolic compounds, particularly the flavonoids, include Magnesol (a synthetic hydrated magnesium acid silicate), polyamide (powdered nylon), silicic acid, silica gel, cellulose powder, carboxymethylcellulose and ion-exchange resins (26,30). The flavonoid compounds are adsorbed by alumina so strongly that they are not readily eluted (30).

Various methods have been used to detect phenolic compounds in the fractions of column eluate, for example, samples of the fractions of eluate may be spotted on filter paper and phenolic compounds detected as in paper chromatography (30), or the effluent may be monitored spectrophotometrically at a selected wavelength (17).

Thin layer chromatography has not been used widely in the separation of phenolic compounds (26,33). As it is inferior to paper in its resolving power for glycosides, Swain (27) doubts "whether it will ever be used in separating components (flavonoid) in sufficient quantity for structural determination". Gas chromatography has had little application to the phenolic compounds (26,34). Counter-current distribution between immiscible solvent pairs has been used in the final purification of some phenolic compounds (30,18).

2.1.3 Characterisation procedures

Natural phenolic compounds occur in living plant tissues usually in combined forms (section 1.2.2) and are isolated unaltered when satisfactory procedures are used. In the identification of combined forms, therefore, three main determinations are involved - identification of the phenolic moiety, identification of the residues linked to it and, finally, identification of the position and the nature of the linkage between the phenolic moiety and the residues.

The compounds may be allocated to certain classes of phenolic compounds on the basis of tests recorded by Seikel (26), and then identified by an appropriate selection from the methods which follow. Paper chromatography and spectroscopic analysis are the basis of micro-methods of identification of compounds isolated either in small quantities or in non-crystalline form (22,29). Alternative identification methods used with larger amounts of crystalline isolates include elemental analysis, the preparation and characterisation of derivatives, characterisation of the products of degradative procedures and the taking of physical measurements including melting points, absorption

spectra and specific rotations (26,35).

Flavonoids. Of the many methods which can be employed in the identification of flavonoid compounds (26,31,36,37), the micro-methods have become firmly established since Nordstrom and Swain (28) reported the identification of compounds using as little as 1 mg of each.

When flavonoid compounds are isolated by paper chromatography, indications of their possible classification and structures are often obtained at the same time (30), since the R_F values on paper chromatograms are generally related to the structures of the compounds (30,31). This relationship between chromatographic behaviour and structure of most flavonoid compounds has been summarised for several solvent systems of widely varying polarity in terms of a linear relationship between the R_M values ($R_M = \log(\frac{1}{R_F} - 1)$) of the compounds and the number of hydroxyl and sugar groups in them (31,38). As the number of phenolic hydroxyl substituents in the flavonoid molecule increases, the R_F in both aqueous and alcoholic solvent systems is decreased. In alcoholic solvents R_F values are slightly increased as a rule when hydroxyl groups are methylated, but the effects with a chelated hydroxyl group are different than with a free one (31). In the benzene-acetic acid-water solvent system the effects of hydroxylation and methylation are similar overall to those in the alcoholic solvents, but the more marked effect of methylation may almost reverse the effect of hydroxylation or exceed the effect of dehydroxylation. The abnormal effect of the chelated hydroxyl groups at the 3- and 5-positions is encountered as expected (38). With all classes of flavonoid aglycones, the R_F values in the n-butanol-acetic acid-water solvents are lowered by glycosylation,

which has the opposite effect on R_F values in water or in dilute aqueous solvents (30). Increased numbers of sugar residues in glycosides are linked with reduced R_F values in n-butanol-acetic acid-water solvents and increased values in the aqueous solvents, except in those cases where an important effect of the position of substitution of the sugar on the aglycone is encountered (30,31). The planar flavonoid aglycones (flavonols, flavones, chalkones, anthocyanidins, aurones) are distinguished by their zero R_F in water from the non-planar aglycones (flavanones, isoflavones, catechins, leucoanthocyanidins and flavanonols), which have a small R_F value in the solvent (30).

Consequently a great number of flavonoid compounds, many closely related, can be distinguished and identified successfully by their R_F values on paper chromatograms developed with selected solvents (30,31). However, since the R_F values of compounds are affected by variations in materials and experimental conditions (26), all paper chromatography for identification purposes should be done with known reference compounds alongside the appropriate unknown compounds on the chromatograms (30).

The class of flavonoid to which an unknown compound belongs may be indicated by a study of the colour reactions on paper chromatograms in natural and ultraviolet light, coupled with the use of ammonia vapour and certain chromogenic reagents (26,30). In the final identification, colour reactions (see Table 6) are compared along with R_F values for known and unknown compounds determined in three or more solvent systems of different polarity. The identity of an unknown with a known compound is supported by identical R_F values and colour reactions

under conditions of co-chromatography. Comparison of the colour reactions and R_F values of an unknown with a closely related known compound may be used to provide some valuable information on the structure of the unknown (30). The presence of certain structural features in a compound may be indicated or confirmed by chromatography on treated papers or by the use of special chromogenic sprays (30,36). Papers are impregnated with aluminium chloride, sodium borate or phosphate and developed with a water-saturated n-butanol solvent (30).

Flavonoids frequently occur as glycosides (section 1.2.2) and many compounds are isolated in the glycosidic form. Glycosides may be shown to have a certain identity by chromatographic comparison with the authentic reference compounds (39). Often direct comparison of glycosides cannot be done and the unknown glycoside is therefore frequently identified stepwise by identifying firstly the aglycone, and then the nature, number and details of attachment of the sugar residues (31).

In the identification of a flavonoid glycoside, up to 1 mg of the unknown compound is hydrolysed by either heating with acid (2N HCl) or treatment with a suitable enzyme (31,40). Harborne (40) has demonstrated the variation in behaviour among flavonoid glycosides to acidic and enzymatic hydrolysis and has demonstrated the value of controlled hydrolysis in the characterisation of some of the more complex glycosides. After acid hydrolysis the aglycone is extracted into ethyl acetate or ether and concentrated before identification is attempted by paper chromatography using the procedures outlined above

(31). This may be relatively easy as the flavonoid glycosides encountered are frequently of common aglycones (Chapter 1). Where the aglycone is found to differ from the known reference compounds available its identity can be established only in conjunction with the use of other methods. For example, alkaline degradation on a micro-scale and paper chromatographic identification of the products of cleavage of the heterocyclic ring, representing ring A and ring B fragments, can be valuable (30,36). The sugars obtained from the acidic hydrolysis are prepared for chromatographic identification by removal of the acid which would otherwise cause interference. Treatment with a solution of di-n-octylmethylamine in chloroform (27), or repeated evaporation of the acidic (HCl) solution under reduced pressure, followed by exposure of the solid residue over potassium hydroxide pellets in a desiccator may be used. Sugars are readily identified by paper chromatography using at least three solvent systems (41).

In the determination of the ratio of sugar to aglycone molecules, the amount of aglycone from the hydrolysis of a sample of the glycoside is determined spectrophotometrically (aglycone identified previously) and, once separated by paper chromatography, each of the sugars may be similarly estimated after development with aniline hydrogen phthalate and elution (31). When several mg of the glycoside are available for hydrolysis, the sugar to aglycone ratio can be determined by comparing the actual weight of the aglycone produced with the theoretical yield for a given monoside, bioside structure etc., containing the sugars previously identified (32). With monosides,

identification is continued by determining the position and nature of the glycoside link in the molecule. However, where more than one molecule of sugar is found for each molecule of aglycone, further studies are required to elucidate the nature and location of glycosylation as well as the linkages which may exist between sugar molecules.

Where di- and trisaccharide residues are to be characterised, controlled acid hydrolysis of the glycoside may enable the intermediate breakdown product to be isolated in part and identified by paper chromatography and hence the order of the constituent monosaccharides to be deduced (31,32). The methods of carbohydrate chemistry have yet to be widely applied in some of this characterisation work (15). Both enzymatic and controlled acid hydrolysis can be employed in conjunction with paper chromatography in the identification of flavonoids glycosylated in more than one position (40). Full methylation of the unknown glycoside prior to acid hydrolysis to remove the sugar substituents has provided a means of determining the position of substitution of the sugar residues by locating the free hydroxyl groups in the methylated aglycone derivative with the aid of paper chromatographic, spectral and colour reaction data (28,31). Certain hydrolytic enzyme preparations have been used to provide valuable information on the configuration of the sugar-aglycone bonds (40) in addition to ~~that on~~ structural details mentioned earlier.

Data obtained by ultraviolet absorption spectroscopy are frequently used to complement and support paper chromatographic data in the identification of flavonoid glycosides and aglycones (26,30,37). Identification on a micro-scale is founded largely on the supporting

evidence from paper chromatographic and ultraviolet spectroscopic studies (26,31). Infrared spectra have, in general, been of less use than ultraviolet spectra in characterising unknown flavonoid compounds (26,32).

While all phenolic compounds absorb radiation strongly in the ultraviolet region (220 - 460 mμ), the contributions to the absorption spectrum of certain associations of structural features in the molecule results in distinct structural classes of compounds having rather characteristic spectra. Further, within a particular class of flavonoid compound, variations in spectral maxima and absorption intensities are related to the differences in the position and nature of substituents (8,37). A variety of reagents is available to complex with or ionise hydroxyl substituents, often selectively, and in a comparison of the normal spectrum and the spectra determined in the presence of a series of these reagents, much structural information may be obtained (37). Absorption spectra are normally determined in alcohol, reagents being added in similar solvent or as solid material. Reagents used are aluminium ion (as $AlCl_3$), as a chelating agent; sodium ethylate, or hydroxide, to ionise all free phenolic hydroxyl groups; sodium acetate, to ionise the more reactive phenolic hydroxyl groups; and boric acid, in the presence of excess sodium acetate, to complex with the o-dihydroxyl groups in the molecules (37). Further details are presented by Jurd (37) of the spectra of various classes of flavonoid compounds, and the effects on the basic spectra of the type and position of the substitution in the molecule, together with an account and documentation in the original literature of the use of

reagents to elucidate certain structural features. That some flavonoid compounds can be identified on the basis of their spectra alone (37) indicates the power of these methods.

Spectral determinations are made on both flavonoid glycosides and aglycones to locate free hydroxyl groups and to indicate the location of sugar substituents in the glycosides (27,29,37). Results may be compared with those obtained either in parallel from the reference compounds used in the chromatographic studies (32,39) or from the literature (29,37). In the preparation of compounds for spectral examination a blank solution for reference purposes must be prepared in a parallel fashion (28,30).

Venkataraman (36) has described the classical procedures for the identification of flavonoid aglycones and glycosides from crystalline isolates. Identification is based on a study of the colour reactions, physical properties and chemical composition of the compound and its derivatives, together with analysis of the results of degradative procedures, chiefly alkaline hydrolysis. Comparisons are made with authentic compounds obtained from existing sources or by synthesis. An example of the application of these methods to the identification of two dihydrochalkone glucosides from Malus species is provided in Williams' work (35) and, to a lesser degree, by Nortjé and Koeppen (32) in the identification of flavonol glycosides.

Cinnamic acid derivatives. These phenolic derivatives are found in plants frequently as esters of a number of compounds (section 1.2.4). Micro-methods of identification, based on paper chromatography and

ultraviolet spectroscopy, are applied to this group of compounds in ways similar to those outlined for the flavonoid compounds (17,26).

The esters may be studied directly and identified with the aid of authentic specimens for comparison. The quinic acid esters of both caffeic and p-coumaric acids are found in isomeric forms (44,45) and straightforward identification of any isomers isolated can best be done by comparison with authentic reference compounds (17). Esters are normally also hydrolysed and the products examined chromatographically to provide further evidence in support of the original identification. Hydrolysis of the esters can be accomplished with alkali (2N NaOH) at room temperature, or with esterase, and in the case of glucose esters, with β-glucosidase (26).

As with the flavonoids, the ultraviolet absorption spectra of the esters and the free acids may be determined in alcohol and in the presence of certain reagents and compared with the spectra of appropriate reference compounds (17,42).

The identity of the phenolic acid obtained from the ester may be determined by the results of co-chromatography in several solvent systems and the colour reactions obtained when the chromatograms are treated with certain chromogenic reagents (5,26,42). A useful range of colour reactions is given in the Hoepfner test (17,42).

In order to complete the identification, the phenolic moiety may be conveniently estimated spectrophotometrically, after hydrolysis, and the non-phenolic moiety estimated by standard procedures (17,44).

The benzoic acid derivatives. These phenolic derivatives have been

characterised chiefly on the basis of a comparison of the paper chromatographic behaviour and colour reactions of known and unknown compounds (5,46). On paper chromatograms these colourless compounds are located with suitable chromogenic sprays (26).

2.2 Phenolic Compounds in the Leaf of Malus pumila (Mill.)

Of the 25 species recorded in the genus Malus by Rehder (47) one, M. pumila (Mill.), accommodates most of our cultivated apple varieties.

The phenolic compounds of the leaf of the cultivated apple have been reviewed by Williams (48). Flavonoid glycosides are the main compounds reported. Phloridzin, the major compound, is accompanied by several 3-glycosides of quercetin and lesser amounts of the same 3-glycosides of kaempferol. These compounds are listed in Table 7 in decreasing order of amount present (48).

In addition to these major compounds, trace amounts of chlorogenic and p-coumaroylquinic acids, epicatechin, catechin and leucoanthocyanins may be found in leaf (48). Chlorogenic acid and epicatechin have been reported in only trace amounts in the leaf (49). While fruitlets have yielded a p-coumaroylquinic acid (45), which has been shown to be 3-O-p-coumaroylquinic acid (17), the leaf tissue has been suspected to contain small amounts of two p-coumaroylquinic acids (50), one of which was chromatographically indistinguishable from the fruitlet isolate (45). Leucoanthocyanin has been detected in the leaf of the McIntosh variety (51). Herrmann (52) has separated and ident-

ified, by paper chromatography, isoquercitrin, astragalin and quercitrin (quercetin-3-glucoside, kaempferol-3-glucoside and quercetin-3-rhamnoside respectively (53)) from extracts of apple leaf.

Table 7

MAIN PHENOLIC COMPOUNDS IN LEAF OF MALUS PUMILA

Class of flavonoid	Aglycone	Glycoside
Dihydrochalkone	Phloretin	2'-glucoside (phloridzin)
Flavonol	Quercetin and Kaempferol	3-galactoside 3-glucoside 3-rhamnoside 3-arabinoside 3-rhamnoglucoside 3-xyloside

Williams has reported (48,54) little change in the pattern of the phenolics present in the leaf tissue of different varieties of apple, although some quantitative variation among varieties in the total and relative amounts of the phenolics has been supported by Flood and Kirkham (56). It is of interest that the hydroxylation patterns represented in these phenolics are those of the A and B ring moieties (section 1.1.4) of kaempferol and quercetin, and none of the myricetin type has been found (54,56). In fact the Rosaceae family, to which Malus belongs, has typically yielded quercetin, kaempferol, leuco-

cyanidin, p-coumaric and caffeic acids as its phenolic compounds (57).

In summary, therefore, the apple leaf can be expected to contain a rather complex mixture of phenolic compounds, chiefly flavonoid glycosides. While Williams has made the major contribution to the study of the main phenolic compounds in the leaf, apparently little detail has been reported (48).

2.3 Objectives of Studies Reported in this Thesis

As a consequence of the potentially complex mixture of glycosides to be found in the apple leaf and the lack of information on the isolation of individual phenolic compounds in combined form from this tissue, it was decided to direct these studies towards an investigation of the extraction, paper chromatographic isolation, and characterisation of phenolic compounds from the leaf of a variety of M. pumila, in order to provide background knowledge of value in projected studies on the possible physiological role of some individual phenolic compounds in this plant. Further, in relation to the metabolism of the phenolic compounds, the incorporation of activity from ^{14}C-glucose into these compounds was investigated in excised leaf tissue. Sturmer Pippin was selected for study because this variety has been the subject of another physiological study in the University orchard (58).

Chapter 3

MATERIALS AND METHODS

3.1 Description of Plant Material and Sampling Procedures

The leaf tissue samples were always of Malus pumila var. Sturmer Pippin, except on one occasion when samples from the varieties Cox's Orange Pippin and Rome Beauty were also included. Samples were taken from cropping trees, in apparent good health, growing in the University's Batchelar Orchard which was operated under typical commercial conditions. All sampling was done during October 1965 except for one batch of samples which was taken in early February 1966. The October samples were collected from one or more of three mature Sturmer Pippin trees and one mature tree of each of the other two varieties while the February samples were collected from four young Sturmer Pippin trees about eight years old. Sample material was removed at random from well-lighted parts of the trees between 10 a.m. and 4 p.m. on days when the foliage was dry and taken immediately in polythene bags to the laboratory where extraction commenced at once.

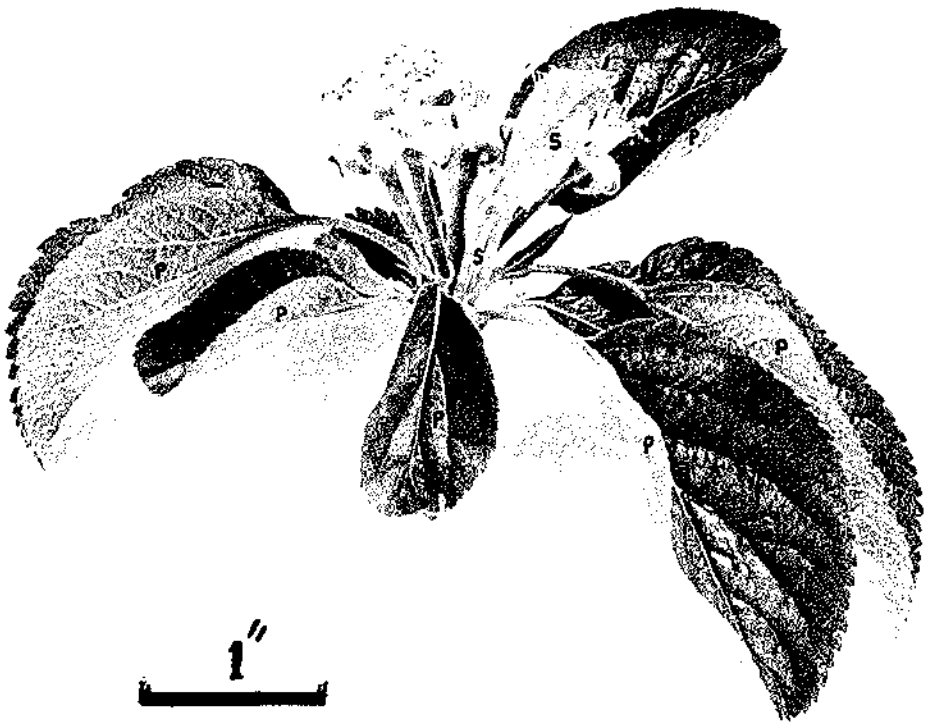
In October the samples were obtained from the spurs (or bourse structures (59)) which were flowering or fruiting. To preserve the freshness of the sample, whole spurs were taken to the laboratory where the leaf sample, unblemished and complete with petioles, was removed. The primary and the secondary spur leaves were always kept

separate. Primary spur leaves, which appear at bud burst in spring, are proximate to the terminal flower cluster on the spur while the secondary spur leaves (or the bourse bud leaves (60)) develop from a bourse bud in the axil of a primary spur leaf soon after the spur has flowered. These leaf types are illustrated in Figures 2 and 3; in Figure 2 the spur structure is shown just after the petals had fallen and the secondary spur leaves had begun to develop and in Figure 3 a later stage of development of the secondary spur leaves is recorded, while in both Figures the primary spur leaves can be seen below the developing fruit clusters. Since the secondary spur leaves were emerging during the October sampling period these leaves from the one set of spurs were on occasions classified according to their stage of development as follows: leaves apparently fully expanded were classified as 'large'; expanding leaves as 'expanding' and those still inrolled at the margins as 'emerged'. When leaves were not readily classified as 'expanding' or 'expanded' on size alone, the appearance of the upper leaf surface, which was lighter green and hairy on the former and glossy green on the latter, was the deciding feature. Secondary spur leaves were conveniently removed from the spur by breaking out the entire axillary structure from which the portion carrying the bourse bud and petiole bases was then excised to separate the leaves. With the primary spur leaves, all unblemished leaves except the very small ones at the base of the spur were included in the sample.

In February, leaves from the terminal portions of elongating shoots were sampled and classified as 'large', 'expanding' and

Figure 2

SPUR OF THE STURMER PIPPIN VARIETY SHOWING THE PRIMARY AND THE DEVELOPING SECONDARY SPUR LEAVES

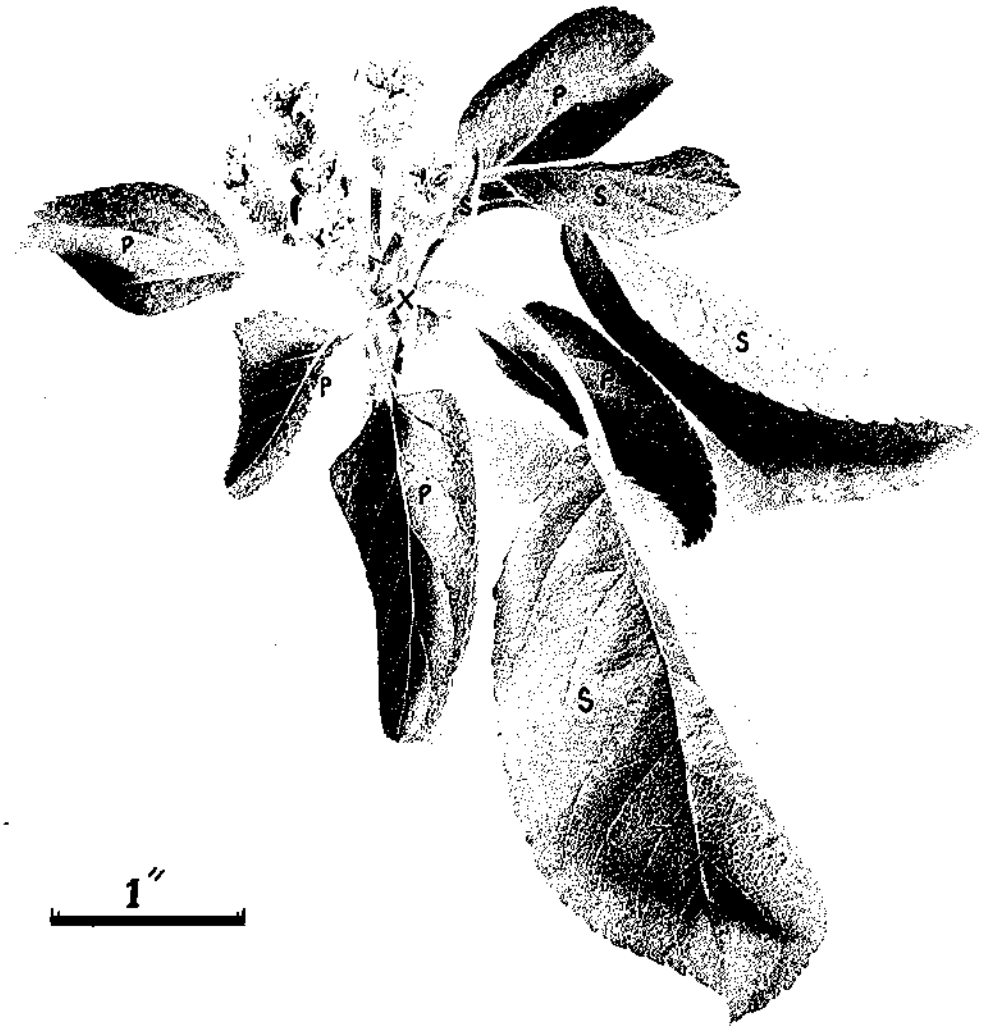


P = primary spur leaves.

S = secondary spur leaves.

Figure 3

SPUR OF THE STURMER PIPPIN VARIETY SHOWING THE PRIMARY AND THE SECONDARY SPUR LEAVES



P = primary spur leaves.

S = secondary spur leaves.

X = axillary structure on which the secondary spur leaves are borne.

'emerged' on the same basis as the secondary spur leaves.

Where dry weight determinations were made, the sub-samples were dried in an oven at 100° for 16-17 hr (61).

3.2 Reagents and Reference Compounds

Reagent grade chemicals were used to prepare the chromogenic sprays and the reagents for the ultraviolet spectral studies. n-Butanol, ethanol, acetone and ethylacetate were distilled in all-glass equipment from reagent grade stocks while the remaining solvents were used in the following grades: acetic acid, AR or reagent grade; pyridine, AR grade; isopropanol, May and Baker R grade and n-hexane, reagent grade. (U - ^{14}C)-glucose, specific activity 361 μc/mg, was purchased from the Radiochemical Centre, Amersham, England.

Samples of kaempferol, d-catechin, quercitrin, rutin, caffeic acid and sinapic acid were kindly provided by Dr E. Wong, Plant Chemistry Division, D.S.I.R., Palmerston North, and the quercetin, phloridzin, l-epicatechin, p-coumaric acid and chlorogenic acid were purchased from Koch-Light Laboratories. Phloretin was prepared from the phloridzin by acid hydrolysis. Standards of D-glucose, D-galactose, D-xylose, L-arabinose and L-rhamnose were obtained from reagent grade stocks. Quercetin, the only reference compound to be purified, was recrystallised from the commercial sample. A portion was dissolved in a 95% ethanol/pyridine (1:1 v/v) solvent and after a dark brown finely-divided insoluble material had settled the solution was decanted carefully and on the addition of an equal volume of distilled water set

aside to crystallise. Samples of all reference compounds were eluted for spectral analysis following paper chromatography in two solvents, normally 6% acetic acid and then n-butanol-acetic acid-water.

3.3 Extraction of Phenolic Compounds from Leaf Tissue

All samples were extracted in the fresh form and wet weights were recorded immediately prior to the extraction which commenced with a minimum delay and often within the hour after the material had been collected in the orchard. The weighed samples were homogenised with not less than 6-7 ml of either 95 or 70% ethanol per gram wet weight of sample, in more than one batch if necessary, in a Waring Blendor operated on 'low' speed to fragment the tissue and then on 'high' speed for three minutes. The homogenate was immediately transferred to a beaker which was placed to the depth of its contents in a water bath at 45-47° and heated for a minimum of 45 minutes to inactivate the liberated enzymes which would attack the phenolic compounds. The homogenate was then filtered, and the residue rinsed with twice its volume of the appropriate ethanol on a Buchner funnel.

The green filtrate, which contained the bulk of the phenolic compounds from the sample, was concentrated in a rotary evaporator at 40° to about one-sixth of its original volume. To remove the bulk of the photosynthetic pigments and lipids, this aqueous concentrate was partitioned in a separating funnel against an equal volume of n-hexane which was first used to rinse the evaporator flask. When necessary a little ethanol was added to improve the separation rate of the aqueous

and organic phases. The aqueous phase was drawn off and after being dried in a rotary evaporator was extracted with a known volume (0.8-1.0 ml/gm wet weight of sample) of 95 or 70% ethanol to yield the main extract of the phenolic compounds which was transferred to a small flask for storage at room temperature until required for chromatography. If an extract was required for chromatography, the n-hexane phase was dried in a rotary evaporator and a known volume (2-4 ml) of ethanol added to prepare the extract.

When the residue on the Buchner funnel was to be re-extracted it was transferred and stirred with three volumes of 70% ethanol in a beaker which was placed in a water bath at 40° for a minimum of 1 hour. The contents were filtered, up to several hours later, as described previously and the filtrate dried and extracted in a small volume of 70% ethanol for chromatography. The residue obtained on this second filtration was normally discarded and only in a detailed investigation of the extraction procedure was it further re-extracted (section 4.1).

3.4 Paper Chromatography

All chromatography was carried out on Whatman No. 1, No. 3 or No. 3MM papers and development was by descending elution normally in stainless steel or glass tanks each large enough to accommodate four standard size (36x57 cm) sheets of chromatography paper. In addition a small glass tank was used for the testing of some solvent systems. The heavier No. 3 and No. 3MM papers were selected for one-dimensional preparative chromatography and the lighter No. 1 paper for one- and

two-dimensional analytical chromatography. Most of the heavier papers were washed with 6% acetic acid (about 100 ml per sheet) and then with 95% ethanol (about 60 ml per sheet) and air-dried before use. To aid solvent run off in the washing process, the bottom edge of each paper was serrated.

Solvent systems employed in the chromatography of phenolic compounds are recorded in Table 8 while those used for the sugars are presented in Table 9. These solvents were normally prepared just long enough before use to allow two-phase systems to equilibrate and separate, although in the preparative chromatography the commonly used 6%HA and BAW were prepared in about 500 ml volumes and used as required over a period of days.

Table 8

SOLVENTS USED IN THE PAPER CHROMATOGRAPHY OF PHENOLIC COMPOUNDS

Components	Proportions*	Identification	Usual time for development
Acetic acid (HA)-water	2% v/v	2%HA	5hr
	6%	6%HA	5hr
	25%	25%HA	5½hr
	40%	40%HA	7½hr
n-Butanol-HA-water	4:1:2.2	BAW	16hr
n-Butanol-EtOH-water	4:1:2.2	BEW	16hr
Benzene-HA-water	125:72:3	BeAW	4½hr
Ethyl acetate-water	saturated	EAW	6hr
Acetone-water	1:1	AW	-
Distilled water		W	5hr
Isopropanol-NH_3-water	20:1:2	IpAW	12hr

* Where two phases separated the upper organic one was used.

The phenolic substances were applied to chromatograms in either 70 or 95% ethanolic solution. For preparative chromatography, solutions were applied as even bands 0.5-0.7 cm wide across the chromatograms 8 cm in from the edge to be inserted in the solvent trough. In one-dimensional analytical chromatography, solutions as spots about 0.5 cm diameter were applied at 2 cm intervals along a line of similar location to the band line. On two-dimensional chromatograms, a single spot of similar size but heavier loading was applied 8 cm in from the edge of the paper. Small aliquots were spotted using a glass tube drawn out to a capillary at one end and larger volumes were banded from a 0.1 ml graduated pipette fitted to a suction filler and slow release air-bleed device. Between the repeated application of solutions which was required to reach the empirically determined loading of the chromatograms, the solvent was evaporated in the warm air-flow from a hair drier. Once loaded, the chromatograms were equilibrated in air for a short time before being placed in the chromatography tank and developed immediately. The chromatograms were normally removed from the tank and air-dried after the solvent front had migrated 35-40 cm beyond the band line and had therefore approached the lower edge of the paper. The solvent front was located on the dried chromatograms and marked in pencil under a Hanovia Fluorescence Model 16 ultraviolet lamp.

Table 9

SOLVENTS USED IN THE PAPER CHROMATOGRAPHY OF SUGARS

Components	Proportions	Identification	Usual time for development
Ethyl acetate-pyridine-water	60:25:20	EaPW	6hr
Isopropanol-water	80:20	IpW	20hr
Isopropanol-n-butanol-water	70:10:20	IpBW	17hr
Ethyl acetate-acetic acid-water	70:15:15	EaAW	7hr

Following the location (see below) and marking of the spots or bands of the phenolic compounds in pencil the analytical chromatograms were preserved for record purposes while the preparative chromatograms were cut with scissors into the outlined bands from each of which the phenolic compounds were recovered. Recovery was effected for each band by immersing the excised chromatography paper in a 60% ethanol/glacial acetic acid (9:1 v/v) solvent for at least six hours. To minimise the volume of solvent needed, the chromatography paper was reduced to pieces about 2 cm square. The resulting solution, together with a rinsing volume of 60% ethanol, was centrifuged up to 3,000 rpm in an MSE bench model centrifuge for about four minutes after which the bulk of it was decanted to leave the matted deposit of small particles of paper in the centrifuge tubes. After evaporation of the decanted solution in a rotary evaporator at 40° the recovered material was

dissolved in a minimum volume of 70 or 95% ethanol and either rebanded for further chromatographic purification or stored in sample tubes at room temperature to await characterisation.

Sugars were spotted in aqueous solution on to No. 1 papers and chromatographed in the manner described for the phenolic compounds.

3.5 Chromogenic Reagents

Chromogenic reagents which were used to detect phenolic compounds on paper chromatograms are detailed in Table 10.

Table 10

CHROMOGENIC REAGENTS USED TO DETECT PHENOLICS ON PAPER CHROMATOGRAMS

Reagent	Concentration	Reference	Compounds detected
Aluminium chloride	1% in ethanol	(30)	Flavonoids
Ammonia vapour	-	(26)	All types
Sodium carbonate	5% aqueous	(30)	All types
Diazotised p-nitroaniline		(62)	Many
Diazotised sulphanilic acid		(63)	Many
Hoepfner reagent		(42)	Hydroxycinnamic acids

Many phenolic compounds were also detected, prior to treatment with the chromogenic reagents, by inspection of the dry chromatograms

under the ultraviolet lamp. The diazotised p-nitroaniline spray was used alone and was not oversprayed with 20% w/v sodium carbonate (62). Sugars were detected on chromatograms with the p-anisidine hydrochloride reagent (64). Extensive use was made of chromogenic reagents in conjunction with analytical chromatography since the colour reactions of phenolic substances with a variety of the reagents were recorded to assist identification. The entire chromatograms were sprayed evenly with the reagents using an all-glass atomiser. Colour reactions noted were those in evidence just after the sprayed chromatograms had dried and were assessed visually without any matching against a colour chart. In the preparative chromatography, chromogenic reagents were normally used only to detect bands of phenolic compounds which were invisible under ultraviolet light alone and in the presence of ammonia vapour. In these cases guide strips of the developed chromatograms were streaked with the selected reagents from glass capillaries and the positions of the bands located were marked on the remainder of the chromatograms in readiness for the recovery of the compounds.

3.6 Ultraviolet Absorption Spectroscopy

Absorption spectra, which were recorded as a plot of absorbance (optical density) against wavelength, were determined within the range 220-440 mμ with a Beckman Model DB recording spectrophotometer using matched 1 cm silica cells containing the samples in 95% ethanolic solution. Besides the normal ethanolic spectrum determined on 2.5 ml of solution, further spectra were determined for each sample after the

addition of reagents which included aluminium chloride (65), anhydrous sodium acetate (66), sodium acetate and boric acid (67) and sodium hydroxide (68). The aluminium chloride reagent was prepared freshly every two days as a 0.6 w/v solution of the anhydrous salt in 95% ethanol of which 0.5 ml was mixed with 2.5 ml of the ethanolic sample solution before the spectrum in the presence of aluminium ion was recorded. The spectrum in the presence of excess sodium acetate was determined after 2.5 ml of the sample solution had been saturated with excess anhydrous sodium acetate. Then 0.5 ml of a saturated solution of boric acid in 95% ethanol was added, the cell shaken and, once the excess solid had settled, the spectrum in the presence of excess sodium acetate and boric acid was recorded. Either three drops (about 0.05 ml) or 0.20 ml (with flavonoid compounds only) of a 2% w/v solution of sodium hydroxide in 95% ethanol, freshly prepared every two days, was mixed with 2.5 ml of the sample solution in a cell and the spectrum in alkali recorded.

A chromatographic procedure was used to prepare the samples and the appropriate blanks for these spectral studies. A suitable portion of each fraction isolated from a leaf tissue extract or reference compound was chromatographed to one side of a sheet of No. 3 or No. 3MM paper, over one-half of which was left blank. From each developed chromatogram a strip containing the band of phenolic substance was removed along with a blank strip of mirror image from the other side of the sheet. These two strips were eluted with 95% ethanol in a sealed chromatography tank for 24-36 hours to provide the sample and the corresponding blank solutions. Spectral studies were then undertaken

with a minimum delay.

3.7 Characterisation Procedures

Characterisation of the fractions which were isolated from the leaf tissue extracts by preparative paper chromatography was based on a study of the paper chromatographic, colour reaction and spectral data obtained for the fractions and their degradative products. Wherever possible, direct comparisons were made with the data obtained from reference compounds.

The paper chromatographic properties of each fraction and reference compound were examined in up to six solvent systems. The phenolic moieties released by acid or alkali hydrolysis of samples of certain fractions were also submitted to similar chromatographic procedures. Sugars released on the acid hydrolysis of glycosides were identified on paper chromatograms by direct comparison with the authentic compounds in four solvent systems. The non-phenolic moieties of alkali-labile fractions were not characterised. Each unknown solution was spotted in two positions on each chromatogram beside the appropriate reference compound which was similarly applied. Colour reactions were determined immediately after the developed chromatograms had dried.

For acid hydrolysis, a sample of each fraction (about 0.25 ml diluted to 2 ml with 95% ethanol) was refluxed for one hour on a boiling water bath with an equal volume of 2N hydrochloric acid. After cooling, the hydrolysate was transferred to a separating funnel, an

equal volume of distilled water added, and extracted twice with ½ volumes of ethyl acetate. The combined ethyl acetate extract was washed with a small volume of distilled water before it was evaporated and the aglycone recovered in the minimum volume of 95% ethanol for chromatography. Evaporation of the aqueous layer in a rotary evaporator was repeated several times before the dried flask was placed in a desiccator over potassium hydroxide and silica gel for 72 hours to remove traces of hydrochloric acid before the sugar was taken up in the minimum volume of water for chromatography.

Alkali hydrolysis of the conjugates in certain fractions was carried out for four hours at room temperature in an atmosphere of nitrogen. A sample, about 0.25 ml, of each fraction was made up to 1 ml with 70% ethanol in a flask which was de-aerated with nitrogen before an equal volume of 2N sodium hydroxide was added and gassed with nitrogen for one minute after which the flask was sealed off. After the hydrolysis, an equal volume of 2N hydrochloric acid was added before the extraction with ethyl acetate was performed to recover the phenolic moiety, as described for the acid hydrolysis. The aqueous phase containing the non-phenolic moiety was discarded.

3.8 Incorporation of ^{14}C into Phenolic Compounds in Leaf Tissue

The incorporation of ^{14}C carbon, supplied as glucose, into the phenolic compounds in young leaves of Sturmer Pippin was investigated. Radioactivity was determined in a Nuclear Enterprises liquid scintillation detector used in conjunction with Philips electronic equipment.

The liquid scintillator was dioxan containing 6 gm of terphenyl per litre and a counting efficiency of 37.5% was obtained. Ilford X-ray film was used in the radioautography.

In early February, whole shoot tips from young Sturmer Pippin trees were taken to the laboratory where five leaves, which had just entered the 'expanding' stage, were selected. These leaves were removed under water by a transverse cut near the base of the petiole, and each was transferred rapidly to a small sample tube which contained 1 μc of the isotope in 0.1 ml of water and placed with the cut end of the petiole below the surface of the liquid. During the five hours the leaves remained in bright natural light in the laboratory, small volumes (approx. 0.1 ml) of distilled water were added 3-4 times to each tube to keep the cut end of the petiole submerged and to promote more complete uptake of the isotope. Following this the leaves were removed, gently blotted dry with filter paper, and weighed prior to the preparation of an extract of the phenolic compounds by the normal procedure using 70% ethanol. The radioactivity in the extract and in four chromatographic fractions, which contained the major phenolic compounds, was determined by liquid scintillation. The two-dimensional chromatograms, prepared from aliquots of the extract developed in BAW and then in 6%HA, were subjected to radioautography.

Chapter 4

EXPERIMENTAL AND RESULTS

4.1 Investigation of Extraction Procedure

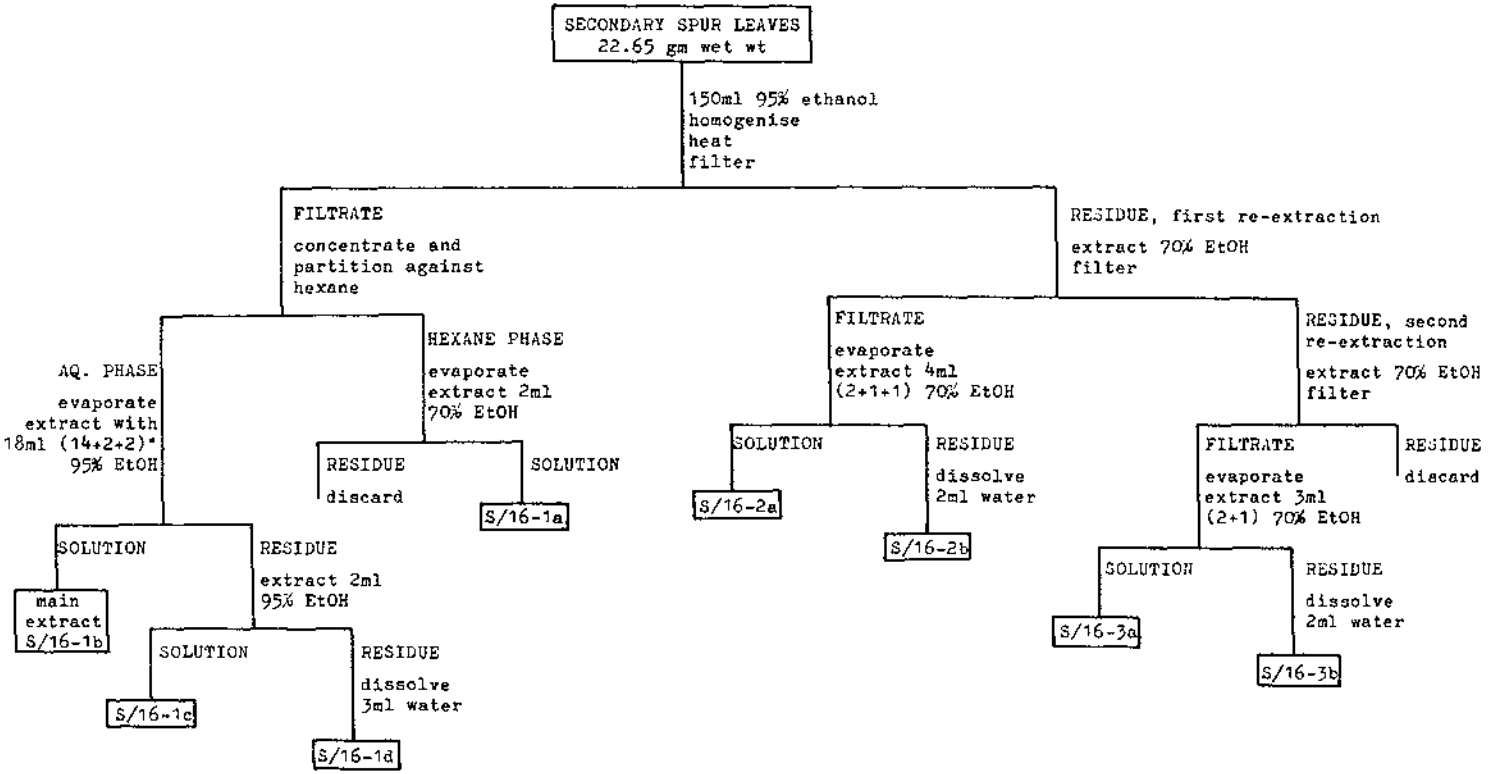
Two procedures for the quantitative extraction of non-bound phenolic compounds from the leaf samples were investigated, the chief difference being the use of either 95 or 70% ethanol in the preparation of the main extracts. The non-bound phenolics were considered to have been extracted quantitatively when the residue of the leaf tissue did not yield detectable amounts of phenolic substances on further re-extraction under the conditions used in the investigation. Of considerable value in the detailed determination of the steps in the two extraction procedures were the results of preliminary studies of the extraction of leaf tissue. For the investigation, secondary spur leaves of Sturmer Pippin were obtained on 29 October when most axillary structures carried four secondary leaves, two of which were fully expanded, so that the leaf tissue was at all stages of development from newly-emerged through fully-expanded (see Figures 2 and 3). This leaf material was mixed and split into two representative samples (S/16 and S/17) from which sub-samples were taken and the dry weight determined (29.7-29.9%). Sample S/16 (22.5 gm wet weight) was extracted with 95% ethanol (150 ml) and sample S/17 (78.63 gm wet weight) with 70% ethanol (500 ml). Details of the subsequent steps in the two

extraction procedures and of the extracts obtained are summarised in Figures 4 and 5. Where the dried filtrates were extracted with an allotted volume of ethanol not in one step but in several, the portion of the total volume used in each step is indicated, in the Figures, in brackets after the total volume.

The first re-extraction of the residue obtained when the homogenate of each sample was filtered was performed in the usual manner (section 3.3) and from the resulting filtrate the indicated (Figures 4 and 5) extracts were prepared. In order to check on the completeness of the extraction of the non-bound phenolics at this stage the residue for each sample was subjected to a second re-extraction. Since there was a marked colour difference in the residues of the S/16 and S/17 samples, both before and after the first re-extraction, only about one-third of the dull orange-brown residue of sample S/17 was submitted to the second re-extraction process in 70% ethanol, which was also applied to the entire light-buff residue of sample S/16. The second re-extraction of a further one-third of the residue of sample S/17 was performed in 95% ethanol to check whether the colour difference in the residues was due to the colouring substances being soluble if 95% ethanol was used in the homogenisation step. The remaining one-third of the sample S/17 residue was discarded. In the second, as in the first re-extraction, the residues which were extracted with 70% ethanol were slurried in three volumes of solvent. The slurries were heated for two hours at 40^{o} in a water bath, left to stand overnight and then filtered. The residues from the filtration were discarded. From the dried filtrates the S/16-3

Figure 4

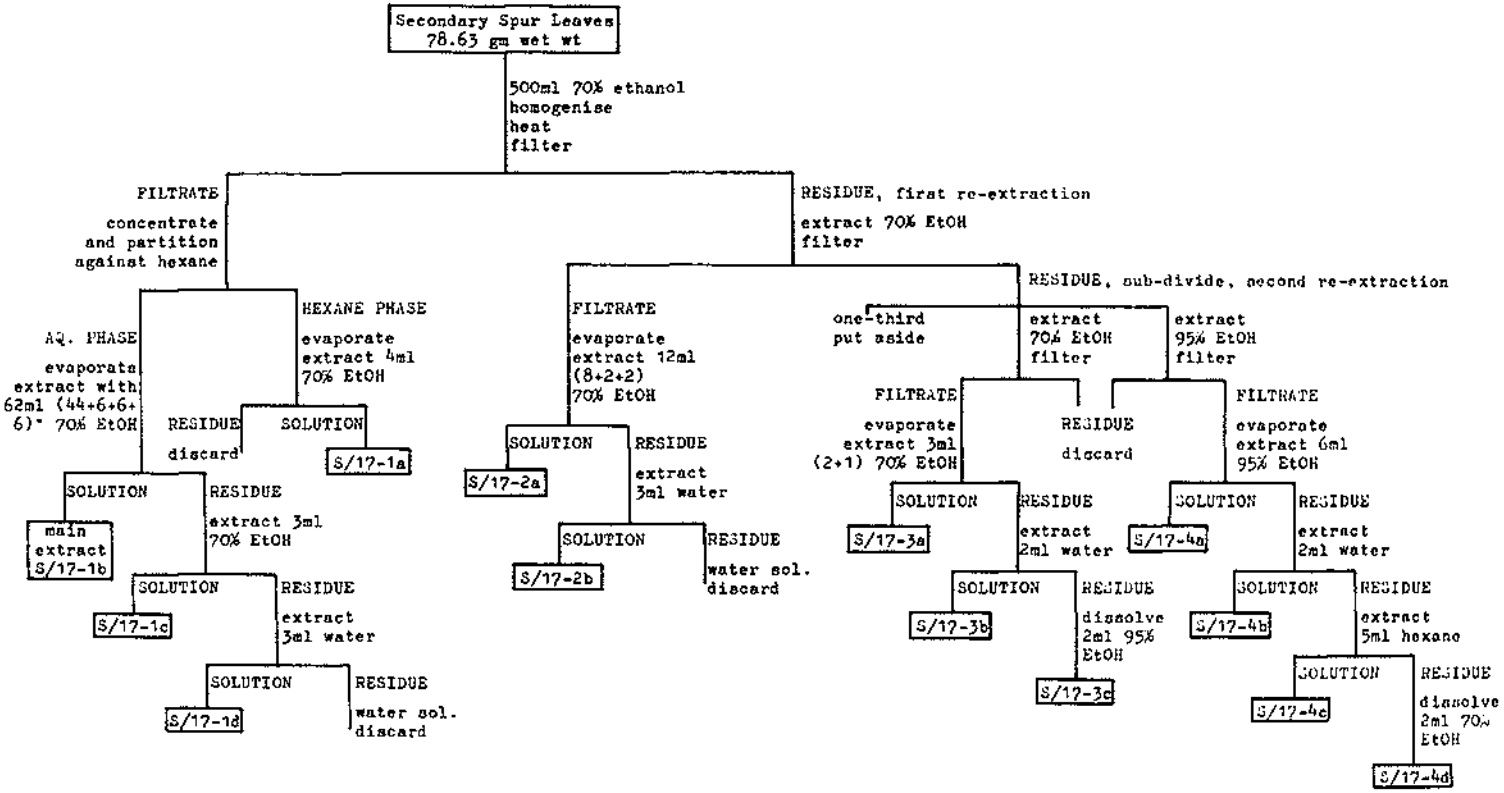
SCHEME OF EXTRACTION OF SECONDARY SPUR LEAVES OF STURMER : SAMPLE S/16



* Indicates portions in which the ethanol was added and the solution removed to prepare total volume of extract.

Figure 5

SCHEME OF EXTRACTION OF SECONDARY SPUR LEAVES OF STURMER : SAMPLE S/17



* See Figure 4.

and S/17-3 extracts were obtained. In the second re-extraction with 95% ethanol, the portion of the S/17 residue was stirred with four volumes of ethanol and left overnight before a further four volumes were added and the mixture heated for 30 minutes at 40° and filtered to yield a light orange filtrate and a buff-coloured residue (discarded). From this dried filtrate the S/17-4 extracts were prepared, and the main one, S/17-4c, stored in darkness at room temperature.

The complement of phenolic compounds in each extract recorded in Figures 4 and 5 was assessed following one-dimensional paper chromatography in 6%HA. This solvent was chosen on account of the wide distribution of R_F values obtained for the phenolic compounds in apple leaf tissue and also for its suitability in the chromatography of extracts likely to contain substantial amounts of sugars which, unless carried to the solvent front, would interfere with the resolution of the phenolic compounds. Besides, the time taken to develop a chromatogram (4½ hours) was relatively short. For all extracts (except the main ones, which were applied at half volume) an equal volume, sufficient to permit the detection of phenolic compounds in a weak solution, was chromatographed in two positions on each of three chromatograms. The complement of phenolic compounds in each extract was then assessed by inspection of the developed chromatograms in ultraviolet light before and after exposure to ammonia vapour and also after spraying with sodium carbonate, aluminium chloride and diazotised p-nitroaniline.

This assessment uncovered some interesting differences in the phenolic content of comparable extracts from the two samples which

were interpreted as follows.

(a) A more complete initial extraction of the phenolics was obtained when the leaf sample was homogenised with 70% rather than 95% ethanol, since extracts S/16-2a and S/16-2b, derived from the first re-extraction of the residue, had a much greater phenolic content than the corresponding S/17- extracts which were similarly obtained.

(b) Insignificant amounts of phenolic substances were removed in the n-hexane phase when lipids and photosynthetic pigments were cleared from the filtrate of the homogenate, since only traces of phenolics were noted in extracts S/16-1a and S/17-1a. By virtue of their low volumes, these extracts would have been saturated with phenolics had appreciable amounts been present in the dried n-hexane phases. Further, the substances removed in the n-hexane phase were found to cause interference in the loading of the chromatograms and in the separation of the phenolic compounds, especially in the less polar solvents.

(c) The extraction of phenolic compounds from the dried filtrate of the homogenate was superior when 70% ethanol was used. Only traces of phenolics were noted in extract S/17-1c and none in extract S/17-1d, while considerable amounts were detected in extract S/16-1d and traces in extract S/16-1c.

(d) The lower solubility in less aqueous ethanol of phenolic compounds which fluoresced blue with ammonia in ultraviolet light and which were located at R_F greater than 0.5, was indicated by several

results. For example, the presence of these compounds in considerable amounts in extract S/16-1d and their absence from extracts S/17-1d and S/16-1c was most notable along with their relatively low level in extract S/16-1b as compared with extract S/17-1b. Further, the presence of considerable amounts of these compounds in particular in extract S/16-2a and of reasonable amounts in both extract S/16-2b and S/16-3a and traces in extract S/16-3b, indicated that a lower proportion was extracted in the homogenisation step with 95% than with 70% ethanol and that the proportion remaining in the residues after the homogenisation in 95% ethanol was not readily extracted even with 70% ethanol.

(e) The non-bound phenolic compounds remaining in the residue obtained when the homogenate was filtered were, in the S/17 extraction, almost completely removed in extract S/17-2a. This was shown by the absence of evidence of phenolic compounds in extract S/17-2b and the presence of only traces, at high R_F, in extract S/17-3a. However in the S/16 extraction, the effective extraction of these compounds from the residue was more difficult and required a second re-extraction of the residue, presumably as a consequence of the lower proportion of certain compounds extracted in the homogenisation step with the 95% ethanol. Thus considerable amounts of phenolic compounds were noted in extract S/16-2a and reasonable amounts in extracts S/16-2b and S/16-3a.

(f) The only extract in the S/17-4 series in which phenolic compounds were detected was S/17-4a where traces were noted about R_F 0.5.

(g) Extracts in which phenolic compounds could not be detected,

e.g., S/17-1d and S/17-2b, would presumably contain low concentrations rather than no phenolics, but the proportion of the total non-bound phenolics of the leaf tissue in these extracts would be very low.

The 70% ethanol was therefore found to be superior to 95% ethanol in both the extraction of the phenolic compounds from the tissue (see (e)) and in the recovery of the extracted compounds from the dried filtrates (see (c)). Overall then, the results indicated that quantitative extraction of the non-bound phenolics of the leaf tissue would be obtained most conveniently by preparing and combining extracts equivalent to S/17-1b and S/17-2a, as outlined in Figure 5.

Thus in this proposed extraction procedure the leaf tissue is homogenised in 70% ethanol (6-7 ml/gm of wet tissue) for three minutes and then heated at 45° for at least 45 minutes to inactivate the enzymes and complete the extraction of the bulk of the non-bound phenolics. The filtrate of the homogenate is concentrated in a rotary evaporator at 40° to about one-sixth of its original volume and partitioned against an equal volume of *n*-hexane before the aqueous phase is dried and extracted with 70% ethanol (0.8-1.0 ml/gm of wet tissue) to recover efficiently the phenolics present in the filtrate. The residue from the homogenate is re-extracted by suspending in three volumes of 70% ethanol and heating at 40° for one hour to render soluble most of the remaining non-bound phenolics which are then recovered efficiently from the dried filtrate in a volume of 70% ethanol (0.15-0.20 ml/gm of wet tissue) and combined with the main extract.

4.2 Effects of the Nature of the Leaf on Levels of Phenolics

The main extracts which were prepared from various types of leaf tissue and included in this study are recorded in Table 11. The leaf tissue homogenate and the main extract examined were prepared with 95% ethanol except for samples S/17-S/20 inclusive, where 70% ethanol was used.

To assess the levels of phenolic compounds in these extracts, two-dimensional chromatograms were prepared from aliquots of each extract, by development firstly in BAW and then in 6%HA. The phenolic compounds were located with the aid of ultraviolet light and chromogenic reagents and the spot intensities assessed visually. A high degree of resolution of the individual phenolic compounds in each extract was best achieved with the above solvent pair. The resolution obtained made visual comparison of the chromatograms a simple and effective method for the detection and assessment of any significant changes in the relative amounts of individual phenolic compounds. The typical two-dimensional chromatographic pattern of phenolic compounds in the primary spur leaf tissue of the Sturmer Pippin variety is reproduced in Figure 6. This pattern did not change over the period 1-23 October that primary spur leaves were sampled.

However, the pattern in secondary spur leaves of this variety was found to change somewhat during the development of the leaf from the 'emerged' to the 'expanded' condition and in this latter condition to resemble that of the primary spur leaf tissue in all respects except for the very low intensity of spot 3c. The change in the

Table 11

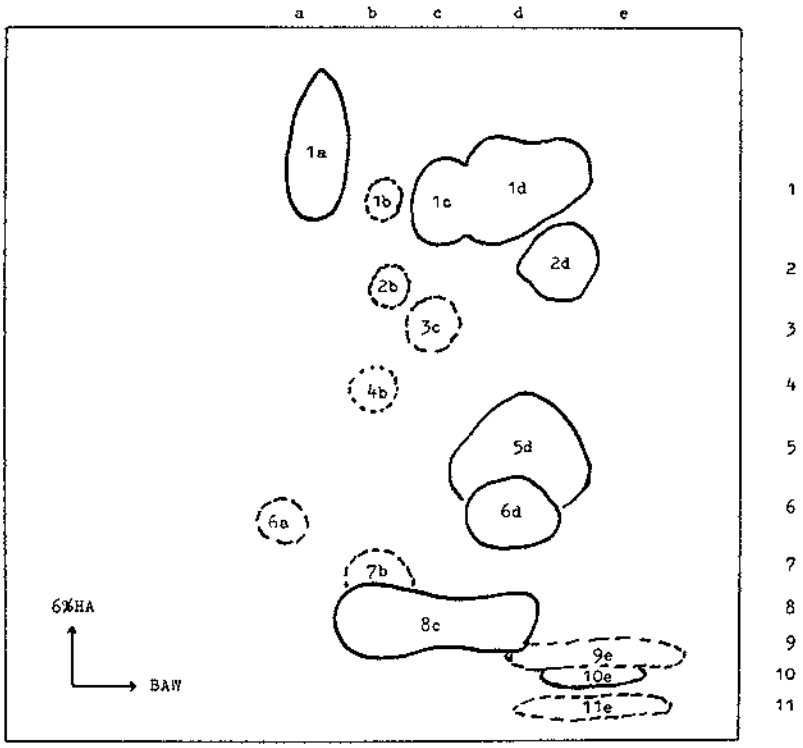
SAMPLES TAKEN AND MAIN EXTRACTS MADE OF APPLE LEAVES

Date	Identification of samples and main extracts	Nature of leaf tissue*	Sample wet weight (gm)	Dry weight (%)
1.10	P/1	1^{ry} Sturmer	13.65	
9.10	P/2	1^{ry} Sturmer	16.38	
	S/3	2^{ry} Sturmer	9.26	
16.10	P/4	1^{ry} Sturmer	15.41	
	S/5	expanded 2^{ry}	2.17	
	S/6	expanding 2^{ry}	7.46	
	S/7	emerged 2^{ry}	4.15	
	S/8	2^{ry} Sturmer	6.52	
22.10	P/9	1^{ry} Sturmer	10.17	
	P/10	1^{ry} Cox's O.P.	10.00	
	P/11	1^{ry} Rome B.	10.75	
23.10	P/12	1^{ry} Sturmer	34.54	28.1
	S/13	expanded 2^{ry}	28.78	30.5
	S/14	expanding 2^{ry}	19.80	29.0
	S/15	emerged 2^{ry}	12.75	26.5
29.10	S/16	2^{ry} Sturmer	22.65	29.8
	S/17	2^{ry} Sturmer	78.63	29.8
5.2	S/18	expanded, shoot	11.20	
	S/19	expanding	4.40	
	S/20	emerged	2.75	

* Except for sample P/10, from Cox's Orange Pippin, and sample P/11, from Rome Beauty, all samples were of the Sturmer variety. Apart from samples taken 5.2 from shoot tips, all were of either primary spur leaves (1^{ry}) or secondary spur leaves (2^{ry}).

Figure 6

TYPICAL PATTERN OF PHENOLICS ON A TWO-DIMENSIONAL CHROMATOGRAM OF A 95% ETHANOL EXTRACT OF PRIMARY SPUR LEAVES OF STURMER



Colours of Spots

Colours are those appearing in ultraviolet light in the presence of ammonia vapour, except for spots 4b, 7b and 10e which, as noted, were visible only after spraying with diazotised p-nitroaniline (p-na).

1a	weak blue	5d	dull red
1b	very weak green-blue	6a	faint yellow
1c	strong green-blue	6d	orange-yellow
1d	light blue	7b	weak positive p-na
2b	very weak green-blue	8c	yellow
2d	bright blue	9e	blue
3c	weak green-blue	10e	weak positive p-na
4b	weak positive p-na	11e	blue

phenolic pattern associated with this development of the secondary spur leaf involved considerable alteration in the intensities of spots 6a and 6d and therefore in the absolute and relative amounts of the phenolic compounds in these spots. On chromatograms of the extracts of 'emerged' leaves 6a was always a major and 6d a barely detectable spot, and as the secondary spur leaf tissue developed and reached the expanded condition the intensities of these two spots changed gradually to those typical of primary spur leaf tissue. The distinctive phenolic pattern of primary and secondary spur leaf tissue was first noted when a sample of primary (P/2) and of secondary spur leaves (S/3), which were obtained from the same lot of spurs, were compared. In extract S/3, 6a was noted to be a major and 6d a minor spot while spot 3c was barely detectable. As sample S/3 consisted of secondary spur leaves at stages of development ranging from newly-emerged to partly-expanded while the primary spur leaves of sample P/2 were fully expanded (their only condition during the entire sampling period), it was considered that the difference in the pattern of phenolics between the two types of leaf tissue from the one lot of spurs was more likely to be associated with the difference in the stage of development rather than with an inherent difference in the leaf types. Thus when fully-expanded secondary spur leaf tissue had developed it was possible to compare, again from the one lot of spurs, the pattern of phenolic compounds in primary spur leaf tissue (sample P/4) and in secondary spur leaf tissue which was classified as 'emerged' (sample S/7), 'expanding' (sample S/6) and 'expanded' (sample S/5). To facilitate direct comparison, extracts of these

samples were prepared in 1 ml ethanol/gm wet weight of tissue and similar aliquots of each were chromatographed. Chromatography showed that in sample S/7, spot 6a was strong and 6d was undetectable, while in extract S/6, these spots were both of moderate intensity and in extract S/5 they were of the normal intensities for primary leaf tissue. Hence as the secondary leaf tissue expanded the change noted was a progressive weakening of spot 6a and the appearance and intensification of spot 6d. In addition to these striking changes, the intensity of spot 3c increased appreciably as the secondary spur leaf tissue expanded. The expanded secondary spur leaf tissue, sample S/5, exhibited a very similar chromatographic pattern of phenolic compounds to the primary spur leaf, sample P/4, except for the very low intensity of spot 3c in the former. Confirmation of these results was obtained in a repeat study a week later when samples of primary (P/12) and of secondary (S/12, S/14 and S/15) spur leaf tissue were obtained from one set of spurs and the pattern of phenolic compounds assessed. Evidence for the constancy of these changes in the levels of certain phenolic compounds as the leaf tissue expanded was obtained when tissue samples of the 'emerged' (S/20), 'expanding' (S/19) and 'expanded' (S/18) categories were obtained from the shoot tips of young trees of the Sturmer Pippin variety and the extracts, on chromatographic examination, found to possess the levels of phenolic compounds expected in extracts of similar secondary spur leaf tissue.

The changes noted in the intensities of spots 6a and 6d in the secondary spur leaf tissue were so marked that the replacement of 95% ethanol, as used in most cases, by 70% ethanol in the extraction

procedure would not have had appreciable effect on the result. However, the much inferior removal and recovery of phenolic compounds in spots 1a-2d inclusive (Figure 6) with the 95% ethanolic extraction could conceivably have resulted in changes in the levels of some of these compounds being overlooked in the S/5-S/7 and S/13-S/15 samples.

4.3 The Phenolic Pattern among Varieties

Extracts (P/9, P/10 and P/11, Table 11) were prepared with 95% ethanol from samples of the primary spur leaf tissue of three apple varieties, Sturmer Pippin (P/9), Cox's Orange Pippin (P/10) and Rome Beauty (P/11) and after two-dimensional chromatography in BAW and in 6%HA, the phenolic patterns were assessed. These patterns were very similar for the Sturmer Pippin and the Cox's Orange Pippin extracts, while the pattern for the Rome Beauty extract was distinguished only by the moderately increased intensity of the weak 6a spot.

4.4 Paper Chromatographic Isolation of Phenolic Compounds

A procedure based on one-dimensional preparative paper chromatography was developed and applied to the isolation of fractions, containing the main phenolic compounds, from a 70% ethanol extract of secondary spur leaf tissue of Sturmer Pippin. The S/17-1b extract was selected for fractionation since it was prepared by the 70% ethanol extraction procedure from secondary spur leaf tissue of all available stages of development which would ensure that not only the bulk of the non-bound phenolics of the leaf tissue but also intermediate levels of

spot 6a and 6d substances were present in the extract used.

A portion of extract 8/17-1b was banded evenly across eight sheets of No. 3 chromatography paper at an average application rate of 1 ml of extract every 21 cm of the band length. In all 13.65 ml of the extract, corresponding to 17.3 gm wet weight of leaf tissue, was chromatographed. Chromatography of an increased volume of extract, while enhancing the amounts of the phenolic compounds available for characterisation, would have led to difficulties in handling the greater number of chromatograms in the purification steps, since all the main bands of the compounds were to be retained.

Following the chromatography of this portion of the extract in 6%HA for five hours, the dried chromatograms were inspected under ultraviolet light in the presence of ammonia to locate the bands of phenolic compounds. Most of the phenolic compounds were present in a broad band which encompassed spots 5d to 8c inclusive. The leading edge of this band was a red-brown colour while the rest was dull yellow. The band was cut lengthwise into four strips, A-D, each of about equal width, and therefore strip A included much of spot 8c while strip D contained much of spots 5d and 6d. Strip F was removed as a narrow 2 cm wide strip of chromatogram immediately ahead of strip D. The presence of presumably spot 4c in strip F was indicated by the positive reaction with diazotised p-nitroaniline. A wide band, which fluoresced blue and blue-green with ammonia in ultraviolet light, extended from the edge of strip F to about 1½ cm from the solvent front. This entire band, except for the 1 cm behind the leading edge where there was heavy contamination with water-soluble non-phenolic

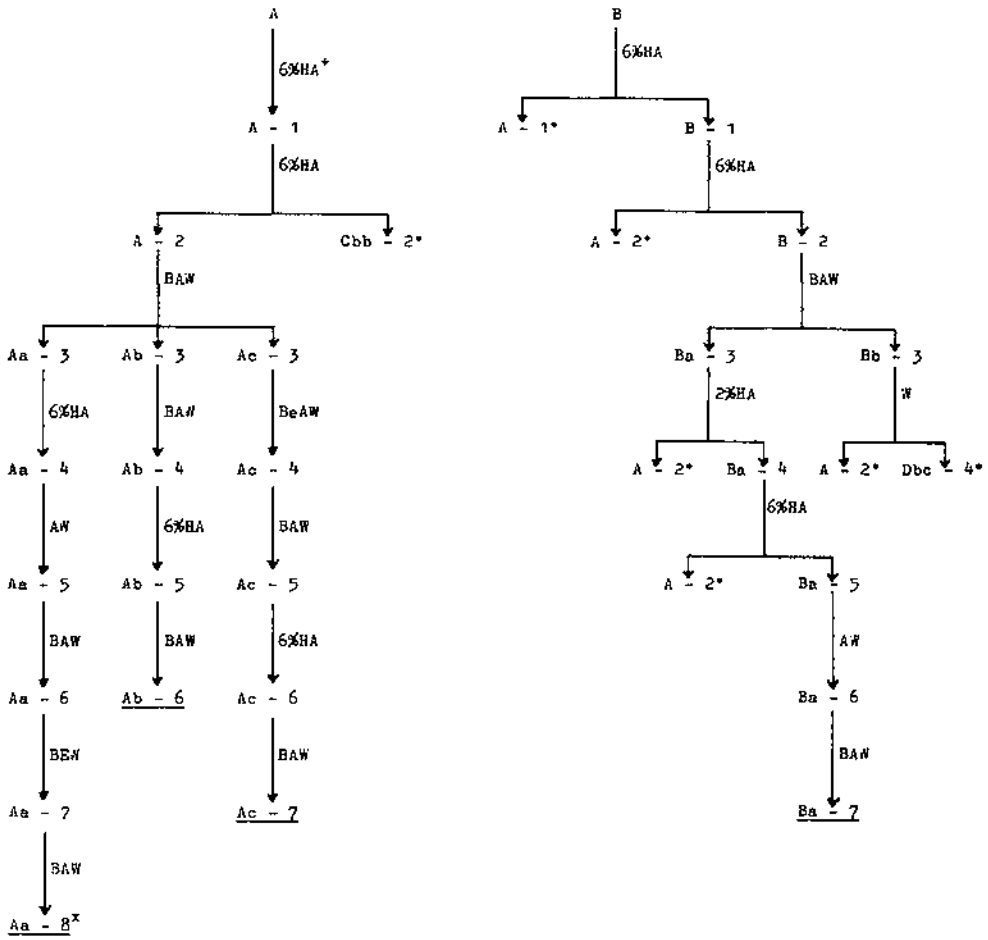
compounds, was removed as strip G. Two bands which fluoresced light blue at low R_F in ultraviolet light in the presence of ammonia, and which corresponded to spots 9e and 11e, were removed as strip Z. This strip therefore extended from almost zero R_F to the edge of strip A. The presence of spot 10e in the middle of strip Z was indicated by the positive reaction to diazotised p-nitroaniline. Thus the original chromatograms were cut into seven contiguous transverse strips which encompassed almost the entire R_F range.

To extract the phenolic compounds, the strips of common identity from the eight chromatograms were cut up and immersed in 60% ethanol/glacial acetic acid (9:1 v/v), except for strip Z which was extracted with BAW on account of the low solubility of the phenolics present in the 60% ethanol/acetic acid. The phenolic compounds extracted from each of the seven designated strips constituted the seven fractions of the same identity; thus fraction A was obtained from strip A, etc. These seven fractions obtained from the extract S/17-1b by chromatography in 6%HA were further fractionated and the phenolic compounds purified by repeated re-chromatography in the steps outlined in Figures 7-10. Washed papers were used in all re-chromatographic steps after the first.

In the Figures the fractions separated and retained in the course of the re-chromatography are identified by both letters and numbers. The capital letter refers to the original fraction from which the bulk of a particular fraction was derived while the lower case letters identify, alphabetically from low to high R_F, the related new fractions obtained on the separation of the previous fraction. The

Figure 7

PAPER CHROMATOGRAPHIC PURIFICATION OF FRACTIONS A AND B



+ See Table 8 for identification of solvent systems.

x The fractions underlined were those submitted to characterisation procedures.

* Combined with main portion of fraction of same identity, Figures 7-10.

Figure 8

PAPER CHROMATOGRAPHIC PURIFICATION OF FRACTIONS C AND D

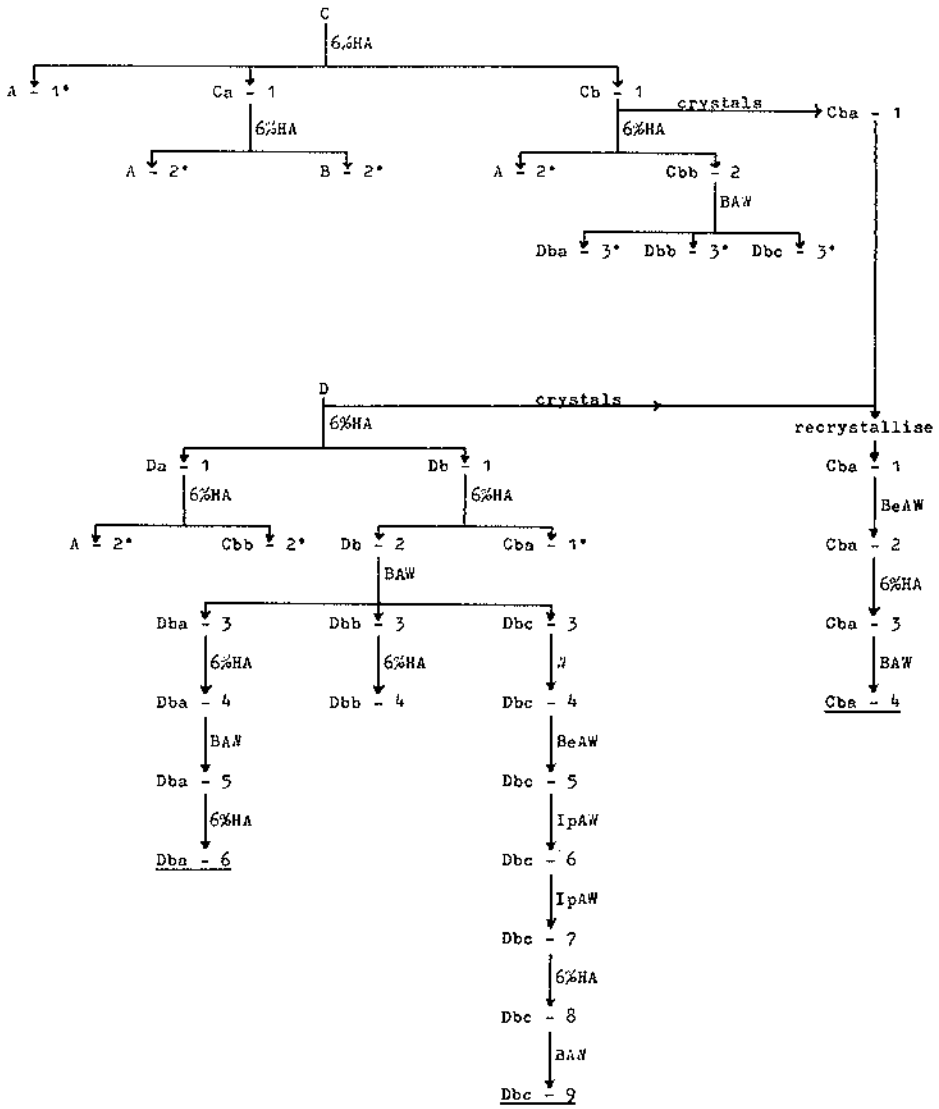
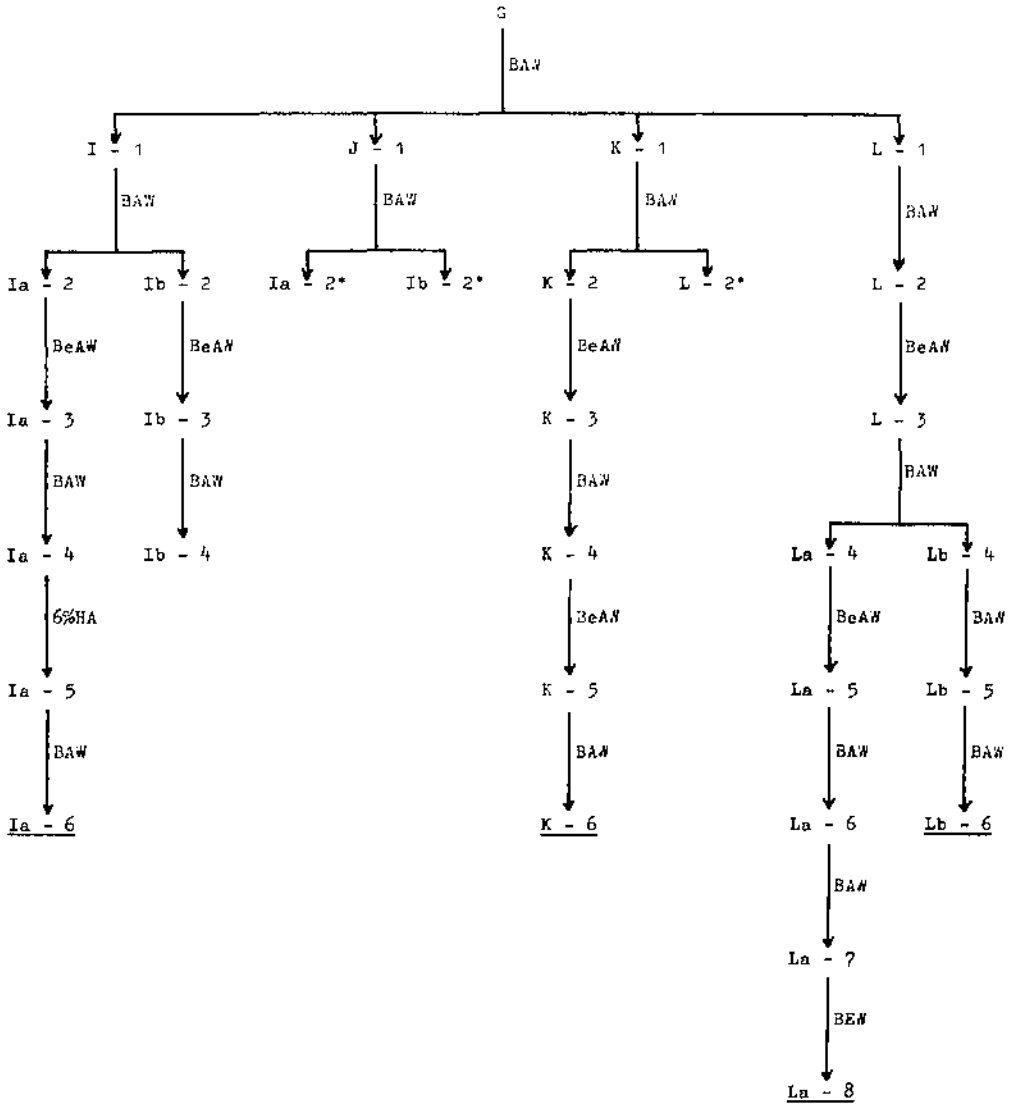


Figure 9

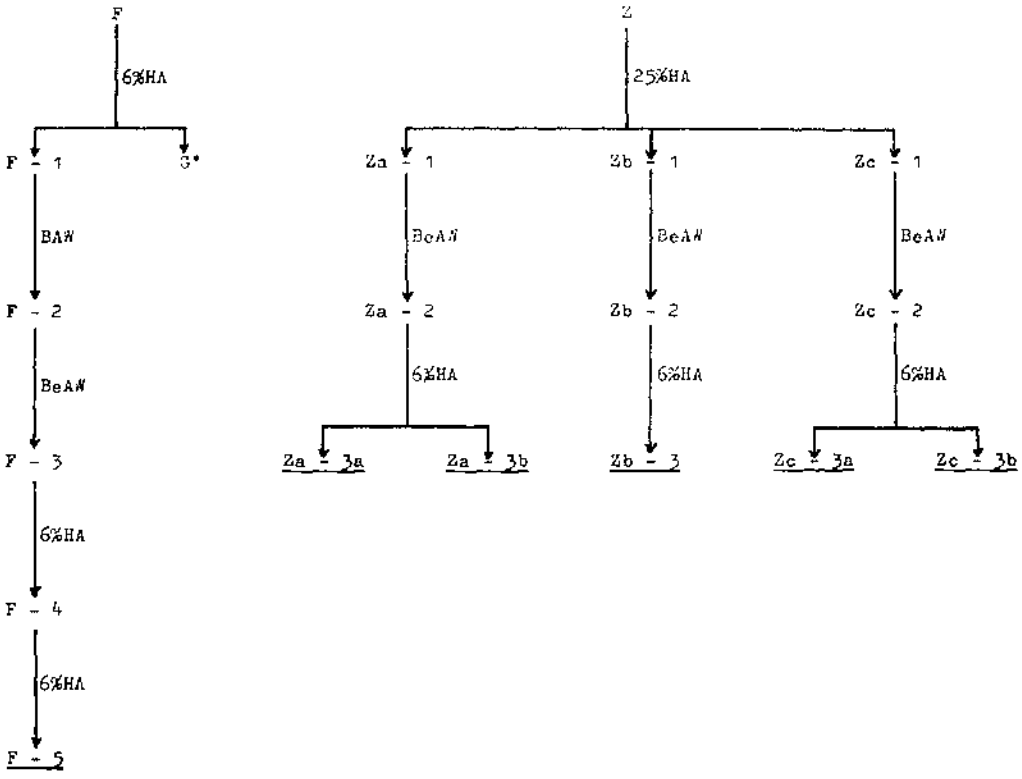
PAPER CHROMATOGRAPHIC PURIFICATION OF FRACTION G



See Figure 7 for details of abbreviations used.

Figure 10

PAPER CHROMATOGRAPHIC PURIFICATION OF FRACTIONS F AND Z



See Figure 7 for details of abbreviations used.

numbers record the number of times a fraction has been submitted alone, or previously in mixed form, to re-chromatography. Where a fraction was identified as being only a minor portion of a fraction already derived from another of the original seven fractions, then that minor portion (indicated by an asterisk) is given the identity of the major portion with which it was combined. The codes for the solvent systems are those recorded in Table 8. The fractions underlined were considered to be chromatographically pure and were submitted to the characterisation procedures. Before the final chromatographic purification step, the portion of each fraction which was used in the spectral studies was separated and chromatographed, in parallel to the rest of the fraction, on a separate sheet from which it could be eluted along with the required blank. Those terminal fractions not underlined were discarded on account of their low content of phenolic compounds.

All the fractions recorded in Figures 7-10 were recovered from the chromatographic strips in the manner described above for the original seven fractions, except that those from fraction Z were recovered in 95% ethanol instead of BAW. Preliminary tests showed that except for the compounds in fraction Z, more complete extraction of the phenolic compounds from the chromatography paper was obtained with the 60% ethanol/glacial acetic acid than with similar volumes of aqueous (60-90%) ethanol. For compounds from fraction Z, BAW was replaced with 95% ethanol since the former was difficult to remove completely in a rotary evaporator. Efficient recovery of the compounds from the chromatography paper was the aim, so that maximum amounts,

especially of the more minor phenolic compounds, would be available for characterisation. To check on the progress and efficiency of the extraction, pieces of the chromatography paper were removed, rinsed, dried and checked for the presence of phenolic compounds under ultraviolet light and with chromogenic reagents. Only traces of phenolic compounds were normally detected on the edges of the pieces of paper after the usual extraction times. The phenolic compounds of each fraction were extracted from the paper in very dilute solution because of the immersion technique employed and therefore, prior to re-chromatography, a concentrated solution of the compounds in a suitable solvent had to be prepared to minimise the time taken to apply each fraction to the chromatograms. Hence each fraction was dried in a rotary evaporator and then taken up in a minimum volume of 70% ethanol (95% ethanol for fraction Z compounds) which was conveniently banded on the chromatograms. While the relatively large volumes of the ethanol/glacial acetic acid which were required in the extraction technique were a disadvantage in the drying step, the efficient recovery of phenolic compounds obtained and the shortage of facilities for the descending elution of compounds from the intact chromatographic strips justified the technique used.

After fractions A-D inclusive and the fractions immediately derived from them had been re-chromatographed in 6%HA, it was apparent that the loading of the extract S/17-1b and some of the crude fractions had been excessively heavy and that initially poor fractionation of the phenolic compounds had resulted. Consequently a number of minor portions had to be combined with the main portions of certain fractions

after many of the early re-chromatographic steps in 6%HA. Combination of these portions was facilitated by the re-chromatography of several of the corresponding earlier fractions, derived in parallel from fractions A-D, on contiguous sections of the one chromatogram, rather than on separate chromatograms, in order that identity in R_F values would be directly determined.

The steps in the purification from fraction A of three fractions, representing spot 8c, are outlined in Figure 7. Repeated chromatography in 6%HA was necessary, because of excessive loading of the chromatograms at first, to obtain spot 8c as a sharp band, detectable in ultraviolet light and extracted as fraction A - 2. A weak band, detected similarly at higher R_F, was extracted with fraction Cbb - 2. Trace bands of other major phenolic compounds were noted in this separation but in keeping with the practice adopted throughout the fractionations, they were discarded. The occurrence of these trace bands pointed to the incomplete resolution of compounds in some chromatographic fractionations at the outset, where loadings were excessive. When chromatographed in BAW, fraction A - 2 was separated into three bands, detected under ultraviolet light, which were extracted as fractions Aa - 3, Ab - 3 and Ac - 3. The phenolic content of fraction Ab - 3, from a weak band, was low. Fraction Aa - 3 yielded fraction Aa - 6 after being chromatographed to remove firstly traces of spot 7b (detected with diazotised p-nitroaniline) and then any compounds of higher R_F in BAW. Chromatography in BEW removed dark brown material which had accumulated with the fraction. There was a tendency for material of this nature to accumulate in certain fractions. It tended to migrate

on the chromatograms along with the phenolic compounds especially when aqueous acetic acid was used and was retained at the origin when BEW and IpAW were employed. These results would be compatible with the derivation of the material from substances which were dissolved from the chromatography paper chiefly by the acetic acid component of the extracting solvent despite the use of washed papers. The purification of fraction Ab - 3 consisted of re-banding in the solvents indicated in Figure 7. For fraction Ac - 3, chromatography in BeAW separated traces of spot 9e which ran in a much elongated form in BAW and thus overlapped fraction Ac - 3. Re-banding of the fraction in BAW and in 6%HA completed the purification.

Fraction B was the source of the small amount of spot 7b which was isolated as fraction Ba - 7 (Figure 7). Most of the phenolics in fraction B were of spot 8c and the initial chromatography in 6%HA removed some of them. In these separations spot 7b was located with diazotised p-nitroaniline. Spots 7b and 8c were resolved in 6%HA, despite repeated chromatography being necessary, since a solvent which would give superior separation was not available. Fraction B - 2 yielded on chromatography two closely-spaced bands of similar colour in ultraviolet light. The lower R_F one was also found to contain spot 7b, and was extracted as fraction Ba - 3, while the other was recovered as fraction Bb - 3. Chromatography of this latter fraction in distilled water resolved two broad diffuse bands from which fractions identified with A - 2 and Dbc - 4 were obtained. For the resolution of these compounds this solvent was inferior to the weak acetic acid solutions with which compact bands were obtained. Further chromatography

commencing with fraction Ba - 3 removed spot 8c to leave finally a small amount of spot 7b in fraction Ba - 7.

In fraction C, spot 5d accounted for most of the phenolic substance present. Chromatography of the fraction in 6%HA (Figure 8) revealed only a broad yellow band with a red-brown leading edge, on inspection in ultraviolet light in the presence of ammonia. This band was cut lengthwise into three strips with the two at lower R_F each accounting for one-quarter of the width of the band. The extract from the strip at lowest R_F was combined with fraction A - 1. The middle strip yielded fraction Ca - 1 and the remaining strip, which contained the greater proportion of the phenolic compounds in fraction C and a very heavy loading of spot 5d, fraction Cb - 1. After fraction Ca - 1 had been chromatographed in 6%HA the band, which was yellow in ultraviolet light with ammonia, was found to overlap a weak band of spot 7b. Hence most of the yellow band was extracted and combined with fraction A - 2 while the remainder which gave the required positive reaction to diazotised p-nitroaniline was included in the strip which carried spot 7b and which was extracted and combined with fraction B - 2. Before fraction Cb - 1 was chromatographed, the bulk of the spot 5d substance which it contained was removed by crystallisation to constitute part of fraction Cba - 1. To accomplish this, fraction Cb - 1 was dried and taken up in a small volume of 95% ethanol to which three volumes of distilled water was added and the solution left to crystallise overnight at room temperature. The crude crystals were then recovered as fraction Cba - 1. This crystallisation step provided a most convenient alternative to chromatography for the separation of

the bulk of spot 5d substance which was by far the most abundant phenolic compound in the tissue extract. The mother liquor from the crystallisation was chromatographed in 6%HA as fraction Cb - 1. Two discrete bands of identical colour reaction were located and the extract from the one at lower R_F was combined with fraction A - 2 while the extract of the other constituted fraction Cbb - 2. The repeated separation of portions of fractions A - 1 and A - 2 during these fractionations reflected the excessive loading of fraction C and the associated effects on the resolution of the constituent compounds into bands. Fraction Cbb - 2 when chromatographed in BAW was separated into three discrete bands of similar colour in ultra-violet light. While the lowest R_F band contained spot 6a and the highest R_F band contained spot 6d and traces of spot 5d, the middle band was of low intensity and did not correspond to any of the spots of Figure 6. The extracts from these three bands were combined with their corresponding main fractions, namely, Dba - 3, Dbb - 3 and Dbc - 3.

Fraction D contained a complement of phenolic compounds similar to fraction C. Before fraction D was chromatographed (Figure 8) much of the spot 5d substance was removed by crystallisation as described previously for fraction Cb - 1. The crude crystals were added to fraction Cba - 1 which, when complete, was dried in a desiccator over silica gel and weighed (0.33 gm). After the crude crystalline product had been purified by re-crystallisation from aqueous ethanol three times, a sample was washed in cold distilled water before being dissolved in 70% ethanol and chromatographed as fraction Cba - 1. The

chromatographic purity of this fraction in three solvents (Figure 8) was noted during the preparation from it of fraction Cba - 4 for characterisation. As with fraction Cb - 1, the mother liquor from the crystallisation constituted the form in which fraction D was chromatographed in 6%HA to reveal a broad band which was yellow in ultraviolet light with ammonia except for the red-brown leading edge. This band was cut lengthwise into two strips from which fractions Da - 1 and Db - 1 were obtained. From the former fraction chromatography in 6%HA separated two bands, the lower R_F one being extracted and combined with fraction A - 2 while the other was extracted and combined with fraction Cbb - 2. Fraction Db - 1 yielded a small crystalline deposit (fraction Cba - 1) when it was treated as described for fraction Cb - 1. Chromatography of the mother liquor in 6%HA yielded a broad band which was extracted as fraction Db - 2. From the three bands of similar colour in ultraviolet light which were resolved when fraction Db - 2 was chromatographed in BAW, fractions Dba - 3, Dbb - 3 and Dbc - 3 were recovered. Spot 6a was contained in fraction Dba - 3 while spot 6d and a small amount of spot 5d were present in fraction Dbc - 3. Fraction Dbb - 3 contained little phenolic material. Purification of fraction Dba - 3 was accomplished by chromatography in 6%HA and in BAW to yield fraction Dba - 6 for characterisation. While fractions Dba - 3 to Dba - 6 always chromatographed as a compact band in these solvents it was noted that the band obtained on chromatography in BAW fluoresced a brighter yellow in the leading than in the tailing portion when viewed in the presence of ammonia in ultraviolet light. After chromatography of fraction Dbb - 3 in 6%HA, fraction Dbb - 4 was

discarded due to the presence of insufficient material to warrant further purification, but not before spots were chromatographed on No. 1 paper and indications obtained of the presence of a component with the colour reactions of spot 6a and R_F values which would place it between spots 6a and 6d in Figure 6. Contaminating amounts of spot 5d were located at the leading edge of the band when fraction Dbc - 3 was chromatographed in distilled water and were best detected in the presence of spot 6d with the aluminium chloride reagent. With this reagent spot 6d appeared yellow and spot 5d an intensely fluorescent blue-green in ultraviolet light. In an attempt to separate the traces of spot 5d, fraction Dbc - 4 was chromatographed in BeAW but the amount of tailing of spot 5d was sufficient to prevent clear separation despite the development of the chromatogram for nine hours. In a search for a more satisfactory solvent system for this purpose the solvents in Table 12 were tested on a pilot scale using strips of No. 1 paper and a small chromatography tank. Authentic phloridzin and quercitrin were spotted on the test strips since at this stage the probable identity of spot 5d and 6d as phloridzin and quercitrin respectively was indicated by a comparison of the properties of the known compounds with those of the fractions representing the two spots. In only the two alkaline solvents, isopropanol-ammonia-water and n-butanol-2N ammonia, were the R_F values of phloridzin and quercitrin sufficiently different and the spots compact enough for these compounds to be separated. The R_F values were always sufficiently similar in the selection of both acidic and neutral solvents tested to produce a zone in which the two compounds overlapped.

Table 12

SOLVENT SYSTEMS TESTED FOR SEPARATION OF PHLORIDZIN AND QUERCITRIN ON PAPER CHROMATOGRAMS

Components	Proportions*
Ethyl acetate-acetic acid-water	50:2:50
Ethyl acetate-acetic acid-water	3:3:1
Acetone-water	1:1
n-Butanol-water	Saturated
Ethanol-acetic acid-water	40:13:30
Ethanol-water	1:3
n-Butanol-ethanol-water	4:1:2.2
n-Butanol-pyridine-water	14:3:3
Isopropanol-ammonia(s.g. 0.91)-water	20:1:2
n-Butanol-2N ammonia	1:1

* Where two layers formed, the upper organic one was used.

Thus fraction Dbc - 5 was chromatographed in the isopropanol-ammonia-water solvent with good results. The main band at low R_F was extracted as fraction Dbc - 6 to leave behind a faint band of spot 5d at higher R_F. Repeat chromatography of fraction Dbc - 6 in this solvent resulted in no spot 5c being detected ahead of the compact band extracted as fraction Dbc - 7. When fraction Dbc - 7 had been rebanded in 6%HA and in BAW it was extracted and characterised as fraction Dbc - 9.

On chromatography in BAW fraction G was resolved into four contiguous bands which were detected under ultraviolet light and extracted

as the four fractions, I - 1, J - 1, K - 1 and L - 1 (Figure 9). In this chromatography of fraction G, some water soluble non-phenolic substances which were present near the solvent front when the S/17-1b extract was chromatographed in 6%HA and which were recovered in the fraction, appeared in the region R_F 0.20-0.30 but the amounts were not sufficient to obviously hinder the migration of the phenolic compounds to higher R_F. However, had the leaf tissue extract not been fractionated initially by chromatography in 6%HA, some preliminary separation of the water soluble sugars, etc. from the phenolic substances prior to chromatography in BAW to separate the above four fractions would probably have been advantageous. Fraction I - 1 was extracted from the lowest R_F band of phenolics, which was invisible in ultraviolet light alone and which fluoresced light blue when ammonia was introduced. From the band, which was a light blue in ultraviolet light and a green-blue when ammonia vapour was present, fraction J - 1 was obtained, while fraction K - 1 was extracted from the next band which exhibited the same colour reactions only more intensely. Colour reactions of the band at highest R_F, from which fraction L - 1 was obtained, were identical to those of the lowest R_F band.

Two closely spaced bands were apparent when fraction I - 1 was chromatographed in BAW. In ultraviolet light, the band which yielded fraction Ia - 2 was a dull dark blue which turned a bright blue on exposure to ammonia vapour, while the higher R_F band, corresponding to fraction Ib - 2, was a dull light blue in ultraviolet light and a bright green-blue when ammonia vapour was present. Fraction Ia - 2 corresponded to spot 1a and fraction Ib - 2 to spots 1b and 2b. When

fraction J - 1 was similarly chromatographed, the same two bands were again detected and on extraction the lower R_F one was added to fraction Ia - 2 and the other to fraction Ib - 2. Fraction Ia - 3, extracted from the broad band which resulted from the chromatography of fraction Ia - 2 in BeAW, was chromatographed in BAW and the compact band extracted as fraction Ia - 4. This fraction chromatographed in 6%HA as a broad band at high R_F which was extracted and chromatographed in BAW as fraction Ia - 5 to produce the expected band and fraction Ia - 6 for characterisation. When both fractions Ia - 3 and Ia - 5 were chromatographed in BAW, in addition to the main band at lower R_F, a faint band, with the colour reactions of the main band, was observed at higher R_F in the location of fraction L - 1. Particularly in the case of fraction Ia - 5 this was not thought to be a further separation of higher R_F compounds in view of not only the earlier chromatography in which they should have been separated but also the absence of traces of all intermediate bands. Fraction Ib - 2, after further chromatography, was discarded as fraction Ib - 4 on account of its low phenolic content.

When fraction K - 1 was chromatographed in BAW, two main bands which overlapped slightly were noted. The lower R_F band which was dull blue in ultraviolet light and green-blue in the presence of ammonia was recovered as fraction K - 2 while from the other band, which was bright blue in ultraviolet light in the presence of ammonia, a minor portion of fraction L - 2 was obtained. Fraction K - 2 represented spot 1c and any of spot 3c which was present. A broad band was obtained, together with a faint band at lower R_F, when fraction K - 2 was chromatographed in BeAW. The chromatogram was developed for nine hours, dried and

inspected before being developed for a further seven hours to improve the separation of the lower R_F band. Fraction K - 3 from this major band was chromatographed in BAW and the main band recovered as fraction K - 4 while the minor contiguous band at higher R_F was discarded. Chromatography again in BeAW separated the main band of fraction K - 4 from a weak lower R_F band which corresponded in its faint blue colour reaction in ultraviolet light with ammonia to the minor contiguous band of the previous separation in BAW. This main band, as fraction K - 5, was chromatographed in BAW to reveal only a compact band recovered for characterisation as fraction K - 6.

Fraction L - 1 chromatographed in BAW as a broad band which fluoresced blue in ultraviolet light in the presence of ammonia and which, as fraction L - 2, chromatographed similarly in BeAW. However, when a lighter loading was used, fraction L - 3 which corresponded to spots 1d and 2d, was resolved by chromatography in BAW into two adjoining bands, the main one at lower R_F being retained as fraction La - 4 and the other as fraction Lb - 4. Following chromatography in BeAW for 18 hours fraction La - 4 appeared as a broad band which was designated as fraction La - 5 except for the weak leading portion discarded because of possible contamination with components of fraction K - 5. In the first of the final steps (Figure 9) in the purification of fraction La - 5, traces of a substance of higher R_F, corresponding to fraction Lb - 5, were removed. Purification of fraction Lb - 4 by chromatography revealed traces of lower R_F substance corresponding to fraction La - 5 after the first but not the second development in BAW.

It was not until the completion of the separations in Figure 9

that the value of the BEW solvent for the resolution of the substances in the fractions was appreciated. This solvent gave good separation of certain of these fractions as compact spots or bands. The solvent would have been a most efficient addition to the BeAW and BAW solvents used in the preparative procedures of Figure 9.

When samples of fractions Ia - 6, K - 6, La - 8 and Lb - 6 were chromatographed in 6%HA on No. 1 paper, two distinct spots of identical colour reaction were observed at high R_F for fraction Lb - 6 while only one elongate spot was noted with each of the other fractions. Since fraction Lb - 6 exhibited the properties characteristic of a certain type of hydroxycinnamic acid conjugate, the occurrence of two spots on chromatography in 6%HA was considered to be the result of isomerisation (69) rather than of the presence of different substances.

The purification of fraction F as fraction F - 5, corresponding to spot 4b, is outlined in Figure 10. After the chromatography of fraction F in 6%HA, the band located with diazotised p-nitroaniline was designated as fraction F - 1 while the band, visible at higher R_F in ultraviolet light, was combined with fraction G. Fraction F - 1 was purified as shown in the Figure to remove traces of substances which fluoresced in ultraviolet light.

Fraction Z, on chromatography in 25%HA (Figure 10), was resolved into three bands. Two of these were light blue in ultraviolet light in the presence of ammonia while the third, which occupied most of the space between these blue bands, was detected with diazotised p-nitroaniline. The lower R_F blue band was recovered as fraction Za - 1 and the higher R_F one as fraction Zc - 1, while the middle band was extracted

as fraction Zb - 1.

Chromatography of fraction Za - 1 in BeAW removed traces of the lower R_F substance of fraction Zb - 1 from the broad main band which was then resolved by chromatography in 6%HA into two similar compact bands identified as fractions Za - 3a (spot 11e) and Za - 3b (spot 9e). Identical chromatographic procedures were applied to fraction Zc - 1 to obtain bands in the same positions and of the same appearance as those from fraction Za - 1. Thus fractions Zc - 3a and Zc - 3b, apparently identical to Za - 3a and Za - 3b respectively, were finally obtained. From these results it became apparent that the substance in spots 9e and 11e was interconvertible between spots when chromatographed in aqueous acetic acid and that in a non-aqueous solvent the four Za - and Zc - fractions would chromatograph as one. Fraction Zb - 1 was freed from traces of other substances by chromatography in BeAW and the compact band detected with diazotised p-nitroaniline was recovered as fraction Zb - 2 which was finally purified by chromatography in 6%HA.

An assessment of the relative abundance of the phenolic compounds in certain fractions which were isolated from the leaf tissue extract by the procedures described above is recorded in Table 13. Because of the losses involved in these fractionations the weights of compounds in the fractions would bear no fixed relationship to the amounts present in the extract and were therefore not determined.

4.5 Characterisation of Fractions Isolated from Extract S/17-1b

The 17 fractions underlined in Figures 7-10 were those on which

Table 13

RELATIVE AMOUNTS OF PHENOLIC COMPOUNDS IN FRACTIONS FROM EXTRACT S/17-1b

Fraction	Relative content of phenolic substances	Source Spot (Figure 6)
Cba - 1	bulk of total	5d
Dbc - 9	x x x x x x	6d
Aa - 8	x x x x x x	8c
Ac - 7	x x x x	8c
Dba - 6	x x x	6a
Ab - 6	x x	8c
Ia - 6	x x	1a
La - 8	x x	1d
Lb - 6	x	1d and 2d
Za - 2	x	11e
Zb - 2	x	10e
Zc - 2	x	9e
K - 6	x	1c
F - 5	trace	4b
Ba - 7	trace	7b

See Figures 7-10 for details of the derivation of fractions.

characterisation studies were undertaken. The results of the first step in characterisation, an investigation of the chromatographic properties of the fractions and of the reference compounds, are presented in Table 14. Besides the R_F values recorded, evidence was obtained of the chromatographic purity of each fraction in the solvents used. The selection of the six solvents used was complicated by the wide range of properties of the compounds to be chromatographed. Both acidic and neutral systems of various polarities were chosen in order that the R_F value of each fraction would differ considerably between certain solvents and that concurrently the chromatographic purity of the fraction would be effectively investigated. Most fractions and reference compounds chromatographed as spots of satisfactory dimensions except in the EAW where many compounds streaked badly. Where this occurred the R_F values are not presented (Table 14). The fractions identified as flavonol glycosides chromatographed as much elongated spots in BeAW while fractions Ia - 6, K - 6, La - 8 and Lb - 6 chromatographed as very compact spots in BEW.

Colour reactions of fractions and reference compounds on the developed chromatograms were determined with the results recorded in Table 15.

Samples of the six fractions which contained flavonoid glycosides were acid hydrolysed and the chromatographic properties (Table 16) and colour reactions (Table 17) of the aglycones obtained were determined along with those of selected reference compounds. Streaking of the flavonoid aglycones occurred in the BEW while satisfactory spot dimensions were obtained in the other systems. Results of the chromatographic

identification of the sugars from these acid hydrolysed samples are presented in Table 18.

The chromatographic and colour reaction data obtained on the phenolic moieties released by alkali hydrolysis of samples of four fractions and on selected reference compounds are presented in Tables 19 and 20.

In Table 21 the data derived from the ultraviolet absorption spectra of fractions and reference compounds are summarised. In addition, the data for the aglycone released on acid hydrolysis of fraction Aa - 8 are presented together with the data for quercetin. The phenolic compounds obtained on the acid or alkali hydrolysis of the other fractions were not examined spectrophotometrically. Many of the absorption spectra which were recorded are reproduced in Figures 11-31. The spectra were determined in 95% ethanol which was a satisfactory solvent for all reference compounds and fractions, except fraction Ia - 6, and also for the reagents at the concentrations required. Spectra of fraction Ia - 6 were determined in 70% ethanol which was used, in place of the ineffective 95% ethanol, to elute the sample of this fraction from the chromatographic strip.

The results presented in Tables 14-21 permitted the phenolic compounds in certain fractions to be identified. Thus phloridzin (phloretin-2'-glucoside) was identified from fraction Cba - 4 on the basis of a comparison of R_F values, colour reactions (Tables 14 and 15) and spectral properties (Table 21, Figures 11, 12) of the fraction and of authentic phloridzin. Confirmation of this identification was

Table 14

PAPER CHROMATOGRAPHIC PROPERTIES OF FRACTIONS OBTAINED FROM EXTRACT 2/'7-1[illegible]

Fractions and ref. compds.	R_F values in solvent systems*†					
	BAW	6%HA	40%HA	BEW	5cAW	2AW
Aa - 8	0.65	0.24	0.66	0.65	0.22	
Ab - 6	0.72	0.20	0.65	0.69	0.21	
Ac - 7	0.80	0.23	0.66	0.79	0.23	
Dba - 6	0.56	0.40	0.76	0.52	0.34	
Rutin	0.52	0.39	0.75	0.44	0.20	
Dbc - 9	0.80	0.36	0.74	0.79	0.28	
Quercitrin	0.80	0.35	0.74	0.79	0.28	
Cba - 4	0.80	0.46	0.79	0.81	0.35	
Phloridzin	0.79	0.46	0.79	0.81	0.34	
K - 6	0.68	0.78	0.87	0.64	0.44	0.18
Chlorogenic acid	0.64	0.64-0.78	0.80	0.20	0.25	0.00
La - 8	0.74	0.83	0.86	0.74	0.34	0.21
Lb - 6	0.78	0.71-0.83	0.87	0.32	0.42	0.00
Ia - 6	0.51	0.92	0.88	0.11	0.20	0.00
Za - 3a	0.92	0.05-0.16	0.58-0.78	0.92	0.62	
Za - 3b	0.93	0.04-0.15	0.58-0.77	0.92	0.62	
Zc - 3a	0.93	0.04-0.14	0.56-0.77	0.92	0.61	
Zc - 3b	0.93	0.04-0.14	0.56-0.77	0.92	0.61	
Zb - 3	0.93	0.12	0.60	0.94	0.48	
Phloretin	0.93	0.13	0.61	0.94	0.49	
F - 5	0.61			0.65	0.08	
d-Catechin	0.68			0.75	0.07	
l-Epicatechin	0.59			0.66	0.05	

* The R_F values of F - 5, d-catechin and l-epicatechin were determined at a different time from the rest of the R_F values in the Table and are therefore not directly comparable with them.

† For details of solvent systems see Table 8.

Table 15

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF FRACTIONS OBTAINED FROM EXTRACT S/17-1b

Fractions and ref. compds.	Colour reactions under given treatments*							
			5% Na_2CO_3		1% $AlCl_3$			
	uv	uv + NH_3	light	uv	light	uv	dp-na	dsa
Aa - 8	P	Y	Y	T	bY	Y	lBr	Y-T
Ab - 6	P	Y	Y	T	bY	Y	lBr	Y-T
Ac - 7	P	Y	Y	T	bY	Y	lBr	Y-T
Dba - 6	P	Y	Y	T	bY	Y	lBr	Y-T
Rutin	P	Y-T	Y	T	bY	Y	lBr	Y-T
Dbc - 9	P	Y-T	Y	T	bY	Y	lBr	Y-T
Quercitrin	P	Y-T	Y	T	bY	Y	lBr	Y-T
Cba - 4	Br	R-Br	N	Br	N	B-G	O-Br	O-Br
Phloridzin	Br	R-Br	N	Br	N	B-G	O-Br	O-Br
K - 6	Gr-B	bB-G	N	G-B	N	lB	-	-
Chlorogenic acid	Gr-B	bB-G	N	Gr-G	N	B-W	-	-
La - 8	N	bB	N	B	N	N	-	R-Pi
Lb - 6	N	B	N	B	N	N	-	R-Pi
Ia - 6	N	bB	N	bB	N	N	-	R-Pi
Za - 3a	N	lB	N	B	N	fGr-B	lBr	lBr
Za - 3b	N	lB	N	B	N	fGr-B	lBr	lBr
Zc - 3a	N	lB	N	B	N	fGr-B	lBr	lBr
Zc - 3b	N	lB	N	B	N	fGr-B	lBr	lBr
Zb - 3	N	N	N	N	N	N	O-Br	Br
Phloretin	N	N	N	N	N	N	O-Br	Br
F - 5	-	-	-	-	-	-	O-Br	O
d-Catechin	-	-	-	-	-	-	O-Br	Y
l-Epicatechin	-	-	-	-	-	-	O-Br	Y

* Codes for the colours are: blue (B), green (G), white (W), grey (Gr), brown (Br), purple (P), red (R), yellow (Y), tan (T), orange (O), pink (Pi), colourless (N), not determined (-), light (l), bright (b) and faint (f).

Abbreviations for the treatments are: ultraviolet light, uv; ultraviolet light in presence of ammonia vapour, uv + NH_3; natural daylight, light; diazotised p-nitroaniline, dp-na; diazotised sulphanilic acid, dsa.

See Table 10 for details of chromogenic sprays.

Table 16

PAPER CHROMATOGRAPHIC PROPERTIES OF THE AGLYCONES OBTAINED FROM THE ACID HYDROLYSIS OF FRACTIONS

Fractions and ref. compds.	R_F values in solvent systems*				
	BAW	6%HA	40%HA	BEW	BeAW
Aa - 8	0.75	0.02	0.21	0.75	0.25
Ab - 6	0.74	0.02	0.19		0.24
Ac - 7	0.75	0.02	0.20		0.24
Dba - 6	0.88	0.03	0.25	0.81	0.23,0.62
Dbc - 9	0.74	0.02	0.19		0.23
Quercetin	0.75	0.02	0.20	0.73	0.24
Kaempferol	0.91	0.03	0.31	0.89	0.55
Cba - 4	0.94	0.15	0.61	0.94	0.49
Phloretin	0.93	0.13	0.61	0.94	0.50

* For details of solvent systems see Table 8.

Table 17

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF THE AGLYCONES OBTAINED FROM THE ACID HYDROLYSIS OF FRACTIONS

Fractions and ref. compds.	Colour reactions under given treatments*							
	uv	uv + NH_3	5% Na_2CO_3 light	5% Na_2CO_3 uv	1% $AlCl_3$ light	1% $AlCl_3$ uv	dp-na	dsa
Aa - 8	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Ab - 6	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Ac - 7	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Dba - 6	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Dbc - 9	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Quercetin	Y	Y	Y	Y	Y-G	bY-G	Br	Y-T
Kaempferol	lY	Y-G	Y	Y	Y-G	bY-G	Br	Y-T
Cba - 4	N	N	N	N	N	N	O-Br	Br
Phloretin	N	N	N	N	N	N	O-Br	Br

* See Table 15 for details of codes for colour reactions and treatments.

Table 12

PAPER CHROMATOGRAPHIC PROPERTIES OF THE SUGARS OBTAINED FROM THE ACID HYDROLYSIS OF FRACTIONS

Fractions and ref. compds.	R_F values in solvent systems*				Colour with p-anisidine hydrochloride
	EaPW	EaAW	IpBW	IpW	
Aa - 8	0.32	0.24	0.34	0.44	Yellow
Cba - 4	0.32	0.24	0.34	0.44	Yellow
D-glucose	0.32	0.24	0.35	0.44	Yellow
D-galactose	0.29	0.25	0.33	0.42	Yellow
D-glucose and D-galactose	0.32	0.26	0.35	0.44	Yellow
Dba - 6	0.32,0.53	0.23,0.43	0.34,0.52	0.44,0.59	Yellow, tan
Dbc - 9	0.54	0.43	0.52	0.58	Tan
L-rhamnose	0.55	0.43	0.53	0.60	Tan
Ab - 6	0.43	0.33	0.45	0.52	Pink
D-xylose	0.45	0.33	0.45	0.51	Pink
Ac - 7	0.37	0.30	0.39	0.47	Pink
L-arabinose	0.38	0.30	0.41	0.47	Pink

* See Table 9 for code and details of solvent systems.

Table 19

PAPER CHROMATOGRAPHIC PROPERTIES OF PHENOLIC MOIETIES OBTAINED FROM ALKALI HYDROLYSIS OF FRACTIONS

Fractions and ref. compds.	R_F values in solvent systems*		
	BeAW	BAW	6%HA
K - 6	0.78	0.89	0.40,0.66
Sinapic acid	0.77	0.88	0.46,0.61
Caffeic acid	0.57	0.86	0.35,0.66
La - 8	0.64	0.90	0.47,0.73
Lb - 6	0.63	0.90	0.47,0.73
Ia - 6	0.64	0.91	0.46,0.72
p-Coumaric acid	0.65	0.91	0.48,0.73

* For details of solvent systems see Table 8.

Table 20

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF PHENOLIC MOIETIES OBTAINED FROM ALKALI HYDROLYSIS OF FRACTIONS

Fractions and ref. compds.	Colour reactions under given treatments*				
	uv	uv + NH_3	dba	dp-na	Hoepfner reagent
K - 6	lB	lB	P-Pi	Pi-Br	Pi-Br
Sinapic acid	B-W	B-G	P-Pi	Pi	lBr
Caffeic acid	lB	B	B-Br	lBr	Pi-Br
La - 8	W	B	W	Br	Y-T
Lb - 6	W	B	W	Br	Y-T
Ia - 6	W	B	B	Br	Y-T
p-Coumaric acid	W	B	B	Br	Y-T

* See Table 15 for details of codes for colour reactions and treatments.

Table 21

SPECTRAL PROPERTIES OF FRACTIONS

Fractions and ref. compds.	Wavelength of absorption maxima in 95% ethanol (mμ)*				
	Alone	0.6% $AlCl_3$ added	NaOH added	Satd. with NaOAc	H_3BO_3 and NaOAc added
Aa - 8	257,(270),361	268,(368),400	281, 405	272, 381	264, 381
Ab - 6	257,(270),359	270,(365),401	278, 408	275, 391	265, 384
Ac - 7	257,(270),357	271,(361),400	275, 411	274, 389	264, 382
Dba - 6	256,(268),359	269,(365),400	281, 410	270, 402	267, 385
Rutin	258,(269),361	271,(368),400	278, 411	271, 402	265, 386
Dbc - 9	258,(265),351	270,(355),398	273, 406	270, 372	262, 372
Quercitrin	259,(265),352	271,(355),398	274, 406	271, 373	264, 374
Cba - 4	285	286	(243),327	326	285,(325)
Phloridzin	284	286	(243),326	325	285,(323)
K - 6	329	327	392	331	331
Chlorogenic acid	327	331	377	330	351
La - 8	317	318	373	319	318
Lb - 6	314	314	367	314	314
Ia - 6*	313	315	364	313	313
Zc - 3a	291,(315)	309	331, 376	(295),325	(293),320
Zb - 3	287		240, 322		289
Phloretin	287		240, 322		289
? - 5	285	286	(243),326	287, 323	286, 321
Ba - 7	286	289	decomp.	(290),326	288,(325)
Aglycone					
Aa - 8	255,(270),373	267,(358),428	decomp.	decomp.	261, 392
quercetin	256,(270),373	265,(360),430	decomp.	decomp.	261, 391

* The wavelengths of secondary peaks and shoulders are given in brackets.
See Chapter 3 for details of the determination of spectra and the use of reagents.

* Spectra of fraction Ia - 6 were determined in 70% ethanol.

Figure 11: Absorption Spectra of Fraction Cba - 4

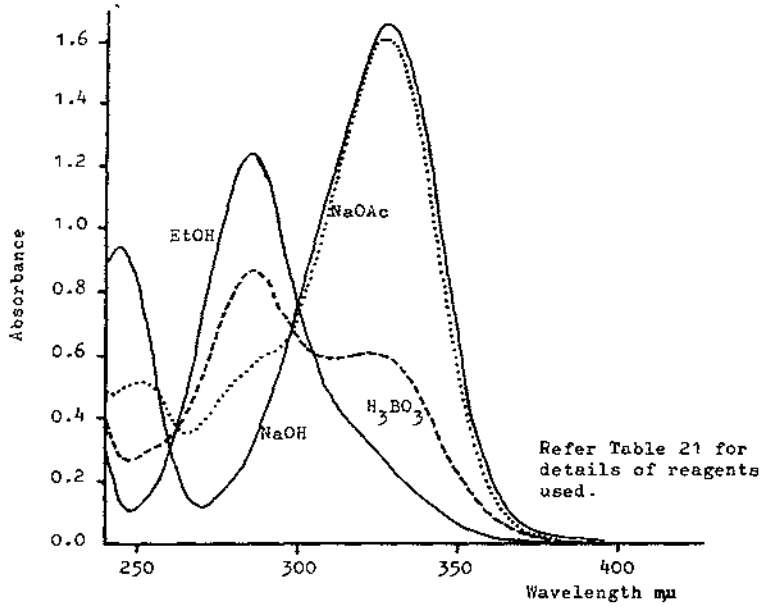


Figure 12:
Absorption Spectra of Phloridzin

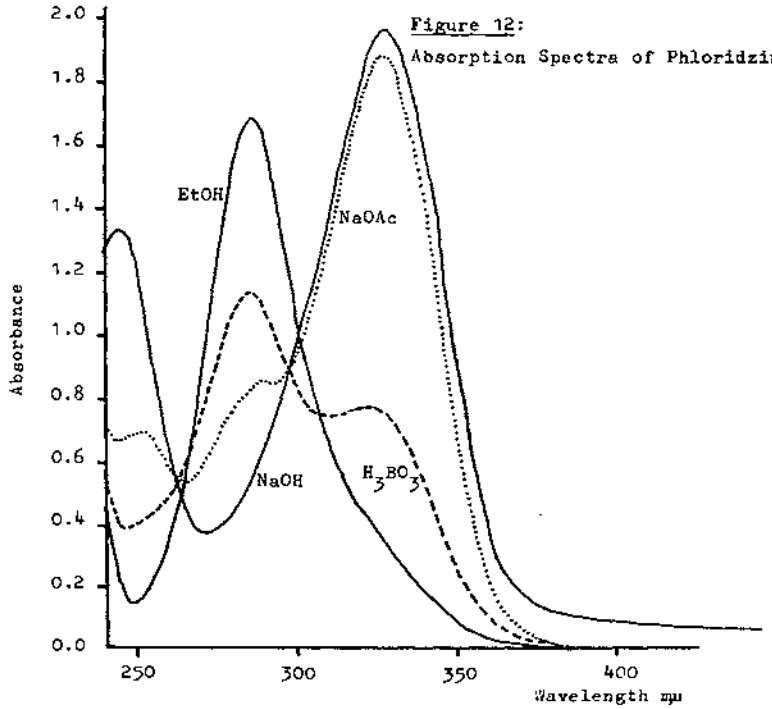


Figure 13: Absorption Spectra of Aglycone of Fraction Aa - 8

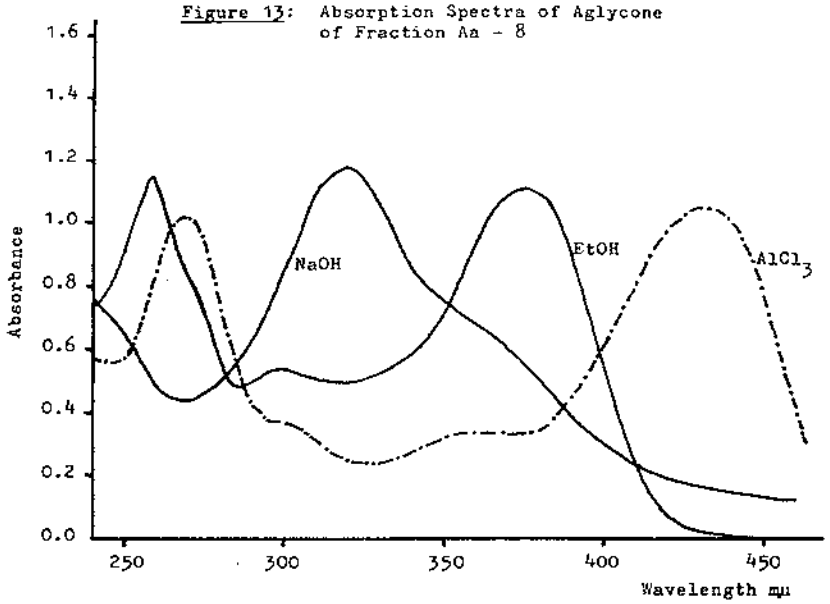


Figure 14: Absorption Spectra of Quercetin

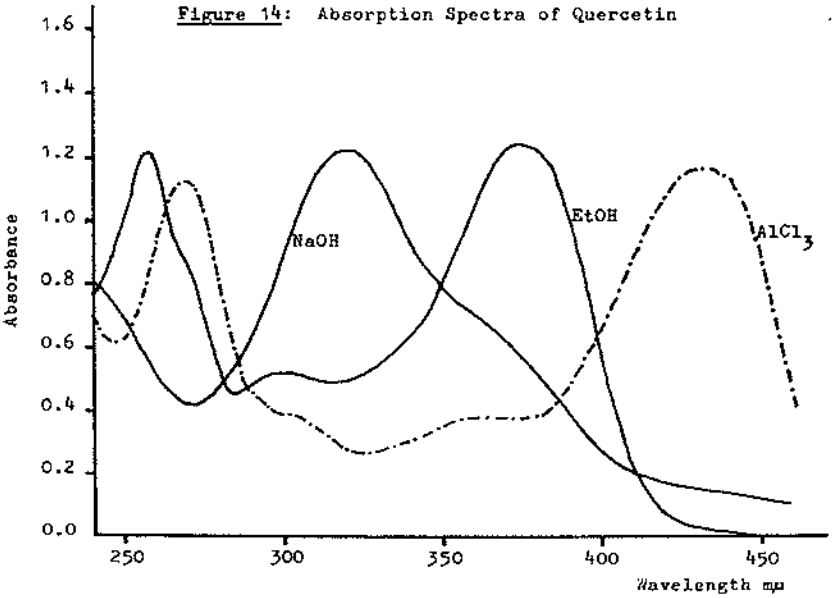


Figure 15: Absorption Spectra of Fraction Aa - 8

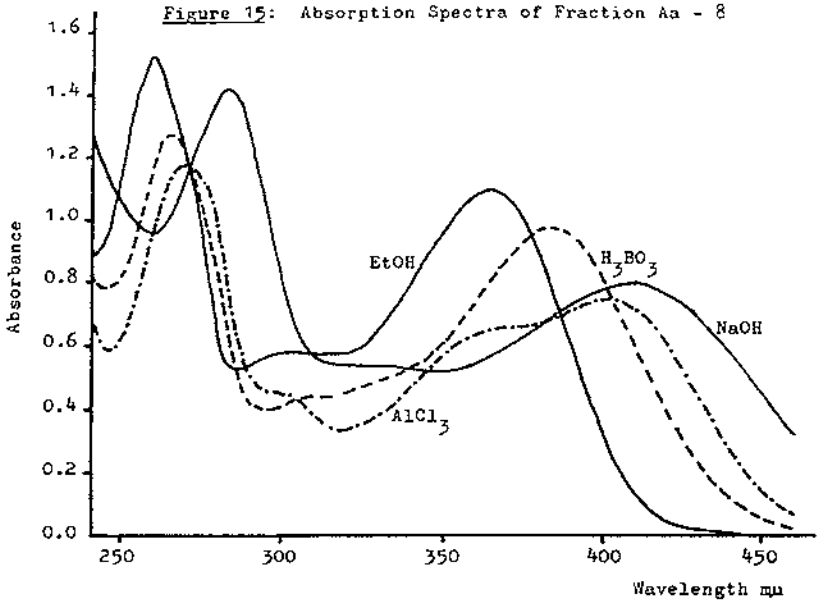


Figure 16: Absorption Spectra of Fraction Ab - 6

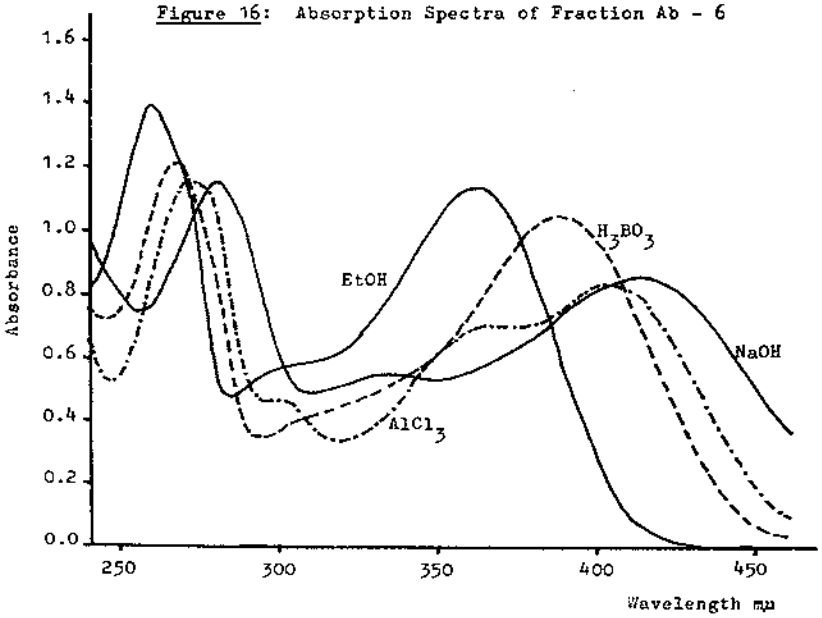


Figure 17: Absorption Spectra of Fraction Dbc - 9

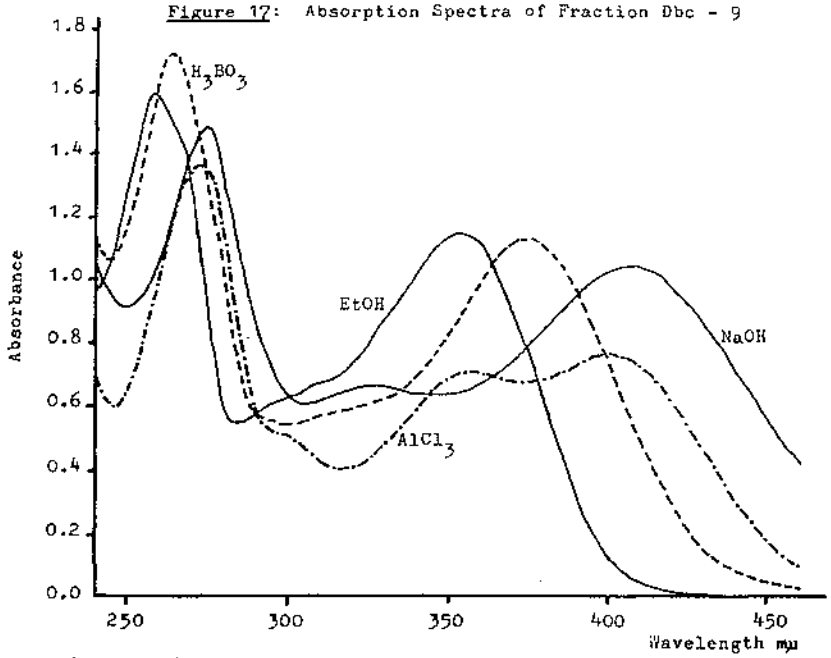


Figure 18: Absorption Spectra of Quercitrin

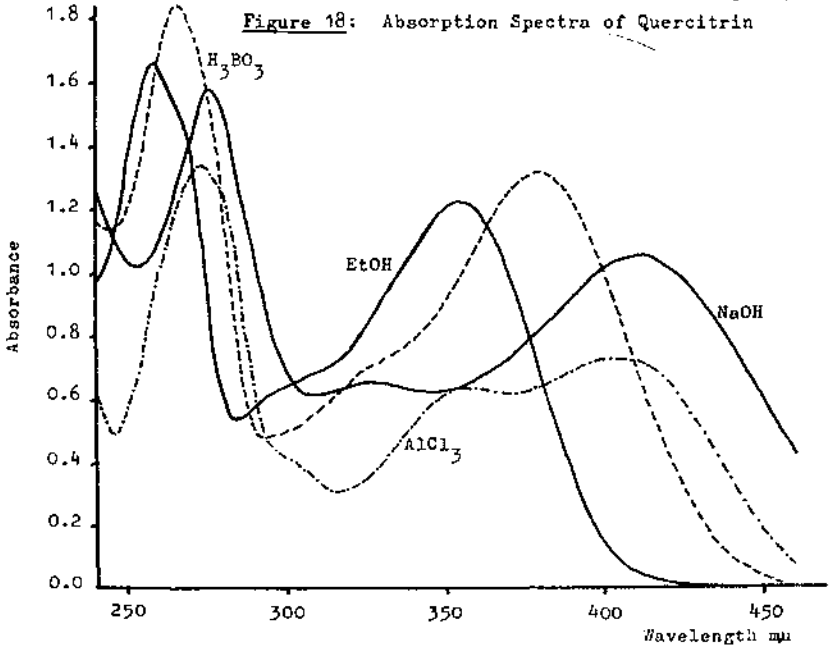


Figure 19: Absorption Spectra of Fraction Dba - 6

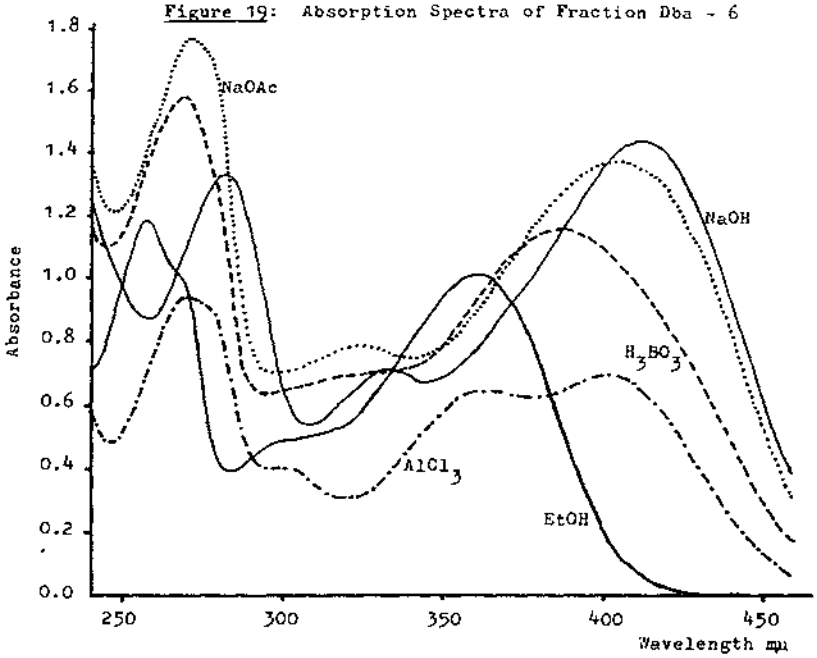
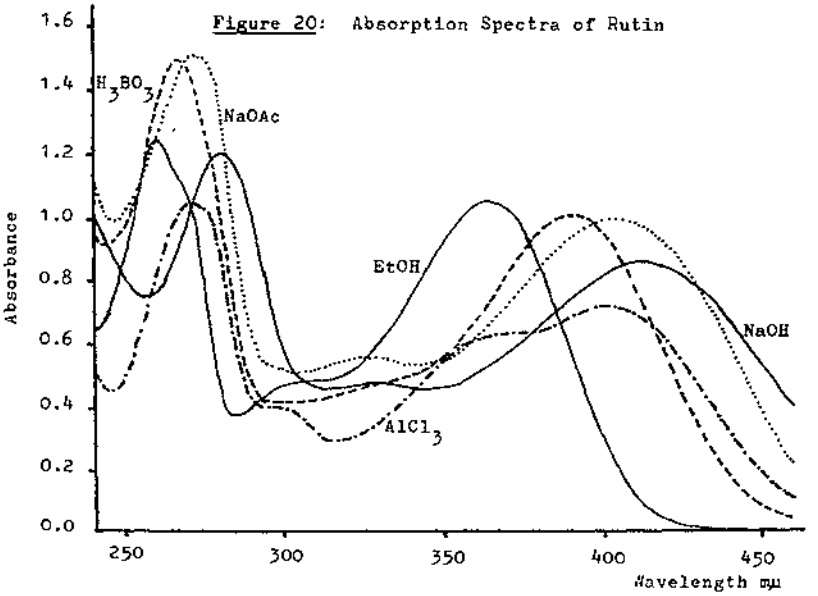


Figure 20: Absorption Spectra of Rutin



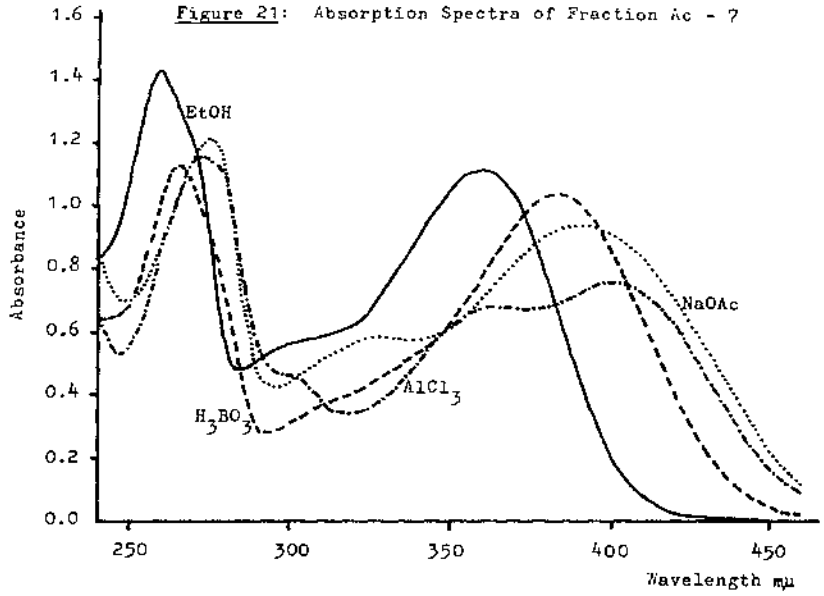


Figure 21: Absorption Spectra of Fraction Ac - 7

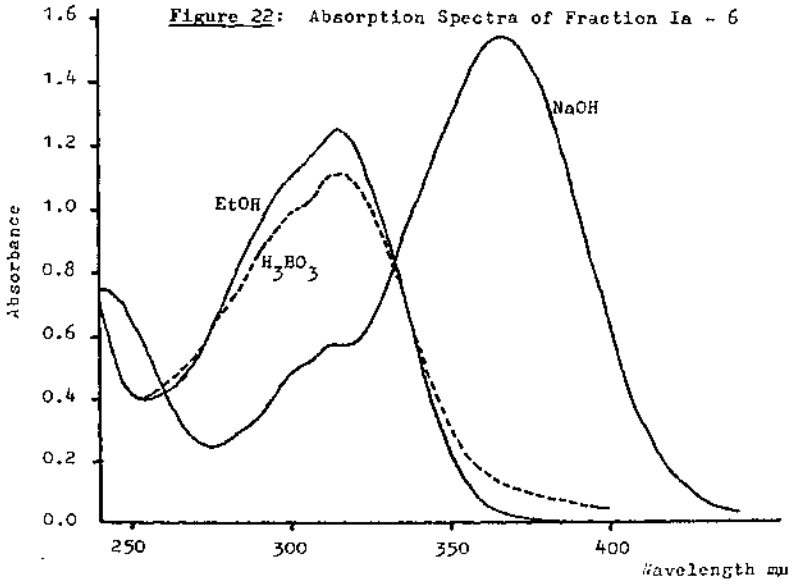


Figure 22: Absorption Spectra of Fraction Ia - 6

Figure 23: Absorption Spectra of Fraction La - 8

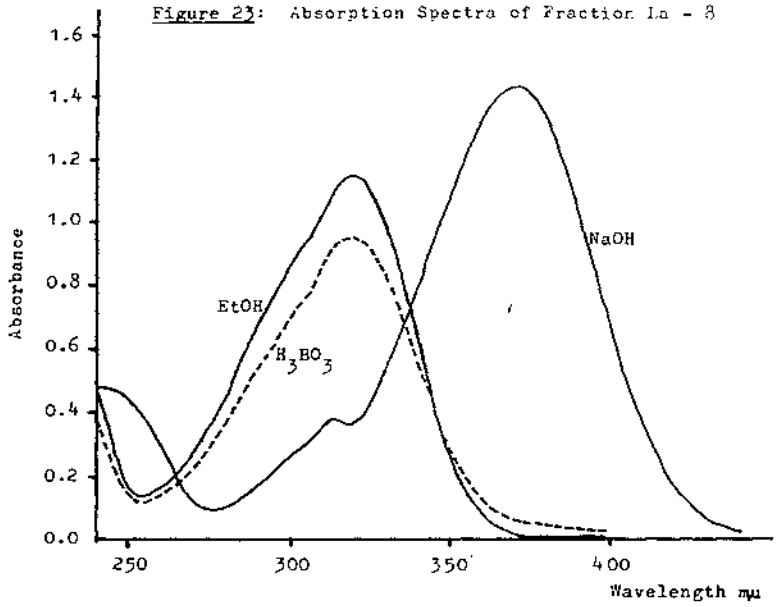
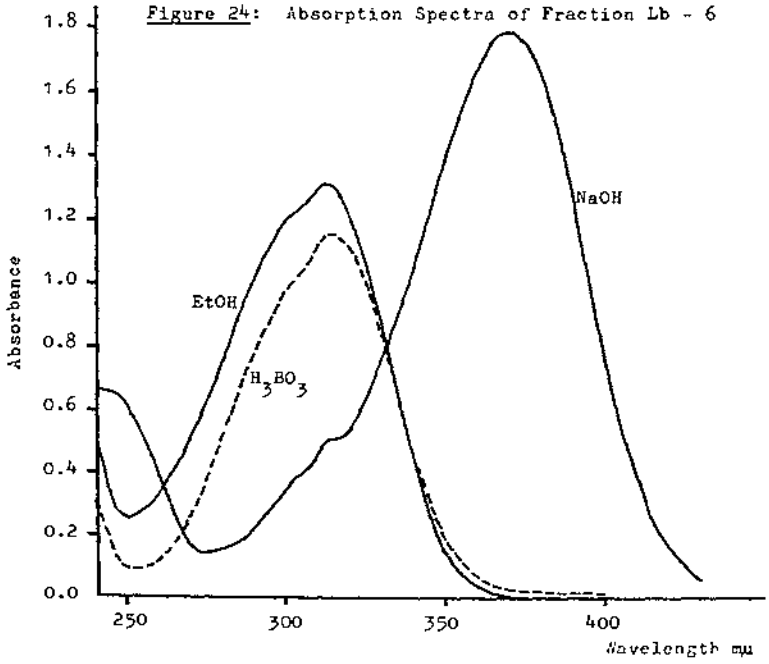


Figure 24: Absorption Spectra of Fraction Lb - 6



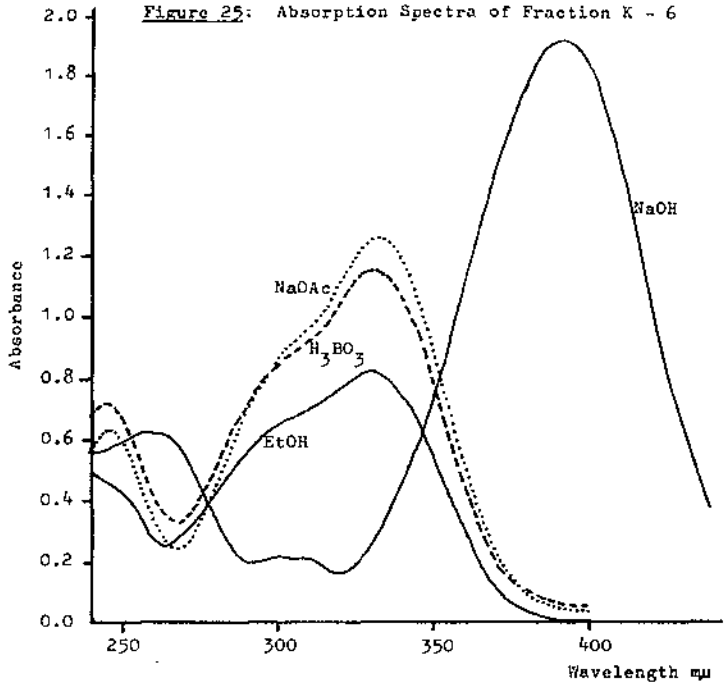


Figure 25: Absorption Spectra of Fraction K - 6

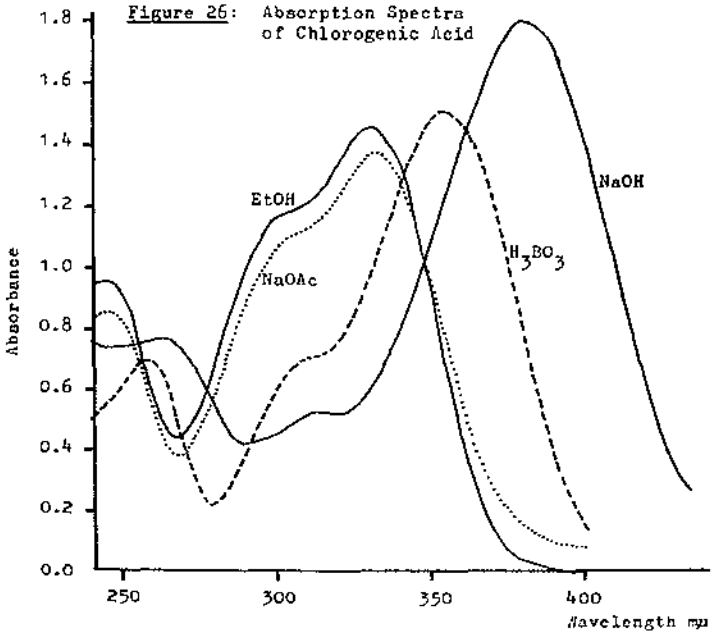


Figure 26: Absorption Spectra of Chlorogenic Acid

Figure 27: Absorption Spectra of Fraction 2b - 3

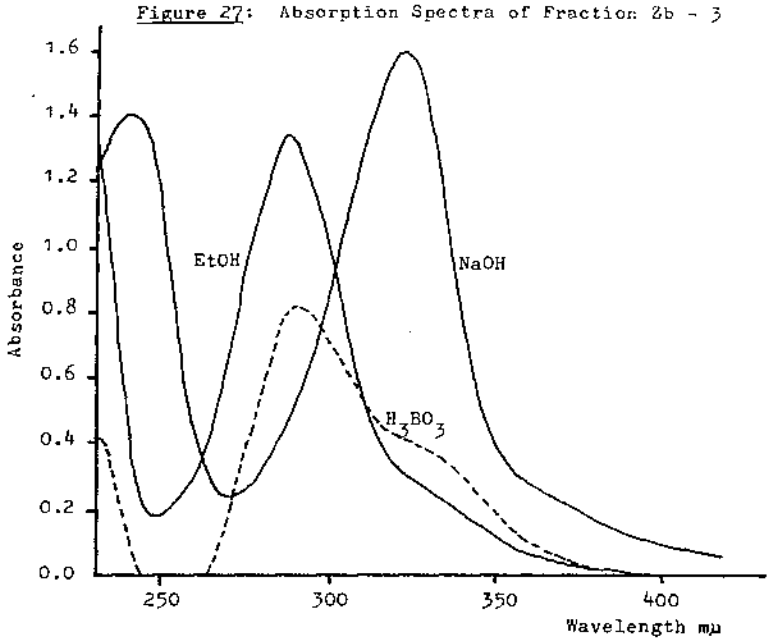


Figure 28: Absorption Spectra of Phloretin

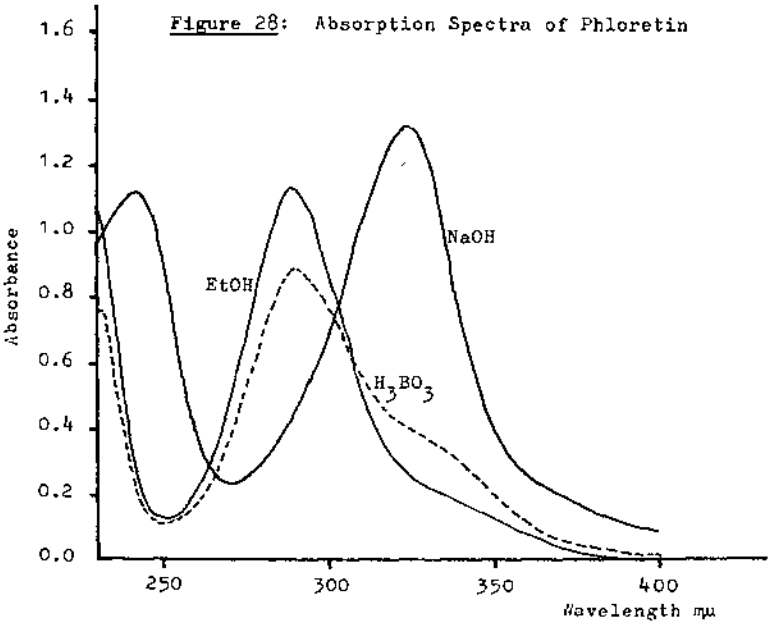


Figure 29: Absorption Spectra of Fraction 2c - 3a

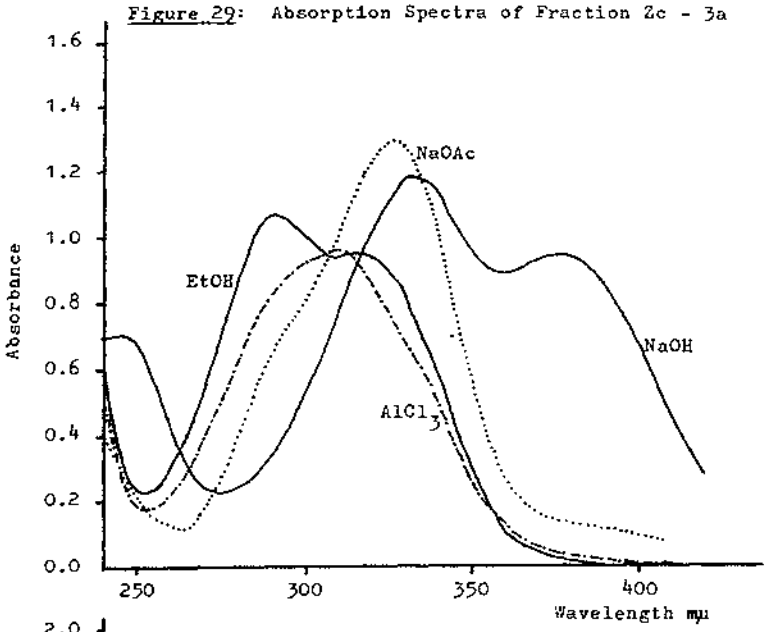


Figure 30: Absorption Spectra of Fraction F - 5

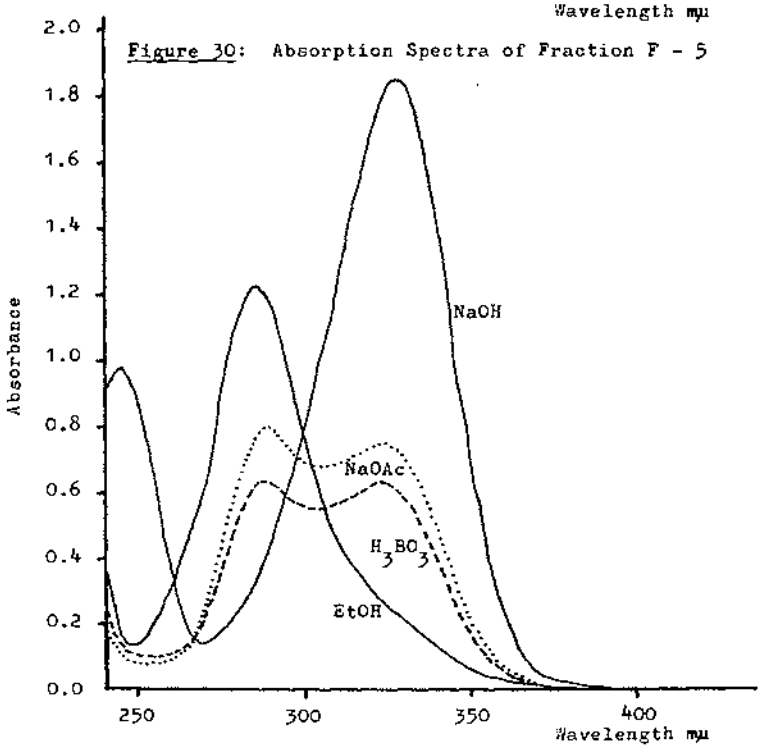


Figure 31: Absorption Spectra of Fraction Ba - 7

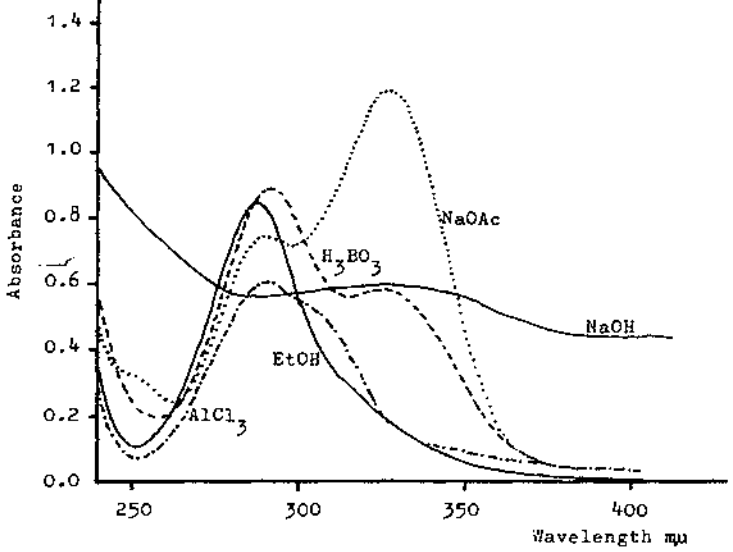
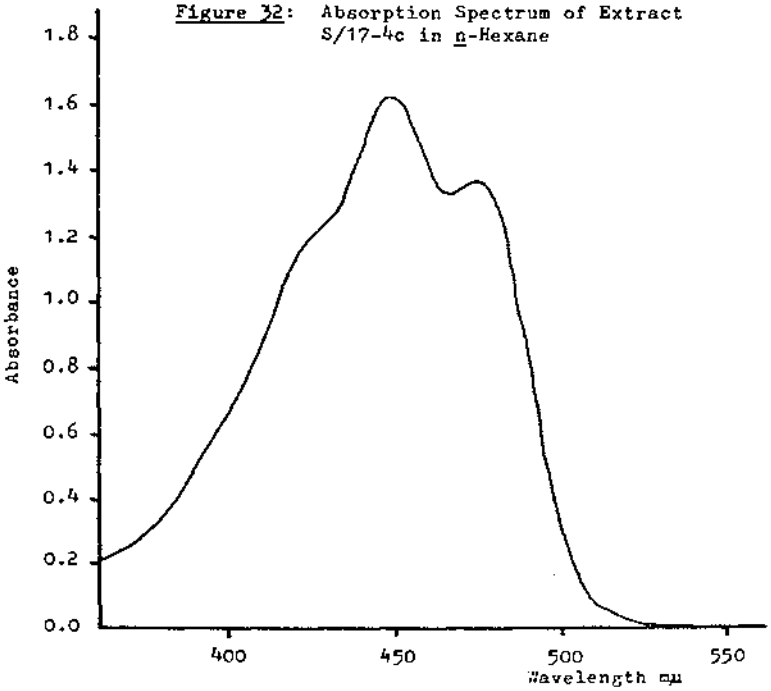


Figure 32: Absorption Spectrum of Extract S/17-4c in n-Hexane



obtained when the products of acid hydrolysis of the fraction were identified chromatographically as phloretin (Tables 16 and 17) and glucose (Table 18) by comparison with authentic specimens.

Chromatographic and colour reaction data for the aglycones (Tables 16 and 17) and the sugars (Table 18) from the acid hydrolysates of fractions Aa - 8, Ab - 6, Ac - 7 and Dbc - 9 indicated that these fractions contained quercetin glycosides. The identification of the aglycones from these fractions as quercetin was further supported by the results (Table 21, Figures 13 and 14) of the comparison of the spectral properties of quercetin with those of the aglycone from fraction Aa - 8 which was indistinguishable (Tables 16 and 17) from the aglycones of the other three fractions. Colour reactions for the fractions (Table 15) were characteristic of quercetin-3-glycosides (39) and were given under identical conditions by the standards, rutin (quercetin-3-rhamnoglucoside) and quercitrin (quercetin-3-rhamnoside). The spectral properties of the fractions (Table 21, Figures 15-18 and 21) are also consistent with the presence of quercetin-3-glycosides. In the neutral spectra the location of Band I and II is consistent with the presence of flavonol glycosides and the inflection on the Band II peak with the presence of a 3',4'-dihydroxyl grouping (37). The bathochromic shifts of Band I, in the presence of boric acid and sodium acetate, are within the 15-20 mμ range expected when an o-dihydroxyl group is present (37). Since instability of a flavonol in alkali normally indicates the presence of a free 3,4'-dihydroxyl grouping, the stability of the spectra in sodium hydroxide solution indicated the protection of one of these groups (37), thus supporting glycosylation at the 3-position.

The bathochromic shifts of Bands I and II with the sodium hydroxide reagent are the result of the ionisation of the phenolic hydroxyl groups. Evidence for the presence of a free 7-hydroxyl group in each fraction is provided by the bathochromic shift of Band I in the presence of sodium acetate being within the 8-20 mμ range. Only when a free 5- or 3-hydroxyl group is present do flavonols form aluminium complexes, which produce bathochromic shifts of Bands I and II and two distinct peaks or inflections for each band. The shift of Band I to Band Ia of the complex is about 60 mμ when a free 3-hydroxyl group is present and significantly less when only a 5-hydroxyl group is free (37); thus the presence of a free 5-hydroxyl group is indicated in the fractions. Overall therefore, the spectral data are consistent with the presence of flavonols with free hydroxyl groups at the 5,7,3' and 4'-positions, i.e., with the presence of quercetin-3-glycosides in the fractions.

The chromatographic identification of sugars obtained from the fractions after acid hydrolysis (Table 18) showed that both glucose and galactose were obtained from fraction Aa - 8. These sugars were best resolved from the mixture by chromatography in EaPW to produce a dumb-bell shaped spot from which it was apparent that the two sugars were present in the mixture in about equal amounts. The identification of fraction Aa - 8 as a mixture of quercetin-3-glucoside and quercetin-3-galactoside rather than as a 3-bioside of glucose and galactose, is supported by the relative R_F values of fraction Aa - 8 and of rutin in several solvents (Table 14). For example, the R_F value of the fraction is higher in BAW and lower in 6%HA than the R_F of rutin, which is the expected relationship in these solvents for the mono- and diglycosides

of an aglycone (30,31). Since Siegelman (39) has reported a partial separation only of a mixture of the 3-glucoside and 3-galactoside of quercetin after the band had been developed repeatedly with a m-cresol-acetic acid solvent, and no evidence of any separation after a single development in several chromatographic solvents, it is to be expected that fraction Aa - 8 would appear chromatographically homogeneous in the solvents used in this study.

Fractions Ab - 6 and Ac - 7, which yielded on acid hydrolysis the monosaccharides D-xylose and L-arabinose respectively, exhibited the chromatographic behaviour consistent with their identification as the 3-monosides of quercetin. L-Rhamnose was the only sugar from the hydrolysate of fraction Dbc - 9; the identification of which as quercetin-3-rhamnoside was supported by direct comparisons throughout with the authentic compound.

Fraction Dba - 6 was a mixture which, on acid hydrolysis, yielded two aglycones, one of which was indistinguishable from quercetin in BeAW (Table 16). In BAW, 40%HA and BEW the aglycone mixture chromatographed as a single spot which was however always more elongate than the quercetin reference spot. Both glucose and rhamnose were identified as sugars from the acid hydrolysate of the fraction. While the fraction resembled rutin with respect to its colour reactions, it differed from rutin in chromatographic (Table 14) and spectral properties (Figures 19 and 20). Though the fraction could not be resolved into two bands in any of the chromatographic systems used, an inspection of the band following preparative chromatography in BAW indicated a leading zone of a brighter yellow colour than the rest of the band when viewed in

the presence of ammonia in ultraviolet light. This colour difference could indicate a partial resolution of the components of the fraction. The presence of rutin as a component of the fraction is supported by various data. For example, on chromatography the components of the fraction always overlapped with rutin. Further, there was a similar overlapping of the aglycones with quercetin except in BeAW which separated them to reveal that one was indistinguishable from quercetin. The spectral properties of the fraction (Table 21, Figure 19) are consistent with the presence of a quercetin-3-glycoside in the mixture. Finally, the identification of glucose and rhamnose as the sugars released on acid hydrolysis of the fraction would be consistent with the presence of rutin in the mixture. The other aglycone, which was present along with quercetin in the acid hydrolysate, could not be identified on account of the insufficient quantity available. However the chromatographic properties of the aglycone mixture indicated it was not kaempferol (Table 16). The colour reactions of this second aglycone and of the fraction Dba - 6 were compatible with the presence of a mixture of two flavonol-3-glycosides in the fraction.

A similarity in the colour reactions (Table 15) of the alkali labile fractions Ia - 6, La - 8 and Lb - 6, indicated the presence of a common phenolic moiety which was supported by other data. The chromatographic properties and colour reactions of the phenolic moiety from each of these fractions were those of p-coumaric acid (Tables 19 and 20). Identification of this acid by mass spectrometry, in a sample of the phenolic moiety of fraction Ia - 6 (70) supported this identification. These fractions possess the properties of esters of

p-coumaric acid. Besides their alkali lability, their blue fluorescence in ultraviolet light only in the presence of ammonia is a property of the p-coumaric acid esters (26). In addition the very similar spectral properties of the fractions (Table 21, Figures 22-24) are those characteristic of the esters of p-coumaric acid (26). One free phenolic hydroxyl group at least is indicated by the bathochromic shifts in alkali while the absence of appreciable bathochromic shifts in boric acid in the presence of sodium acetate supports the absence of an o-dihydroxyl grouping (42). The occurrence of two spots of identical colour reaction when fraction Lb - 6 was chromatographed in 6%HA and only one spot when chromatographed in the other solvents (Table 14) is typical of an ester of p-coumaric acid and is the result of cis - trans isomerisation (17,45). Only elongate spots were obtained when the other two fractions were chromatographed in 6%HA. Indications were obtained during the analytical chromatography that some of the compound of fraction Ia - 6 was being altered under the conditions of storage or chromatography. Thus in addition to the main spot, a faint spot with the chromatographic properties and colour reactions in ultraviolet light of fraction Lb - 6 was noted when fraction Ia - 6 was chromatographed in BAW and in BEW. In the final step in the purification of fraction Ia - 5 (Figure 9), chromatography in BAW also revealed a similar trace component which was then not considered to indicate an incomplete earlier separation since no trace of an intermediate band, corresponding to fraction La - 6, was detected.

The absence of an appreciable bathochromic shift in the absorption maximum in the presence of boric acid and sodium acetate indicated that

the compound present in fraction K - 6 did not contain the phenolic o-dihydroxyl grouping of chlorogenic acid (42). Yet the bathochromic shift with alkali indicated that at least one free phenolic hydroxyl was present in the phenolic compound of the fraction (Figures 25 and 26). The spectra in alkali of fractions K - 6, Ia - 6, La - 8 and Lb - 6 and of chlorogenic acid were recorded immediately as the absorbance at the absorption maximum was decreasing slowly with time. In the case of chlorogenic acid this decrease was more rapid than with fraction K - 6. On treatment of chromatograms with the Hoepfner reagent chlorogenic acid was orange-yellow while the component of the fraction was pink. The chromatographic properties (Table 19) and colour reactions (Table 20) of the phenolic moiety from the alkali hydrolysis of the fraction were distinct from those of p-coumaric and caffeic acids and slightly different from those of sinapic acid. The presence of two components on chromatography of the phenolic moiety in 6%HA was typical of a hydroxycinnamic acid (17). The identification of ferulic acid in the mass spectrum of a sample of the phenolic moiety (70) was consistent with these results.

The identity of fraction Zb - 3 with phloretin was indicated by a comparison of chromatographic behaviour (Table 14), colour reactions (Table 15) and spectral properties (Table 21, Figures 27 and 28).

In the properties examined, fractions Za - 3a, Za - 3b, Zc - 3a and Zc - 3b were identical. The compound in these fractions exhibited the same chromatographic behaviour as the cis - trans isomers of the hydroxycinnamic acids (17). The spectra for fraction Zc - 3a are presented in Figure 29. Overall the data did not permit positive

identification.

Likewise, data obtained on fractions F - 5 and Ba - 7 did not permit identifications to be made. However, if these fractions contained flavonoid compounds, their spectra would indicate (Figures 30 and 31) an absence of A and B ring conjugation in the molecule (37) which would be consistent with the exhibition of colour reactions typical of flavans.

In summary therefore the following compounds were identified in the fractions isolated. Fraction Cba - 4 (phloridzin), fraction Aa - 8 (quercetin-3-glucoside and quercetin-3-galactoside), fraction Ab - 6 (quercetin-3-xyloside), fraction Ab - 7 (quercetin-3-arabinoside), fraction Dbc - 9 (quercetin-3-rhamnoside), fractions Ia - 6, La - 8 and Lb - 6 (alkali labile conjugates of p-coumaric acid), fraction K - 6 (alkali labile conjugate of ferulic acid) and fraction Dbc - 6 (rutin tentatively identified in the mixture).

In addition to the characterisation of the phenolic compounds in the fractions isolated from extract S/17-1b, the nature of the orange-yellow colouring matter in extract S/17-4c (Figure 5) was also investigated. This colouring matter was of low solubility in 70% ethanol and of higher solubility in 95% ethanol and therefore in the S/17 extraction procedure it remained to colour the residues of the leaf tissue in contrast to the S/16 extraction procedure in which it was extracted to leave light-coloured residues (section 4.1). Aliquots of extract S/17-4c chromatographed in both BAW and BeAW as orange spots R_F 1.00 in keeping with the solubility of the coloured substance in n-hexane. The spectrum of a sample of the extract was recorded in

n-hexane with a result (Figure 32) typical of a carotenoid extract (71). The carotenoid nature of this colouring matter would be consistent with its observed solubility properties and would account for the difference in the colour of the residues in the S/16 and S/17 extraction procedures.

4.6 Incorporation of ^{14}C into Phenolic Compounds in Leaf Tissue

The leaf tissue used for this feeding - young (1-2 weeks old) Sturmer Pippin leaves which in early February were present only at the shoot tips of young trees - was selected because earlier results (section 4.2) indicated that the relative levels of some phenolics were changing in this type of tissue. The radicactivity recovered in a 70% ethanolic extract (2.4 ml) of the phenolic compounds of the leaf tissue (1.2 gm wet weight), which was prepared immediately after the feeding period, is recorded in Table 22. Aliquots of this extract were chromatographed in two-dimensions in the standard solvents and the radioautograms developed after 125 days' exposure to the chromatograms showed that phloridzin (spot 5c, Figure 6) was the only phenolic compound which was sufficiently radioactive to be detected. There were several other substances of more intense activity noted in the region where soluble sugars would be expected. Four fractions containing the main phenolic compounds of the leaf tissue (phloridzin and quercetin glycosides) were prepared, by chromatographing a 0.85 ml portion of the extract in 6%HA, eluted and counted (Table 22). Fraction 4, which contained much of the phloridzin, was the most active as anticipated from the results of the radioautography. Much of the activity in

Table 22

INCORPORATION OF ^{14}C ACTIVITY INTO PHENOLIC COMPOUNDS IN LEAF TISSUE ADMINISTERED (U - ^{14}C)-GLUCOSE

Item	Radioactivity (cpm)*	% of Total Activity	
		Administered	Chromatographed
Incorporation into extract			
Total activity administered	4.118×10^6	100	
Activity taken up	4.070×10^6	99	
Residual activity in tubes	45,800	1	
Activity in 70% ethanol extract	679,400	16.5	
Activity in n-hexane phase	44,600	1	
Activity taken up not recovered	3.347×10^6	81	
Incorporation into fractions of extract			
Activity (0.85 ml extract) chromatographed	241,000		100
Activity not in main phenolics	220,930		91.7
Activity in fractions 1-4 (main phenolics)	20,070	0.5	8.3
Activity in fraction 1	1,480		0.6
Activity in fraction 2	750		0.3
Activity in fraction 3	4,760		2.0
Activity in fraction 4	13,080	0.3	5.4

* Counting efficiency 37.5%.

fraction 3 would also be due to phloridzin. The relatively low activity in fractions 1 and 2 was also in keeping with the result of the radioautography. About 8% of the activity of the extract and about 0.5% of the activity administered to the leaf tissue was found to be present in these four fractions.

Chapter 5

DISCUSSION

5.1 Investigation of Extraction Procedure

Investigation of the extraction procedure for the removal of phenolics from apple leaf tissue showed that quantitative extraction was more efficient with 70% ethanol than with 95% ethanol. This is to be expected (26,30,31) since the phenolic compounds occur in the leaf tissue mainly in glycosidic and alkali labile forms (section 4.5). It is now apparent that these alkali labile forms were the most difficult to extract with 95% ethanol and were responsible for the higher R_F blue fluorescence in ultraviolet light when, in the investigation of the extraction procedure, extracts were chromatographed in 6%HA to ascertain their phenolic content. Fraction Ia - 6, corresponding to spot 1a (Figure 6), no doubt contained the compound most difficult to extract in 95% ethanol and possibly also in 70% ethanol. The ineffective elution of this fraction from the chromatography paper with 95% ethanol was compatible with the behaviour of the compound not only in the extraction procedure but also on chromatography in BAW and in 6%HA. While solvents more aqueous than 70% ethanol may have facilitated the extraction of this compound, the use of such solvents would probably have been precluded by the need to compromise when a number of phenolic compounds, possessing the range of solubility

properties of those present in apple leaf tissue, had to be extracted together.

5.2 Effect of the Nature of the Leaf on Level of Phenolics

The changes noted in the levels of certain phenolics in secondary spur leaf tissue during the period from leaf emergence to full expansion were most striking and consistent. The constancy of the changes during these stages of leaf development was indicated by the occurrence of similar changes in leaf tissue obtained from actively growing shoot tips. Thus the pattern of change remained unaltered despite firstly the difference in the nature of the shoot systems which carried the secondary spur and the shoot tip leaves and secondly the secondary spur leaves having grown in a spring environment and the leaves from the shoot tips in a summer environment. The secondary spur leaves are carried as a rosette on a very short shoot, the terminal meristem of which ceases to produce further emerging leaves from early November, when a resting bud with scale leaves is formed terminally. However, on the normal shoots, the growth of which has stopped by early February except on young trees, the leaves are spaced apart due to the elongation of the internodes, a process which does not occur appreciably with the shoots which carry the secondary spur leaves. The constancy of the pattern of change in the levels of the phenolic compounds of the leaf tissue between spring and summer suggested that variation in the environmental conditions had an insignificant effect on the changes, which contrasts with the reported effect on certain plants (15,72).

Hence the constancy of these changes in the face of changes in environmental conditions and in elongation of the associated shoot system, indicated that they are more closely linked with the processes of leaf expansion than with other factors. Variation in the level of flavonols with leaf age has previously been noted (15).

The habit of leaf growth of the spur system (60,73,74) precluded a more direct and extensive investigation of the influence of environmental conditions on the changes in the pattern of phenolic compounds in expanding leaf tissue. The first spur leaves to appear in spring, the primary spur leaves, expand very rapidly after bud burst and no samples of these expanding leaves were obtained. Further, the period in which rapidly expanding secondary spur leaves are present is restricted to about one month from mid-October and hence to a time in which only limited changes in environmental conditions occur. The only source therefore of rapidly expanding leaf tissue for comparison in late spring and summer is that from shoot tips. It would be of interest then to check on the apparent lack of environmental influence on the pattern of change in the phenolic compounds in rapidly expanding leaf tissue using leaves obtained from shoot tips over a period of several months.

Little can be said about the stability of the pattern of phenolics in fully expanded leaf tissue over time and under different environmental conditions. However, the lack of any detectable change during the three-week period that expanded primary spur leaves were sampled would indicate that there may be no significant change in the pattern as the healthy leaves age for a time. Further, the lack of

any major difference in the phenolic pattern of these primary spur leaves and the expanded secondary spur leaves would suggest that this may also apply to expanded secondary spur leaves.

5.3 The Phenolic Pattern among Varieties

The contention of Williams (48) that there is little obvious varietal influence on the pattern of phenolics in the leaf has been largely borne out by the results of the very limited present investigation. However, the noted indication of a quantitative variation in the intensity of spot 6a is in agreement with the report of Flood and Kirkham (55) that quantitative differences in the phenolics exist among varieties.

In any detailed determination of the pattern and amounts of the individual phenolic compounds in the leaf of a range of varieties, it would be important to compare leaf samples of similar stages of development since it is to be expected that the changing pattern noted in the Sturmer Pippin variety would be exhibited to some degree by all varieties.

5.4 Paper Chromatographic Isolation of Phenolic Compounds

The successful isolation of several quercetin-3-glycosides from the leaf tissue extract in amounts sufficient for characterisation by micro-methods further demonstrated the suitability of paper chromatography for the isolation of micro-amounts of phenolic compounds from

complex mixtures (26,30). In the course of the isolation of many of the fractions, BAW proved as expected (30) to be an extremely useful solvent. While BeAW is most suited to the separation of non-glycosidic flavonoids (38), it nevertheless proved effective in the isolation procedures for the purification of selected fractions which chromatographed as broad rather than as compact bands, on account of the phenolic compounds being present in glycosidic or other combined forms. The resolution of quercetin-3-glycosides as very broad bands when chromatographed in water was somewhat unexpected since Siegelman (39) has reported the successful application of this solvent to the preliminary separation of these glycosides.

The composition of the ethanol/acetic acid mixture which was found to extract efficiently the bands of phenolic compounds in the preparative chromatography compares favourably with solvents suggested in the literature. For example, Siekel (30) has reported the use of 40-70% ethanol for the elution of flavonoid glycosides, while Harborne (31) has recommended the addition of acetic acid (10% by volume) to the eluting solvent for strongly absorbed pigments. During chromatography, acetic acid has been reported (75) to limit atmospheric oxidation of phenolic compounds and this may be an added advantage of its presence in the eluting solvent when the phenolic compounds present are unknown. The importance of using a solvent which would maximise the efficiency of recovery of the phenolic compounds was important in view of the number of times each compound was chromatographed and the reported efficiency of recovery of certain phenolic compounds from paper chromatograms (28,68,76). The drying of

chromatograms at room temperature could also be expected to improve the recovery of flavonoid compounds (28).

Crystallisation was a most convenient method for the isolation of much of the phloridzin present in the leaf tissue extract and at the same time it reduced considerably the scale of the paper chromatography required to purify the quercitrin. Since the paper chromatographic separation of phloridzin and quercitrin necessitated development with an alkaline solvent, the isolation of a sample of phloridzin by the method employed may have had the added advantage that the phloridzin was not chromatographed in an alkaline system in which it is unstable (77).

5.5 Characterisation of Fractions Isolated from Extract S/17-1b

Compounds isolated and identified from the S/17-1b extract of apple leaf tissue are largely in agreement with those reported earlier (section 2.2). Thus phloridzin accounted for the major portion of the phenolic substances detected in the extract with about 20 mg/gm fresh weight of leaf tissue being isolated as crude crystalline material (Cba - 1, Figure 8). The amount in the leaf of certain fruiting varieties of apple has been reported to be between 28 and 58 mg/gm fresh weight of leaf (78). Clearly the yield of phloridzin obtained in this study would be appreciably below that extracted from the leaf tissue on account of the losses in chromatography and crystallisation. Traces of phloretin which were identified in the extract (fraction Zb - 3) were probably produced by the action of a glucosidase on the

phloridzin during the initial step of the extraction. A phloridzin glucosidase, which showed a high degree of specificity towards phloridzin, has been obtained from a homogenate of apple leaf tissue in ethanol (79). The enzyme was reported to release phloretin when the leaves were homogenised in ethanol but not when they were plunged directly into boiling alcohol. No trace of flavonol aglycones could be detected however on two-dimensional chromatograms of the extracts prepared in this study. This would indicate that the flavonol glycosides were not attacked significantly before the glycosidases released on homogenisation of the leaf tissue were inactivated.

The quercetin-3-glycosides which were identified are in agreement with those reported for leaf tissue by Williams (48) and for fruit tissue by Siegelman (39). Further, the tentative identification of rutin as a component of fraction Dba - 6 is supported by its reported occurrence in both leaf (48) and fruit (39) tissue. While the quercetin-3-glucoside and quercetin-3-galactoside were inseparable in the solvents used, a solvent such as phenol-water may however have separated them. Solvents containing phenol were purposely avoided on account of the difficulty in preventing interference from traces of this compound in the spectral studies. It is also of note that the closely related 3-glucoside and 3-galactoside of kaempferol have been reported to be inseparable in the normal range of solvents (80). Though the nature of the linkage between the aglycone and the sugars in these quercetin-3-glycosides was not investigated, the quercetin-3-arabinoside may be avicularin (quercetin-3-α-L-arabinofuranoside) since this one of the several known quercetin-3-arabinosides (53) has been reported in apple

fruit (39).

The absence of any glycosides of kaempferol in the phenolic compounds isolated is noteworthy in view of their reported occurrence (48). This is in agreement with the result of a preliminary examination of acid-hydrolysed samples of extracts P/4 and S/8 by two-dimensional chromatography which indicated that only one spot of flavonol aglycone component could be detected. It would thus appear that there was not an appreciable amount of kaempferol glycosides in either extract. The limited evidence available would suggest that the component of fraction Dba - 6, apart from the compound tentatively identified as rutin, may be a bioside of a second flavonol aglycone which, on chromatographic evidence, is not kaempferol. Further, since the sugars obtained on acid hydrolysis of the fraction were glucose and rhamnose, it is reasonable on the basis of chromatographic behaviour to expect the second flavonol to be a rhamnoglucoside corresponding to rutin. Fraction Dbc - 6 could not have contained kaempferol-3-rhamnoglucoside since this compound would have been separated from rutin by chromatography in BAW as the R_F values given by Harborne (31) for these compounds are 0.54 and 0.45 respectively. The R_F values of this second aglycone are intermediate between those of quercetin and kaempferol in several solvents and greater than both compounds in BeAW. A flavonol such as isorhamnetin (quercetin-3'-methyl ether) could be expected to exhibit these chromatographic properties (30,31,38). When the phenolic compounds reported to occur in both the apple and the pear (Pyrus communis) are considered, the probability of isorhamnetin occurring in the apple is strengthened. In the two plants the similarity in the

phenolic compounds, which are of the kaempferol and quercetin hydroxylation patterns (54), is illustrated by the occurrence in the leaf tissue of both of several 3-glycosides of kaempferol and quercetin (48). This similarity is presumably an expression of the close botanical relationship of the apple and the pear which are both members of the sub-family Pomoideae in the family Rosaceae (48). Of considerable interest therefore in relation to a possible further similarity in the phenolic compounds is the more recent report of the isolation of isorhamnetin glycosides and a quercetin glycoside, but no kaempferol glycosides, from the fruit of the pear, variety Bon Chretien (32). The occurrence of kaempferol in the apple leaf is supported by the report of Williams (48) and the identification of astragalin (kaempferol-3-glucoside) along with quercetin glycosides by Herrmann (52), but not by the identification of quercetin and not of kaempferol glycosides in an examination of the flavonoids of the leaves of 65 wild members of the genus Malus (81). Further, Williams (48) has reported several 3-glycosides of both quercetin and kaempferol in the apple fruit, yet Siegelman (39) has identified only quercetin-3-glycosides in the flavonoid complex of Grimes Golden apple skin.

Although the form in which p-coumaric acid was combined in fractions La - 8 and Lb - 6 was not determined, the R_F values and colour reactions of the fractions would support their identification as isomers of p-coumaroylquinic acid which have been previously reported in apple leaf and fruit tissue (45,50). If correct this would mean that fraction Lb - 6 contained 3-O-p-coumaroylquinic acid (17,45). While the nature of the p-coumaric acid derivative in fraction Ia - 6 is not known

it is clearly a molecule with low solubility in non-aqueous solvents. When fraction Ia - 6 was chromatographed, the appearance of traces of a substance identical in colour reaction and chromatographic properties to fraction Lb - 6 may be explained by the presence of a common non-phenolic moiety in the two fractions.

The occurrence of a conjugate of ferulic acid (fraction K - 6) in the apple leaf is not unexpected. Small amounts of ferulic acid have been found in hydrolysates of leaf tissue of some Rosaceous plants although caffeic and p-coumaric acids are more typical (57). The presence of ferulic acid rather than sinapic acid would be in agreement with the reported absence of trihydroxy derivatives in the phenolic compounds of the Pomoideae (56). Traces of ferulic acid have been reported in alkali but not in acid hydrolysates of core tissue extracts of McIntosh apples (82) and radioactive ferulic acid has been found in the acid hydrolysate of ethanolic extracts of leaf disks of Malus baccata or M. mandshurica which had been fed ^{14}C-cinnamate (83). Ferulic acid has been found combined with quinic acid in several plants (17,84) and its presence in this form in fraction K - 6 would be a logical possibility since conjugates of both p-coumaric and caffeic acids with quinic acid have been reported in apple leaf (section 2.2).

Any chlorogenic acid present in the leaf extract may have been discarded in fraction Ib - 4 which contained the spots 1b and 2b with the properties characteristic of traces of chlorogenic acid. Since Taylor and Zucker (20) have reported the possibility of extensive oxidation of caffeoyl esters when fresh Xanthium leaf was ground in methanol, any chlorogenic acid present in the apple leaf may have been

largely destroyed in the course of the extraction. Since lyophyllisation of the Xanthium leaf tissue prior to extraction prevented any destruction of this type (20) it would be of interest to test this extraction procedure with the apple leaf tissue.

While no catechins or leucoanthocyanins were identified in the leaf extract, representatives of the flavans may have been present in fractions F - 5 and Ba - 7. The spectral properties of the compounds in these fractions would not rule this out (37). When two-dimensional chromatograms of leaf extracts were sprayed with 5% sodium carbonate and left overnight, a compact faint brown spot was observed within the boundaries of spot 5d. This was interpreted as an indication of the possible presence of d-catechin along with the main compound, phloridzin, in spot 5d. However, with fraction Cba - 4 this reaction with 5% sodium carbonate could not be detected. A study of R_F values of the reference compounds showed that any d-catechin present in fraction Dbc - 5 would have been carried ahead of the quercitrin and phloridzin on chromatography in IpAW (Figure 8).

If rutin was indeed a component of fraction Dba - 6, then rutin and quercitrin were two of the compounds involved in the changing intensities of spots 6a and 6d as the leaf tissue expanded (section 4.2). These findings are of interest when viewed in relation to the biosynthesis of flavonoid glycosides. Glycosylation has been held to be possibly the last step in the biosynthesis of a flavonoid glycoside and a diglycoside to be synthesised stepwise via the monoglycoside (13). For example, the stepwise synthesis of rutin from quercetin in the presence of an enzyme preparation from mung bean leaves has been

shown to progress as follows (85).

NDP-glucose + Quercetin ⟶ Quercetin-3-β-D-glucoside + NDP

NDP-L-rhamnose + Quercetin-glucoside ⟶ Rutin + NDP

(NDP = nucleotide diphosphate)

Thus if quercitrin and rutin were synthesised in this manner in apple leaf, it would be possible to explain the synthesis of predominantly one or other of these glycosides in a particular type of leaf tissue by postulating that the NDP-L-rhamnose supply was utilised in the 'emerged' leaf tissue for the synthesis of rutin and in the 'expanded' tissue for the synthesis of quercitrin. Arising from a change of this nature in glycoside synthesis could also be a change in the level of quercetin-3-glucoside. While a change in the level of this glycoside would not have been readily noted on a two-dimensional chromatogram because of the presence of several quercetin-3-glycosides in spot 8c, it may have nonetheless occurred and contributed to the observed intensification of spot 8c as the leaf tissue expanded. Since genetic control of the levels of certain flavonoid pigments in plant tissues is recognised (9), the constancy of the pattern of glycosides noted in the apple leaf of a particular stage of development, whether in spring or in summer, would possibly be best interpreted in terms of strict genetic controls on the metabolism of the glycosides in the leaf. In the synthesis of rutin and quercitrin for example these controls could operate by directing as suggested above the utilisation of NDP-L-rhamnose.

While no hydroxybenzoic acid derivatives were identified in the leaf extract some may have been present since Ibrahim and Towers (5) have identified five hydroxybenzoic acids in acid hydrolysed leaf extracts of Malus robusta. However, it would appear from the results of this present study that none of the clearly discernible spots on a two-dimensional chromatogram is a hydroxybenzoic acid derivative and that such compounds, if present in the Sturmer Pippin leaf sample, occur at levels or in forms not readily detected by the methods employed.

5.6 Incorporation of ^{14}C into Phenolic Compounds in Leaf Tissue

Of the major phenolic compounds, phloridzin was shown to be the only one into which appreciable radioactivity was incorporated, the activity of the quercetin glycosides being undetectable by radio-autography. A similar degree of labelling of the phloridzin and the quercetin glycosides has been previously reported (83,86) after a number of ^{14}C-compounds were fed tc excised leaf tissue of various Malus species. The percentage of the radioactivity of the 70% ethanol extract which was present in the phloridzin (about 8%, Table 22) was somewhat greater than the values up to 1% reported for similar experiments with Malus leaf disks (86).

It is of interest that an effect of the age of the leaf tissue, and therefore presumably of its metabolic state, on the utilisation of both phenylalanine and cinnamic acid in the synthesis of phloridzin has been reported (83). The utilisation occurred only in young leaf

tissue, under six weeks old. While glucose is apparently utilised in phloridzin synthesis in older leaf tissue as well (83,86) it may be that with younger tissue, of the age used in this study, greater utilisation would be obtained. The changes in the capacity of the leaf tissue to utilise certain compounds in the synthesis of phloridzin pointed to the changing pattern of metabolism of the phenolic compounds as the leaf developed. A facet of this changing pattern of metabolism was also evident in these studies when the amounts of certain phenolic compounds were found to alter consistently as the leaf tissue expanded. Consequently in recognition of this changing metabolism and to control its effect on results the tissue employed in all investigations of the occurrence and metabolism of phenolic compounds in the apple leaf should be of a definite type and stage of development.

Chapter 6

SUMMARY

1. An investigation of the quantitative extraction of non-bound phenolics from apple leaf tissue (Malus pumila) demonstrated the superiority of 70% ethanol over 95% ethanol in similar procedures. A method of extraction using 70% ethanol was therefore developed.

2. A paper chromatographic procedure was developed and applied to the isolation of 17 fractions containing the main phenolic compounds present in a 70% ethanolic extract of apple leaf tissue.

3. Phenolic compounds identified from these fractions by micro-methods based on paper chromatography and ultraviolet spectroscopy were phloridzin, phloretin, quercetin-3-glycoside, quercetin-3-galactoside, quercetin-3-arabinoside, quercetin-3-rhamnoside, quercetin-3-xyloside, p-coumaric acid combined in three alkali labile conjugates and ferulic acid in one such conjugate. Rutin was tentatively identified in a mixed fraction. These results are discussed in relation to the reported occurrence of phenolics in the apple.

4. Certain phenolic glycosides were found to change in amount as the secondary spur leaf tissue of apple developed in spring from emergence through to approximately full expansion. The occurrence

of the same pattern of change in comparable tissue collected from shoot tips in summer suggested the pattern may be genetically controlled.

5. Young excised leaf tissue fed (U - ^{14}C)-glucose incorporated the label into phloridzin but not appreciably into the other phenolic compounds.

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