THE PHENOLIC COMPOUNDS OF GREEN AND ROASTED COFFEE BEANS

by

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PREFACE

The roasting of coffee beans has been practised for about 700 years. For just as many years men have doubtless wondered what happens during roasting and why different coffee beans have given products differing in taste and aroma. For at least 130 years scientists have attempted to solve this problem. Recently, sophisticated techniques have yielded masses of analytical data, but not a complete answer. Indeed, a further phenomenon has been uncovered - that green and roasted coffee beans show so many similarities with green and roasted barley, cacao beans and groundnuts, but yet have such subtle differences of taste and aroma.

It has been shown that sugars and amino acids are important precursors of the aroma of roasted cacao and coffee beans. The relative proportions of these two components have been found of importance in determining the quality of coffee beans. Phenolic compounds have also been named as aroma precursors and it is proposed to investigate the behaviour of the coffee bean phenolic compounds during roasting, and their role in determining the quality of the roasted bean.

ABBREVIATIONS

1. Chlorogenic Acids.

Chlorogenic acids are esters of a cinnamic acid and quinic acid.

Nomenclature of the chlorogenic acids is non-systematic, confusing, and to the uninitiated gives no indication of the structure, eg Isochlorogenic acid 'A', Band 510.

To avoid these shortcomings the following abbreviations (or occasionally the non-abbreviated form) have been used throughout this thesis.

First Cypher:- A figure or figures indicating the position at which the cinnamic acids are attached to the quinic acid.

Second Cypher:- A group of letters indicating which cinnamic acid is attached to the quinic acid.

eg C = caffeic acid.
F = ferulic acid.
Co = 4-hydroxycinnamic acid.
DC = two caffeic acid substituents.

Therefore Isochlorogenic acid 'A' = 3, 4DCQ = 3,4-dicaffeoylquinic acid. Band 510 = 4CQ = 4-caffeoylquinic acid.
3FQ = 3-feruloylquinic acid.

2. Other Compounds with a benzene nucleus.

The trivial names used for many of these compounds give no indication of the structure, and the following systematic abbreviations have been used to indicate the structure where this is of particular significance.

First Cypher:- A figure or figures indicating the position of substituents on the benzene nucleus.

Second Cypher:- A letter or letters indicating the nature of the substituents.

Bz = Benzene.

BA = Benzoic acid.

BzAl = Benzaldehyde.

CA = Cinnamic acid.

CAl = Cinnamaldehyde.

M = Methoxy.

H = Hydroxy.

MD = Methylenedioxy.

D = Di-

T = Tri-

eg Syringealdehyde = 3,5DM,4HBzAl = 3,5-dimethoxy 4-hydroxy benzaldehyde.

eg Caffeic acid = 3,4-DHCA = 3,4-dihydroxycinnamic acid.

eg Pyrogallol = 3,4,5-THBz = 3,4,5-trihydroxybenzene.

Exceptions:-

The following abbreviations are in common usage, and their usage has been retained:-

PHE = Phenylalanine.

TYR = Tyrosine = 4-hydroxyphenylalanine.

DOPA = Ortho-dihydroxyphenylalanine = 3,4-dihydroxy phenylalanine.

3. Miscellaneous Abbreviations.

Normal abbreviations (arranged in alphabetical order) come before the hieroglyphs.

AOAC = Association of Official Analytical Chemists.
BPt = Boiling point.
BS = British Standard.
cm = Centimetre.
dmb = Dry matter basis.
DNPH = Dinitrophenylhydrazine or dinitrophenylhydrazone.
DSA = Diazotised sulphanilic acid.
eg = For example.
fwb = Fresh weight basis.
g = Gram.
GLC = Gas-liquid chromatography.
HMW = High molecular weight.
ie = That is.
Kg = Kilogram.
m = Mole.
MEC = Molar extinction coefficient.
mg = Milligram.
min = Minute.
ml = Millilitre.
MPt = Melting point.
nm = Nanometre.
NMR = Nuclear magnetic resonance.
PC = Paper chromatography.
ppm = Parts per million.

PVP = Poly-n-vinyl pyrrolidone.

Sat = Saturated.

TLC = Thin-layer chromatography.

USDA = United States Department of Agriculture.

UV = Ultra-violet.

° = Degrees Centigrade.

μg = Microgram.

μl = Microlitre.

μm = Micromole.

% $^{v}/_{v}$ = Percent, volume for volume.

% $^{w}/_{v}$ = Percent, weight for volume.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

A BRIEF HISTORY OF COFFEE AND AN INTRODUCTION TO ITS PROCESSING AND ROASTING

It seems that coffee was first known in Arabia, where roasting of the bean had begun as early as the 13th Century (1). Its consumption spread into Turkey in the 16th Century and into Europe and the USA in the 17th. In London, the earliest known Coffee House was opened at St. Michael's Abbey, Cornhill, in 1652 (2). The first cultivation of coffee is credited to Muslim Pilgrims in India around 1600, and by 1750 plantations were also well established in Haiti, Jamaica and Cuba, and the Dutch East Indies (3). A progressive increase in coffee trading made this commodity an important factor in world trade by about 1850. World production of green coffee exceeded 3 billion Kg in 1962 (4), and in 1968 was expected to be of the order of 4 billion Kg (5).

The plants concerned are evergreens, within the family Rubiaceae, and genus Coffea (2). The coffees of commerce are drawn from the species Coffea arabica Linn. var arabica or var bourbon (commonly known as Arabica); C. canephora (commonly known as Robusta); C. liberica and C. excelsa (6). The last two are of lesser importance, and in addition there are several wild species, probably of no commercial value, eg C. mauritiana and C. resinosa (7).

Robusta and Arabica beans are fairly easily distinguished by differences of size, shape and colour, and there are important differences in chemical composition. Commercially a distinction is made between "low grown" and "high grown" coffees, the latter reputed to be of better quality. Milds and Santos are terms applied to two

Arabicas which differ in their method of processing, ie removal of the green beans from within the cherries.

Coffee trees produce mature fruits six to nine months after flowering, and these remain at their prime for about one week. The fruit is red when ripe and contains two seeds (ie coffee beans), which are separately enclosed in a thin membrane or silverskin. This is surrounded by a layer of mucilage immediately inside the 'fleshy' part of the cherry.

There are two main methods of processing. The natural process is applied mainly to Robusta coffee, particularly where water is in short supply. Drying from about 70% to 12% moisture content is carried out either in the sun or more rapidly by hot air driers. The quicker method is preferable because it reduces the risk of microbiological spoilage. After drying, the outer skin, pulp and parchment are removed in one mechanical process known as hulling.

Wet processing is thought to produce a better quality bean, but it is essential that the beans are just ripe when processing is begun. A slight degree of over-ripeness resulting in lower moisture content is acceptable for dry processing. The skin and pulp are removed by water, and then the undamaged parchment usually by a natural fermentation process. The final stage is drying either in the sun or by forced hot air circulation.

Roasting is still commonly a batch process and charges of up to a quarter of a ton or larger may be used (6). Fluidised bed and microwave roasting are claimed to have some advantages. Commonly a blend of green beans is roasted. The time of roasting should never exceed twenty minutes and the air temperature is in the range 180-200° (6). However, roasting is an exothermic process and the temperature within the bean may be considerably higher. Initially, free and bound water are driven off followed by a range of products from pyrolytic reactions. The beans gradually darken, increase in volume by 50-100%, and finally coffee oil seeps to the surface of the bean (6, 8). Determination of roast end-point is a difficult subjective evaluation requiring great skill if it is to be entirely successful. A light roast shows a weight loss of about 13% (dmb) and a severe roast a weight loss of 22% (dmb) or more. The weight loss and colour may be taken as indices of the degree of roast (9, 10). According to Kaufman, green coffee beans have no aroma and only a bitter taste - roasting develops the well known aroma and flavour (11). The roasting process is terminated by exposure to cool air or a water drench. The roasted beans may be further blended at this stage to maintain a particular quality (12).

THE COMPOSITION OF GREEN AND ROASTED COFFEE BEANS.

(a) Lipids.

Thaler and Gaigl (13) reported that the oil content of green Arabica beans is about 17%, Robusta about 11% and Liberica about 12%. Hartmann et al (14) reported the following crude composition for coffee oil.

Table 1 Coffee oil Composition (Hartmann et al (1969) (14)

Compound	% Coffee oil
Triglyceride	75.0
Diterpene, esters	18.5
Diterpene, free	1.0
Phosphatides	4.0
Phytosterol esters	1.5

The main diterpenes are caffestol and kahweol, both containing one primary and one tertiary alcohol group (14). Traces of lanosterol, dihydrolanosterol and squalene have also been reported (15). Several workers have investigated the fatty acids present in the triglycerides.

Table 2 The Fatty Acid Composition of Coffee Oil Triglycerides.

Component Fatty Acids	Coffee Oil (%)				
Myristic	trace	trace	trace	-	0.14
Palmitic	35.3	33.7	35.2-38.6	38.2-46.5	35.47
Palmitoleic	trace	trace	trace	-	-
Stearic	6.6	7.9	6.6-8.4	6.4-7.0	8.31
Oleic	7.6	10.1	7.5-10.9	5.6-14.3	9.35
Linoleic	45.8	43.5	38.4-43.0	30.0-40.9	41.88
Linolenic	1.6	1.1	trace	-	1.64
Arachidic	2.7	3.3	4.0-4.8	-	0.17
Gadoleic	trace	trace	-	-	-
Margaric	trace	trace	-	-	-
Reference	(16)	(16)	(17)	(18)	(35)

No varietal differences have been detected. The early information about the effects of roasting on the coffee oil is sparse, conflicting, and lacks precision. Navellier (19) reported that "lipids" fall by 3% on roasting, but Bressani et al (20) reported an increase in the ether extract during roasting, and equated this with lipid. Kaufman and Hamsager reported that the content of free diterpenes increased at the expense of the diterpene esters, but in a later publication reported that content of both fell during roasting. Content of triglyceride also fell (21, 22). Fabe et al (35) reported that the ether extract increased from 15% to 18% (dmb), the free fatty acids increased from 1% to 3% and the unsaponifiable matter fell from 11% to 9% (dmb green bean) during roasting. There was very little change in the triglyceride fatty acid composition.

(b) Minerals.

Derbessey et al (18) reported that green coffee beans contained ash at levels of 3.1-3.6% (dmb). A more detailed investigation was made by Winton and Winton (23) who reported about 4% (dmb). The major consituent was potassium oxide (60%) followed by magnesium oxide (10%), calcium oxide (5%), ferric oxide (1%), silica (1%), sodium oxide (0.5%), and traces of aluminium, copper and manganese oxides. The anions were phosphate (15%), sulphate (5%), and chloride (1%).

Other workers have reported the content of rubidium to vary between 9 and 141 mg/Kg (dmb) and of manganese between 8 and 45 mg/Kg (dmb) (24, 25). Tepley and Prior reported moderate calcium and iron, but low sodium and fluorine (26). Over 90% of the ash is water-soluble, and represents about 14% of the soluble components of roasted coffee beans (27, 28).

Most of the minerals are associated with other coffee bean constituents; part of the potassium, and perhaps some of the magnesium is bound to the depsides (29, 30). Northmore (30) believed that the quality of the green bean was in some way related to the content of magnesium chlorogenate, such that the content of magnesium is a quality-limiting factor.

The influence of the minerals on the chemical reactions of roasting, and on the quality of the roasted bean have not been investigated. Smith (28) reported that the potassium salts of the depsides were more heat stable than the free acids.

(c) Carbohydrates.

(i) Oligosaccharides and Monosaccharides.

In 1956 Plunkett (31) reported the presence of sucrose in green coffee beans. Wolfrom et al (32) reported 6-7% (dmb) of sucrose and a trace of glucose in green beans, and smaller quantities of sucrose were found in roasted beans. Glucose and fructose were present after roasting and they deduced that sucrose hydrolysis occurred during roasting. Sivetz (33) reported that green coffee contained about 1% of reducing sugars. Barbirolli (34) examined ten green coffees and found that all samples contained sucrose 5-8% (dmb) glucose 0.5-1.0% (dmb) in five samples, and fructose 0.3% (dmb) in one sample. All roasted coffees contained considerably less sucrose 1.0-1.5% (dmb) and maltose 0.2-0.3% (dmb), which was produced during roasting. Glucose and fructose were usually detectable after mild roasting but were lost after more severe treatment. Fabe et al (35) reported 5.45% (dmb) total sugars present in green beans and these rose to 7.75% at 10% roast loss, and then fell progressively to 3.27% at a 16% total roasting loss. At 18% and 20% total roasting loss slight increases in sugar content were noted.

Navellier (19) reported that sucrose was the most abundant sugar in green coffee (7-8%) and that other sugars were only found in traces if precautions were taken to prevent acid and/or enzymic hydrolysis during extraction.

In 1963 Courtois et al (36) reported three oligosaccharides to be present in Robusta beans. They were sucrose, the major component, raffinose, and stachyose. Shadaksharaswamy and Ramachandra (37) reported the same oligosaccharides present in Arabicas. Sucrose always represented more than 93% of the total oligosaccharides, and Arabicas contained more than Robustas. However, Robustas contained approximately double the quantity of raffinose and stachyose.

(ii) Polysaccharides.

Joly (38) reported in 1951 that green coffee contained 38% cellulose, but this was the reult of a proximate analysis which did not measure cellulose alone. In 1957 Thaler (34) reported that the polysaccharides of green coffee contained glucose, galactose, mannose and arabinose. In 1960 Laver (40) isolated a 10% potassium hydroxide-soluble "hemicellulose" and a 10% potassium hydroxide-insoluble "holocellulose" which contained L-arabinose, D-galactose, D-mannose, and D-glucose in the ratio 1:2:6:2. Further purification yielded a fraction containing 95% mannose. Wolfrom et al (32) reported that this "hemicellulose" increased during roasting, probably at the expense of the "holocellulose." They found little, if any, cellulose, lignin or pectin in the green bean. However, in a later publication Wolfrom and Patin (41) reported that the main structural polysaccharide was a 1-β-4 D-mannan. This was structurally associated with a glucan which was shown to be cellulose and which represented about 5% (dmb) of the green bean.

Courtois et al (36) reported a cold-water-soluble gluco-galacto-mannan and a boiling-water-soluble arabino-galactan. Patin later reported a similar arabino-galactan (42), and suspected that some difficulty in hydrolysis was due to the presence of aromatic esters. Ferulic acid has been reported present in some wheat flour pentosans (43).

Thaler and Arneth (44, 45) reported that raw Arabicas contain four fractions of polysaccharide which differ in solubility. One of these polymers was soluble in cold water; a second in hot water; a third fraction precipitated when the green coffee bean was treated with chlorine dioxide, (holocellulose); a fourth fraction, which was solubilized by the chlorine dioxide treatment, was called "degradation polysaccharide." Roasted coffee contained the hot-water-soluble fraction, the "holocellulose" and the "degradation polysaccharide." They reported that the cellulose was very stable during roasting, the mannan only slightly affected, the galactan partially destroyed, and the araban markedly destroyed, in proportion to the severity of the roast. The four Arabicas examined showed little variation. Traces of xylose, rhamnose and uronic acid were also released by hydrolysis of these four polysaccharide fractions.

Wolfrom and Andersen (46) reported an arabino-galactan was present in instant coffee. This pentose had lower arabinose content than the arabino-galactan found in the green bean and they suggested that easily cleaved terminal arabinose groups had been lost during brewing. There have been no reports of free arabinose. The same instant coffee also contained a galacto-mannan similar to that reported in the green bean by Wolfrom (41). It contained less galactose than the galacto-mannan reported by Thaler (44). Maier et al (47) isolated a mannan from the brown pigments of roasted coffee. This substance formed 3% of the brown pigments and resembled the mannan reported by Thaler (44).

In 1968 Shadaksharaswamy and Ramachandra extracted and analysed the acid-soluble polysaccharides of green coffee beans. This material contained rhamnose (1 part), arabinose (3 parts), mannose (3 parts), galactose (2 parts) and was present at about the same level in Arabicas and Robustas. They believed that some non-protein nitrogen was present in this polysaccharide.

(d) Nitrogen Content.

(i) Amino Acids and Proteins.

Protein content of green beans has been reported in the range 10-14% (dmb) (35, 38, 49) of which about 3% (dmb) is soluble in water. The alkali-soluble protein has been reported at levels of 2% and 4% of the green bean (dmb) (49, 50). No alcohol-soluble protein was found (44). Underwood and Deatherage (49) reported that the water-soluble proteins of Santos and Columbia coffees contained about 15% nitrogen and had an isoelectric point at pH 4.6-4.7. They showed that aqueous extracts of green and roasted coffee contained an appreciable quantity of non-protein, amino acid-containing compounds. These were probably proteoses, peptones and peptides, and in the roast bean, products of various browning reactions. The water extractable nitrogen fell rapidly on roasting (51).

Centi Grossi *et al* (52) examined green coffee bean water-soluble proteins by electrophoresis and separated twelve fractions. Arabicas and Robustas showed essentially identical patterns.

In 1956 Plunkett reported eleven free amino acids in green coffee beans, but found that they were completely lost on roasting (31).

Hydrolyses of green and roasted coffee beans, of extracted protein, and of pigments from the roasted beans have yielded 18 amino acids (13, 47, 51, 53-56). Thaler and Gaigl (13) reported that there were no significant differences between Arabicas and Robustas. They also reported that arginine was completely destroyed on roasting, and that cystine, lysine, serine and threonine showed considerable loss. Glutamic acid, leucine, valine, phenylalanine, and proline increased.

(ii) Alkaloid Nitrogen

According to Sivetz (57) the main alkaloids are caffeine, trigonelline and nicotinic acid. Theophylline and theobromine have also been reported in most green coffee beans (58-59). The wild species of coffee are reported to contain no caffeine, but instead a substance called cafemarine the structure of which has not yet been determined. The wild species do contain trigonelline (7).

Much of the stimulant activity of coffee has been ascribed to caffeine and trigonelline, but 'fat' and chlorogenic acid are also active (60, 61).

Hughes and Smith (62) reported that green coffee beans contain 16-44 mg/g nicotinic acid. During roasting, trigonelline demethylates to nicotinic acid (6, 11) and Tepley and Prior (26) reported about 100 g nicotinic acid in one gram of coffee. Sivetz (63) reported between 95-263μg/g. A cup of coffee probably contains about 1/10 the human daily dietary requirement of nicotinic acid, in an easily available form (64). (Tepley and Prior (26) reported that roast coffee also contained traces of seven other B vitamins).

Trigonelline has been reported in green coffee at various levels. Thaler and Gaigl (13) showed that Arabicas contained 1.0-1.2%, Robustas 0.6-0.7%, and Libericas 0.2-0.3%. They also reported (53) that loss in normal roasting was 30-60% but up to 80% in severely roasted Robustas. Trigonelline at fairly high concentrations has a bitter taste (66).

Severe roasting converts nicotinic acid to pyridine (6, 11, 53, 61) which has an objectional taste (11). Pyridine, and 2-methyl, 3-methyl and 3-ethyl pyridines have been reported in coffee volatiles (67).

Caffeine content of green coffee beans has been reported in the range 0.9-2.6%, Arabicas containing 0.9-1.3%, Robustas 1.6-2.6% and Libericas 1.2-1.5% (13, 29, 35, 38, 53). Roasting produces a progressive loss of up to 20% (35, 68), believed to be due to sublimation (at 176°), rather than decomposition, since it can be recovered from roaster flues (69). The comparison of the progressive loss of caffeine with the loss of chlorogenic acids has been used as an index of the degree of roast (70, 71). Caffeine is highly soluble in water and may form about 4.5% of a coffee brew (10% w/v) (28). Sivetz (57) reported that cups of coffee made from Arabicas and Robustas respectively contain about 100 mg and 200 mg of caffeine.

Some of the caffeine in the green bean has been reported in the free form, and some as a complex of 3CQ and 5CQ (72, 73).

(e) Non-Phenolic Acids

In 1959 Lentner and Deatherage reported that green coffee contained acetic, butyric, citric, malic, propionic and valeric acids (74). Sivetz (75) reported citric, malic, oxalic, pyruvic and tartaric acids at concentrations of 0.2-0.5% (dmb), in total about 1.5% (dmb). Roasted coffee has been reported to contain in addition citraconic, formic, fumaric, 2-furoic, iso-valeric, itaconic, lactic, maleic and mesaconic acids, and two which were not identified (74, 76-80). Lentner and Deatherage (74) reported that formic and acetic acids increased during roasting, and that destruction of citric acid was 33-56%, and destruction of malic acid 16-40%. Kaufman, (11) and Lentner and Deatherage (24) both reported that roasting caused a decrease in acidity of coffee bean aqueous extracts by one pH unit, followed by a slight pH rise. They believed that production of acids from sugars was responsible for the pH fall, and that decarboxylation of acids was responsible for the pH rise observed. Woodman et al (80) reported a rapid increase in the content of non-phenolic acids at a 15% total roast loss. The quantity of these acids did not decrease at high roasts and loss could not, therefore, be responsible for the pH rise. These workers believed that loss of chlorogenic acids was responsible for the pH rise.

Fumaric acid and 2-furoic acid content increased throughout the roast, and were probably produced from malic acid and carbohydrate respectively. Citraconic, mesaconic and itaconic acids were probably formed from citric acid.

(f) The Phenolic Compounds

(i) The Phenolic Compounds of Green Coffee Beans

Probably the earliest cited reference is to work by Robiquet and Boutron (82). They reported that in green coffee beans there was an acid which turned green when mixed with ferric chloride. Work began on the extraction of this acid (84), and in 1846 it was isolated as a caffeine complex, and named chlorogenic acid (85).

In 1907 attempts were made to analyse this substance, and successful alkaline fusion yielded caffeic acid (86). It was suggested that chlorogenic acid consisted of caffeic acid and a sugar. In 1920 Freudenberg (87) demonstrated the presence of quinic acid, and deduced that chlorogenic acid was a caffeoylquinic acid. Twelve years later Fischer and Dangschat (28) demonstrated that chlorogenic acid was 3-caffeoylquinic acid.

In 1950 Barnes et al (89) reported the isolation of "Isochlorogenic acid" from green beans, using countercurrent distribution and silicic acid column chromatography. They announced that "Isochlorogenic acid" was 5CQ, probably in equilibrium with its lactone. In 1956 Plunkett (31) confirmed the presence of 3CQ and "Barnes' Isochlorogenic acid".

In 1958 Smith (90) reported that there was no free caffeic acid in green coffee beans, and that the lower quality Robustas contained more chlorogenic acids than Arabicas. In 1958 Sondheimer (91) reported a new chlorogenic acid isomer in green beans, and called it "Band 510" because of its time of elution from a silicic acid column. He also made the first report of "Neochlorogenic acid" in green coffee beans, although it had previously been reported in fruit by Corse (92).

In 1959 Lentner and Deatherage (74) confirmed that there were only traces of free caffeic acid and free quinic acid in coffee beans. In 1960 Krasemann reported the presence of at least six phenolic acids, and doubted that caffeic acid was one of them. She did identify 3CQ, "Isochlorogenic acid" and "Band 510" (93). The same year Pictet and Brandenburger (94) reported the presence of 3CQ, "Isochlorogenic acid," "Neochlorogenic acid," and three FQ acids in green Arabicas and Robustas. In Robustas they also found 4HCA and/or one of its depsides with quinic acid. They stressed that the presence of free caffeic or ferulic acids were artefacts caused by careless handling of the samples. In 1962 Corse et al (95) isolated 3FQ from green Robusta and Arabica beans. Robustas contained about 0.1% (dmb), and Arabicas considerably less.

In 1963 Scarpatti and Guiso (96) reported that the fraction "Barnes' Isochlorogenic acid" was not 5CQ, but a mixture of three DCQ acids. They subsequently reported that "Neochlorogenic acid" was 5CQ, and that "Band 510" was 4CQ (73). They reported that the components of "Barnes' Isochlorogenic acid"were 3, 4 and 3, 5 and 4, 5 DCQ (97) and these results were confirmed by Nakanishi (98) and Corse et al (99), who also reported the presence of a mixture of the 3' methyl ethers of 3, 5 DCQ.

Kung et al (100) quantitated the CQ and DCQ acids of green Arabica beans by GLC. Table 3 summarises the quantitative results of several workers. Corse and Patterson (101) examined green beans for the presence of sinapoylquinic acids, but concluded that they were not present.

Table 3 The Content of Depsides in Green Coffee Beans.

Depside	Content % (dmb)					
3CQ	-	2.85	4.81	-	-	5.56
4CQ	-	0.39	-	-	-	0.41
5CQ	-	0.40	-	-	-	0.88
3FQ	-	-	-	-	less than 0.1	
3,4DCQ	-	-	-	-	-	0.28
3,5DCQ	-	-	-	-	-	0.21
4,5DCQ	-	-	-	-	-	0.11
Total	6.86-8.05	3.65	6.47	6.75	-	7.45
References	(68)	(91)	(93)	(94)	(95)	(100)

Table 4 The Content of Depsides in Roasted Coffee Beans.

Depside	Content % (dmb)*			
3CQ	-	1.22	-	1.96
4CQ	-	-	-	0.21
5CQ	-	-	-	1.02
3,4DCQ	-	-	-	0.01
3,5DCQ	-	-	-	0.09
4,5DCQ	-	-	-	0.01
Total	3.95-5.24	1.58	3.55	3.33
References	(68)	(93)	(94)	(100)

*Content in roasted bean - no correction for total dry matter lost during roasting.

Some of these phenolic compounds are thought to be present in the bean in more than one form. In 1958 Lindner (102) reported that green bean chlorogenic acids were present as the free acids, the potassium salts, and the potassium caffeine complexes. Griebel (103), and Smith (29, 90) suggested that there were no free acids. The existence of the caffeine complexes of 3CQ and 5CQ has been confirmed (72, 73), but there have been no reports of other caffeine complexes. Northmore thought that magnesium chlorogenates were present in the green bean (30).

In 1968, Thier *et al* (103) reported that Robustas contained 0.3-0.4mg % scopoletin, about four times as much as Arabicas. It is generally believed that Robustas also contain a greater quantity of chlorogenic acids than Arabicas (6).

(ii) The Influence of the Phenolic Compounds on the quality of the Green Beans.

Northmore (30) reported that the colour of green Kenyan coffee beans was related to the liquoring quality after roasting. In order of decreasing quality blue, green, brown and yellow colours are encountered. He reported that the oxidation of alkaline magnesium chlorogenate produced a blue colour similar to that of the higher quality green bean.

De Amorim and Silva (104) reported that the higher the green bean Polyphenol Oxidase activity, the higher the quality. Forsyth (105) has reported that the quinones formed by this enzyme are self-inhibitory. It has been reported that polyphenols and acids such as the chlorogenic acids and caffeic acid act as antioxidants for aldehydes, and it has been suggested that the higher the aldehyde content of the green bean, the higher the liquoring quality after roasting (104, 106).

It is suggested on the basis of this information that if Polyphenol Oxidase attacks the chlorogenic acids, quinones are formed, and the aldehydes are no longer protected. The aldehydes are lost, and the bean quality falls. The quinones inhibit the enzyme and cause the low Polyphenol Oxidase activity associated with low quality beans. Other workers (107, 108) have shown that the quinones produced by Polyphenol Oxidase take part in non-enzymic reactions that produce insoluble brown pigments. Such pigments could be involved in the brown and yellow colours that Northmore (30) reported to be typical of low quality beans.

(iii) The Effects of Roasting.

It has been reported (6) that after roasting, Robustas and Arabicas contain about the same quantity of chlorogenic acids, despite the fact that green Robustas contain more than green Arabicas.

Kaufman (11) believed that the lower acidity of coffee solubles extracted from relatively severe roasts was as a result of chlorogenic acids decarboxylation and more recently Woodman et al (80) confirmed this. Kaufman believed that some of the minor components of the aroma were produced by this degradation.

In 1955 Lindner (102) reported that the chlorogenate caffeine complexes were the least stable form of chlorogenic acids during roasting, but Smith (29, 90) reported that they were the most stable. In 1958 Smith (90) reported that the greatest loss of chlorogenic acids occurred at the stage when the coffee aroma and flavour were developing, and stressed in 1963 (29) that depside and cinnamic acid pyrolysis should be studied to determine its connection with flavour development.

Lentner and Deatherage (74) reported that an increase in quinic acid content occurred during roasting. They assumed that this was due to depside hydrolysis, but reported that the observed increase was not sufficient to account for the recorded loss of depsidic quinic acid. They suggested that quinic acid degradation occurred, perhaps via its lactone. They reported that 30-50% of green bean chlorogenic acids were destroyed during roasting, the loss being directly related to the severity of the roast. Merrit and Proctor (68) essentially confirmed these results, by recording losses of 24-45%. Lentner and Deatherage purified roast bean extracts by paper chromatography, eluted the phenolic components and quantitated these by titration against alkali. They stressed that direct UV spectrophotometry, eg the AOAC technique (109) was not suitable for quantitating chlorogenic acids in roast bean extracts because of possible interference by non-phenolic products of roasting. The AOAC technique was used by Merrit and Proctor. Pictet and Brandenburger (94) reported a 47% loss of chlorogenic acids from an Arabica Colombia bean. The phenolic components were purified by paper chromatography and quantitated by direct UV spectrophotometry.

Krasemann (110) reported that roast beans contained seven phenolic components compared with five in the green bean (93). She reported that 3CQ when roasted was immediately converted to the other four green bean phenolic compounds, plus two new products. A seventh product was only found in roasts of medium severity. Kung et al (100) reported that during roasting 3CQ was 65% destroyed, 4CQ was 42%

destroyed and that 5CQ increased to 116% of its green bean content. They thought that this was due to inter-isomerisation of the other CQ or DCQ acids. Two of the DCQ acids were almost completely destroyed (more than 90%) but 3,5DCQ was much more stable and about 50% survived roasting. A summary of the quantitative results is given in Table 4.

Hogl (111), and Smith (29) reported the presence of quinol, pyrogallol, protocatechuic aldehyde and catechol in roasted coffee. It was suggested that quinol and pyrogallol were produced from the quinic acid component of chlorogenic acids, and that protocatechuic aldehyde and catechol were produced from the caffeic acid component.

Klocking <u>et al</u> (55) isolated the "humic acids" of roasted coffee by lead acetate precipitation from aqueous extract. After removal of the low molecular weight components by gel filtration chromatography, the "humic acids" were hydrolysed. Six phenolic compounds were isolated and caffeic acid, catechol and 4-hydroxybenzoic acid were identified. Maier <u>et al</u> (47) isolated eight fractions from water-soluble roast coffee pigments by gel filtration. Four of these fractions were reported to contain phenolic compounds.

(iv) <u>The Effect of Brewing.</u>

Natarajan <u>et al</u> (112) examined 5% $^{w}/_{v}$ brews of coffee which were stored in glass and brass containers at 25° and 70° for up to 16 hours. No loss of chlorogenic acids was observed. Segall and Proctor (113) reported a 12% loss of chlorogenic acids from coffee brews held at 73° for 6 hours. This was greater than the loss from solutions of pure 3CQ held under the same conditions. Segall and Proctor (113) did not refer to the work of Natarajan <u>et al</u> (112), and these contrasting results have never been explained.

By means of a Taste Panel Ordynsky (66) established that 3CQ in coffee brew has a bitter taste.

(g) Coffee Aroma and Coffee Volatiles.

In 1969 Walter and Weidemann (67) published a list of substances that have been identified in coffee volatiles. This excellent review lists each reported component by its empirical formula, and by classification as alcohol, ether, aldehyde, ketone, diketone, ester, phenol, sulphur compound or furan. There is also a chronological list showing the date of the first report of any component. To summarise, 355 components have been identified, 44 of which were known before 1929, and 71 before 1960. A summary of the major classes is shown in Table 5.

Table 5 The Components of Coffee Aroma (67).

Alcohols	23	Ethers	16	Aldehydes	30
S compounds	41	Esters	32	Ketones	64
Furans	58	Phenols	26	Diketones	25
Pyrazines	26	Pyridines	4		
				Total 345	

Major progress has only been made since the introduction of gas chromatography, mass spectrometry, and nuclear magnetic resonance spectrometry as aids to separation and identification. The achievements of the earliest workers, Bernheimer 1880, (114) Monari and Scoccianti 1895 (115), Jaeckle 1898 (116), Erdmann 1902 (117) and Staudinger and Reichstein 1928 (14) deserved special mention since they had only classical, and indeed primitive, techniques and equipment available, and yet all their discoveries have since been confirmed.

There is nothing to be gained by giving full details of each worker's contribution between 1930 and 1963 since that is available from the review (67), but brief mention will be made of certain more interesting points. Hughes and Smith (119) probably made the first quantitative study of coffee volatiles in 19~~6~~59, and Rhoades (120) in 1968 was probably the first person to apply gas chromatography to coffee volatiles. Merit et al (120) commented on the possible production of artefacts during the sampling of the volatiles when heat, steam, water or a solvent were used in the extraction. They used an inert carrier gas to sweep the volatiles from roasted coffee at room temperature, and condensed them in a series of cold traps. They suggested that furan and 2-methyl furan were of particular significance in coffee aroma.

Most workers examined only components of low boiling point, but Radtke (122-124) divided the components into 3 groups - those with boiling points below 100° (Fraction A), those in the range 100°-220° (Fraction B) and those above 220° (Fraction C). The components of Fraction B were subdivided into monocarboxylic acids, phenols, carbonyls and nitrogenous compounds.

Removal of only the phenols from fraction B left a 'roast aroma', but it was definitely not a coffee aroma. Removal of only the nitrogenous compounds altered the aroma, but it was still distinctly coffee like. Removal of only the carbonyls made the greatest alteration to the aroma.

Remixing of the isolated carbonyls and nitrogenous compounds, or remixing of the isolated phenols and carbonyls, produced coffee-like aromas. Addition of the isolated nitrogenous compounds to the phenols and carbonyls produced quite a realistic coffee aroma.

When the aroma concentrates were stored very marked colour changes occurred in a short time, but the pattern of volatiles separated by GLC showed no significant changes. The techniques employed were unable to detect the vital changes that were responsible for staling.

Bondarovitch et al reported that certain dihydropyrazines had aromas reminiscent of roasted peanuts or popcorn and they believed that similar compounds could be of importance in the aroma of many cooked or roasted foods. Unfortunately dihydropyrazines are rather unstable and as a result their isolation is difficult (125), but the presence of pyrazines has been established. Twentysix pyrazines have been reported in coffee aroma (67), and 19 in roasted cacao aroma, of which six are also present in coffee aroma (126, 127), and a range of pyrazines have also been found in roasted peanut aroma (128). Such compounds were almost certainly present in Radtke's 'nitrogenous compound' group of volatiles.

The wide range of volatile compounds that has been reported (See Table 5) arises from a range of complex pyrolytic reactions that occur during roasting. Pyrazines, pyridines and imidazoles also (in certain cases) have been found amongst the products of reactions between sugars and ammonia (129-132), carbohydrates and amino acids (133), and pyrolysis of serine (134).

Pyrolyses of sugars and polysaccharides give rise to a wide range of volatiles including aliphatic carbonyls, alcohols and acids, furans, and cyclic diketones (135-141), and further interaction with amino acids in Maillard type ractions extends the range of products

(142). Pyrroles could be produced by similar mechanisms to furans, but involving a nitrogen-containing fragment (134, 142, 143). The pyrolysis of sulphur-containing amino acids has been shown to produce a wide range of volatiles, many of which contain sulphur. Since only traces of thiamine are present (26) it can probably be assumed that the sulphur-containing coffee volatiles are derived from these amino acids.

A range of phenolic substances have been reported amongst coffee volatiles (See Table 6). Gautschi et al (134) believed that these phenols were produced mainly from chlorogenic acid, but thought that certain amino acids might also be involved. A few phenolic compounds have also been reported as pyrolysis products of lignin, cellulose, sugars, caffeic acid and ferulic acid (139, 145-148). Phenolic compounds have also been reported amongst the volatiles of roasted cacao and barley (126, 149). (See Table 6).

Table 6 Phenolic compounds found amongst the volatiles from roasted coffee beans and their occurrence amongst volatiles from other pyrolyses

Present in Coffee Volatiles	Caffeic Acid	Ferulic Acid	Lignin	Cellulose	Sugars	Barley	Cacao
	Produced by roasting						
Phenol	+		+	+	+	+	+
2-cresol	+		+	+			
3-cresol	+		+	(+)		+	
4-cresol	+		+				
2,3-dimethyl phenol							
2,5-dimethyl phenol							
2,6-dimethyl phenol							
3,5-dimethyl phenol							
2-ethyl phenol	+						+
4-ethyl phenol							
2,3,5-trimethyl phenol							
Guaiacol			+		+		+
4-ethyl guaiacol		+					
4-vinyl guaiacol		+					
Eugenol							
2-hydroxybenzaldehyde							
Methyl-(2-hydroxy phenyl) ketone							+
Methyl-(5-methyl 2-hydroxy phenyl) ketone							
Methyl vanilloyl ketone		+					
Methyl salicylate							
Catechol	+					+	
Resorcinol						+	
Quinol			+				
4-vinyl catechol							
Methyl-(2,3-dihydroxy phenyl) ketone							

THE PRECURSORS OF FOOD FLAVOURS

In the preceding section, the results of many model systems were summarised and mechanisms suggested to account for the production of the wide range of compounds that have been identified in roasted coffee volatiles. It is interesting to note that examinations of raw beef (150, 151), raw chicken (152), raw lamb (153), raw cacao (154) and raw barley (155) have shown that the water-soluble low molecular weight substances, when roasted, are capable of producing an aroma typical of the whole roasted foodstuff. In the case of raw groundnut (133, 156-7) and raw coffee (158) the workers believed that proteins and possibly water-soluble polysaccharides were also involved in aroma production. Rohan (159) reported that the precursors of cacao aroma included quercetin, quercetin-3-glucoside, caffeic acid, 4-hydroxycinnamic acid and 3CQ. Wang and Sakurai (155) did not consider the phenolic components of barley to be essential for aroma production, but Shimizu *et al* (149) identified 27 phenolic compounds in roasted barley volatiles, which suggests that phenolic compounds make some contribution to the aroma. Hassan (158) stated that the aroma fraction of green coffee beans included phenolic compounds.

Since there are indications that phenolic compounds are involved in the aroma production of roasted barley, cacao and coffee, it was decided to investigate the behaviour of coffee bean phenolic compounds during roasting, in more detail and with greater accuracy than has previously been possible.

CHAPTER TWO

EXPERIMENTAL, RESULTS AND DISCUSSION

CHAPTER TWO

SECTION ONE

EXTRACTION OF THE PHENOLIC COMPOUNDS FROM GREEN AND ROASTED COFFEE BEANS

1. EXTRACTION OF PHENOLIC COMPOUNDS FROM GREEN AND ROASTED COFFEE BEANS.

Introduction.

To facilitate extraction, the beans must be reduced in size. Waiss (109) reported that grinding of green coffee beans caused a loss of chlorogenic acid, the loss being related to the degree of size-reduction. He also reported that the relationship between degree of extraction and particle size was obscure.

Reference to the literature showed that previous workers in this field had used an aqueous alcohol as the extraction solvent. Lehmann and Hahn (65) used aqueous methanol; Plunkett (31), Smith (90) and Kraseman (110)used aqueous ethanol; Barnes, Feldman and White (89), Sondheimer (91), Pictet and Brandenburger (94), Sondheimer, Szymanski and Corse (72), Gnagy (160), Khana *et al* (161) used aqueous 60-70% propan-2-ol.

Discussion.

The loss of chlorogenic acid during grinding is due, partially, to the heat generated. Green Arabica beans are extremely hard, and the energy expended and heat generated during their grinding is not inconsiderable. To keep any chlorogenic acid loss to a minimum, a short grinding period is desirable. A relatively long extraction is then desirable to compensate for the relatively larger particle. Typical of the conditions previously employed are those reported by Gnagy (160), ie grind to pass 100 mesh, one extraction by shaking 5.0g with 100ml 70% propan-2-ol for three hours.

Most other workers have used aqueous propan-2-ol and it was considered suitable for this project.

The method adopted was as follows:-

Beans (0.5Kg) were ground by gradually passing once through a Minikek Pin Mill (Kek Ltd, Macclesfield, Cheshire) operating at steady speed conditions. The powdered beans were collected in a calico bag and fractionated on a series of BS Sieves. That fraction passing through 20 mesh and remaining on 40 mesh was used for all extractions. The mill was dismantled and cleaned thoroughly between samples.

Extraction.

Duplicate 10.0g samples of ground bean were weighed into 500ml round-bottom glass-stoppered flasks and shaken for 30 minutes with 100ml 70% propan-2-ol. The suspension was allowed to settle and decanted onto a Whatman No. 1 filter paper. The residue in the flask was re-extracted four times.

The filtered extracts were bulked and made up to a volume of 1 litre. These extracts were stored in the dark at -10^{o}. Before use the extracts were allowed to come to room temperature and were thoroughly shaken to redistribute any material which had been precipitated at the low storage temperature.

CHAPTER TWO

SECTION TWO

QUANTITATION OF CHLOROGENIC ACIDS

QUANTITATION OF CHLOROGENIC ACIDS.

Introduction.

Quantitative methods for phenolic compounds have been reviewed by Swain and Goldstein (112). They described two basic methods for the quantitation of chlorogenic acids.

1. Methods utilising the strong UV absorption that these compounds exhibit.
2. Methods utilising chromogenic reagents and colorimetry.

1. UV spectrophotometry.

Chlorogenic acids and closely related substances show UV absorption maxima in the range 305-330nm. The AOAC Standard Method for coffee beans compares the absorption of a coffee bean extract at 328nm with the absorption of standard 3CQ at the same wavelength. It is not possible to take a blank reading with this technique.

Vicinal dihydroxy phenols are able to chelate certain ions such as Al^{3+}, Fe^{3+}, borate and acetate. These ions cause a bathochromic shift in the spectrum. Krupinkova (163) reported that ethanolic aluminium chloride produced a shift of 35nm in the spectra of 3CQ, 5CQ and caffeic acid. Monohydroxy derivatives such as 3FQ, 5FQ and 3CoQ and 5CoQ showed shifts of only 2nm or 3nm.

The bathochromic shift can be utilised for determination of vicinal dihydroxy compounds, eg if the 'total' phenols are determined by UV absorption at a particular wavelength as in the AOAC method and measured again at the same wavelength after addition of eg aluminium chloride, the nett change in the absorption is directly proportional to the content of vicinal dihydroxy compounds. To ensure accurate results, the pH must be standardised, and competing ligands such as citric acid must be excluded (164).

Molybdate (MoO_4^{2-}) also produces a bathochromic shift, sufficient to move the absorption into the visible region, and this will be dealt with in the next section.

2. Use of chromogenic reagents.

Phenolic compounds are many and varied in their physical and chemical properties. As a result, some of the methods described by Swain and Goldstein are not applicable to chlorogenic acids. These include the use of Gibbs Reagent, 4-aminophenazone, and vanillin. The first two reagents must be used in alkaline conditions, and in alkali, vicinal dihydroxy compounds are unstable (165). The Vanillin Reagent (in strong acid) is reported to be selective for 2,4-dihydroxy compounds (162), whereas chlorogenic acids are 3,4 substituted.

Nitrosating reagents.

The commonest nitrosating reagents are reported as p-nitroaniline and sulphanilic acid (162). The product of the reaction is a nitrosophenol, but the wavelength and intensity of maximum absorption are dependent upon the phenol. All reagents must be freshly prepared, and it may be necessary to carry out the reaction at low temperature to ensure stability of the colour (162, 166).

This type of reagent is used extensively for location of phenolic compounds on chromatograms. The range of colours produced with different compounds is of some diagnostic value (167, 168, 169). For example, diazotised sulphanilic acid produces a violet colour with ferulic acid, brown with caffeic, and red with 4-coumaric acid (168).

Phosphomolybdate and phosphotungstate reagents.

There are several reagents differing slightly in composition. The principle involved is the reduction by a phenol of a mixture of phosphomolybdate and phosphotungstate to a complex mixture of hydrated molybdenum and tungsten oxides. This mixture, often known as molybdenum or tungsten blue, has a wide absorption maximum in the region of 725 nm.

Sodium Molybdate reagent.

A complex is produced between this reagent and vicinal di- and trihydroxy compounds. The molybdate produces a bathochromic shift and maximum absorption is just outside the visible region. The peak is broad and an intense yellow or orange colour is produced, the exact colour depending on the pH and concentration of the reactants. Swain and Hillis (170) used 5% sodium molybdate at pH 6.5 (phosphate buffer). The increase in absorption, at the wavelength of maximum absorption of the complex, is proportional to the amount of sensitive phenolic compound present.

Discussion.

As reviewed in Chapter I, the major phenolic components of the coffee bean are the CQ acids and DCQ acids. There are also some reports of a trace of FQ acids. The ideal quantitative method would be one which gave equal emphasis to the three CQ acids, the DCQ acids and the three FQ acids, but which was not subject to interference by non-phenolic compounds.

Gibbs Reagent, 4-aminophenazone and the Vanillin Reagent are quite unsuitable for the chlorogenic acids. The direct UV spectrophotometric method can give equal emphasis to all the components only if they have the same specific extinction at the chosen wavelength. According to Krupnikova the range of wavelength for maximum absorption for the FQ acids and CQ acids is between 315 and 330nm. The CoQ acids are further outside this range (163). As a result it is unlikely that this type of technique could give equal emphasis to all the components.

A greater disadvantage is the sensitivity to non-phenolic compounds which absorb at the chosen wavelength, but for which no blank value can be taken. Hodge (171) in his review of non-enzymic browning showed that many products of this group of reactions absorb strongly in the UV. Interference from this source could become very significant when examining roasted beans where the chlorogenic acid content had decreased and the browning products were present at a high level.

The use of an agent producing a bathochromic shift overcomes interference from non-phenolic compounds. However, it is specific for vicinal dihydroxy compounds and would not allow measurement of the FQ acids. Ligands competitive for Fe^{3+} or Al^{3+} might be present in the roasted beans, causing interference which could give rise to anomalous results. The use of an excess of the ion might overcome that difficulty. Molybdate, acetate and borate are probably less prone to this type of interference since no mention of it appears to have been made in the literature. The use of molybdate will, therefore, allow specific determination of the CQ acids by a method which should be free from interference by non-phenolic compounds.

Nitrosating reagents require careful control of reaction conditions, particularly temperature, to give reliable results. The nitrosophenols vary considerably in colour and this fact makes the reagent useful for location and identification of spots on chromatograms. In the case of a quantitative technique, because of the range of colours, it would be very difficult to choose a suitable wavelength at which to measure total chlorogenic acids.

When the phosphomolybdate and phosphotungstate reagents are used, colour is produced when the reagents are reduced; there is the possibility of interference from reducing agents other than phenols. Results published by Swain and Hillis (170) show that colour intensity varies considerably with variation of the hydroxylation pattern of the phenol involved. Vicinal dihydroxy compounds generally gave a strong response, but phenolic compounds which are more difficult to oxidise, eg monohydroxyphenols and 2,4-dihydroxyphenols gave considerably less response. Thus a weak response could be expected from the FQ acids.

Since all these established techniques have their limitations when applied to the accurate measurement of mixtures of CQ acids and FQ acids, it was decided to consider whether alternative methods might be available.

All these aforementioned techniques measure the phenolic portion of the depside ie caffeic acid, ferulic acid, 4-cinnamic acid or occasionally sinapic acid or gallic acid; problems arise because of this variation. The structural characteristic common to all depsides is the presence of quinic acid. It was felt that measurement by reaction with quinic acid might be more satisfactory and allow equal emphasis to be given to all depsides.

Cartwright and Roberts (172) had reported a method for location on chromatogram of quinic acid, and other closely related compounds. This locating reagent was examined with the intention of producing a colorimetric reagent for quinic acid depsides.

CHAPTER TWO

SECTION THREE

CARTWRIGHT AND ROBERTS' REAGENT

CARTWRIGHT AND ROBERTS' REAGENT.

Introduction.

During the course of work on determining the structure of theogallin, Cartwright and Roberts (172) developed a spray reagent for detecting quinic acid, quinic acid lactone (quinide) and shikimik acid (dehydroquinic acid) on chromatograms. The spots were located by spraying the chromatogram with a one third-saturated solution of sodium metaperiodate, and twenty minutes later spraying with a solution of sodium nitroprusside (0.4% $^{W}/_{v}$) and piperazine (0.4% $^{W}/_{v}$) in 85% ethanol. The chromatogram was then heated for five minutes at 100°. Quinic acid was detected as an orange spot, sometimes with a green edge, and shikimik acid was grey.

No explanation was offered for the mechanism, but periodate is widely used for rupturing glycol linkages. Two such linkages are present in quinic acid, and shikimik acid.

This reagent was applied to the location of a range of quinic acid-containing depsides.

LOCATION OF QUINIC ACID DEPSIDES BY CARTWRIGHT AND ROBERTS' REAGENT.

Materials.

Solutions (5mg/ml) of quinic acid, 3CQ, 5CQ, 3CQ caffeine potassium salt and 3FQ were prepared in 70% propan-2-ol. They were stored in stoppered glass vials.

Saturated sodium metaperiodate solution was prepared by gradually adding the analytical grade reagent to warm distilled water, and stirring until no more would dissolve. The solution was cooled and agitated to aid crystallisation. The cooled reagent was filtered and diluted with twice its own volume of distilled water.

The solution containing sodium nitroprusside (0.4% $^{w}/_{v}$) and piperazine (0.4% $^{w}/_{v}$) was prepared by dissolving the appropriate weight of reagents in 85% ethanol.

Procedure.

The depside solutions (2-3µl) were applied to Whatman No. 1 chromatography paper using a micro-pipette. Solvent was removed by a stream of warm air. Location was by the technique of Cartwright and Roberts (172). (See preceding section).

Results and discussion.

It was unnecessary to apply Cartwright and Roberts' full technique for location of the depsides. Spraying with one third-saturated periodate rapidly produced an orange-yellow spot. Quinic acid was not located by periodate alone, but on completing the technique an orange spot was produced, without any particular changes in the appearance of the depside spots. This suggested that the phenolic component of the depside was involved in colour production with periodate.

Two possible explanations were considered:-

1. The periodate oxidised the phenolic compound, and was itself reduced to iodine, the iodine being responsible for the yellow colour.
2. The yellow colour was the oxidation product of the phenol, iodine may also have been present.

The location of the 3FQ in addition to the CQ acids was of interest because it suggested a possible basis for a quantitative method for both FQ and CQ acids.

An experiment was performed to examine the reaction mixtures for the presence of iodine.

EXAMINATION OF THE DEPSIDE OXIDATION PRODUCTS FOR THE PRESENCE OF IODINE.

Materials.

Solutions of depsides as previously described.

Starch solution (1% $^w/_v$), prepared by adding 1g of soluble starch to 100ml of deionised water, and boiling until gelatinisation had taken place. This was allowed to cool before use.

Periodate solution, as previously described.

Procedure.

Depside solution (1ml) was added to periodate (5ml) in a stoppered test tube. The reagents were mixed by shaking and the yellow-orange colour allowed to develop. A few drops of starch solution were added and the mixture shaken again.

Results and discussion.

Addition of starch solution had no detectable effect on the colour. There was no suggestion of the purple clathrate compound that is formed by starch and iodine. This suggests that iodine is not responsible for the yellow colour. Slight fading of the colour was noticed over a period of about ten minutes, suggesting the possibility of over-oxidation.

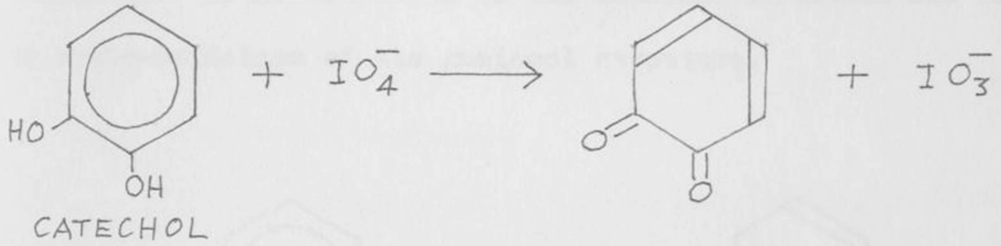
If the colour was due to oxidation of the phenolic compound, it was felt that this reagent could be valuable for colorimetric determination of those phenols. Such a technique would not be subject to interference by substances which did not react, or which reduced periodate but did not produce the yellow colour. The ability to detect FQ acids in addition to CQ acids would be a valuable asset of such a technique.

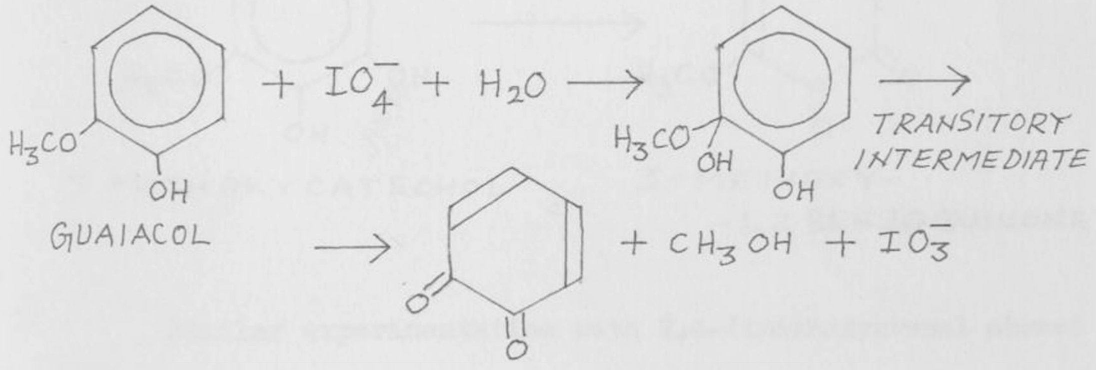
It was decided to investigate this reaction in more detail.

THE PERIODATE OXIDATION OF PHENOLIC COMPOUNDS.

Introduction.

Adler (173) and Adler *et al* (174) reported on the effect of aqueous periodate on guaiacol and catechol. They proposed a reaction mechanism and reported that the yield of methanol from guaiacol was almost stoichiometric. Estimation of the methanol was used to determine the degree of methylation of lignins.



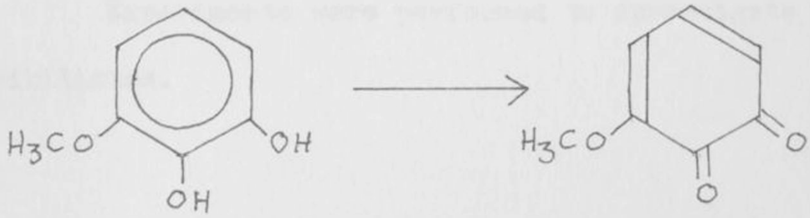


The use of O^{18} labelled water showed that when a new hydroxyl group was inserted in the ortho position it had been derived from the water (175).

When 3-methoxycatechol, and 2,6-dimethoxyphenol were treated with periodate, the products isolated were the ortho-benzoquinones, para-benzoquinones; dimers thereof; and 3,8-dimethoxy-1,2-naphthoquinone and 3,5-dimethyoxy-1,2-naphthoquinone. The dimeric ortho-benzoquinones and dimeric para-benzoquinones could be converted into the naphthoquinones by further periodate oxidation (176). The use of an organic solvent favoured production of the violet 3,5-dimethoxy-naphthoquinone; use of an aqueous solvent favoured production of the red 3,8-dimethoxy-naphthoquinone.

They also found that increasing the phenol concentration relative to the periodate, or increasing the time or temperature of the reaction increased the yield of the naphthoquinones at the expense of the benzoquinones.

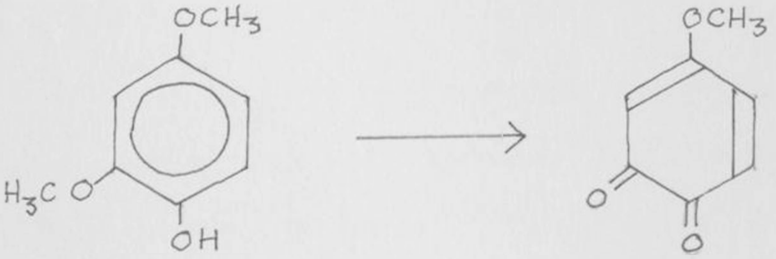
Examination of the products of the oxidation of 3-methoxycatechol showed a 60% yield of 3-methoxy-1,2-benzoquinone. This showed that ortho-oxidation of the catechol structure was favoured to ortho-oxidation of the guaiacol structure.



3 METHOXYCATECHOL

3-METHOXY-
-1,2 BENZOQUINONE

Similar experimentation with 2,4-dimethoxyphenol showed that ortho-oxidation of the guaiacol group was favoured to para-oxidation.



2,4 DIMETHOXY PHENOL

4-METHOXY-
-1,2 BENZOQUINONE

The UV spectra of the purified products was examined; all had maxima in the range 320-450nm.

This series of observations by Adler and his colleagues suggested that periodate could be used as a locating reagent for ortho- or para-dihydroxy phenols and their monomethyl ethers.

Of greater importance, was the possibility of developing a quantitative colorimetric technique which simultaneously measured ortho-dihydroxy phenols and their monomethyl ethers, eg CQ and FQ acids. Moreover, since the products from the dihydroxy compound and its monomethyl ether were reported to be essentially the same it was very possible that such a technique would give equal emphasis to both components.

Experiments were performed to investigate these possibilities.

THE EXAMINATION OF AQUEOUS METAPERIODATE AS A LOCATING REAGENT FOR PHENOLIC COMPOUNDS WITH VARIOUS SUBSTITUTION PATTERNS.

Materials.

Each available phenolic compound (See Table 7) was dissolved in 70% propan-2-ol at a concentration of 5mg/ml and the solutions stored in stoppered vials.

Aqueous sodium metaperiodate solutions were prepared at the following concentrations:- (a) saturated; (b) 5% $^w/_v$; (c) 0.5% $^w/_v$.

Three activated Silica Gel G TLC plates were prepared in the conventional manner. (See Appendix B).

Procedure.

One TLC plate was sprayed with one of the periodate solutions in the manner normally used for location, and the plate allowed to dry. Each phenolic solution was applied to each plate by means of a micropipette at levels of 1 μg, 5 μg and 10 μg. Each set of spots was examined for colour development before proceeding to the next phenolic compound.

Results (Table 7) were recorded as negative when no detectable colour was produced within ten minutes. Positive results were recorded as the lowest quantity of phenolic compound (μg) to be readily detected.

Results and discussion.

Ortho and para-dihydroxy compounds and their monomethyl ethers

Table 7 Sensitivity of Phenolic Compounds to the Periodate Reagent.

Phenolic Compound	Strength of Periodate			Phenolic Compound	Strength of Periodate		
	Sat.	5%	0.5%		Sat.	5%	0.5%
MONOHYDROXY							
2HBA	-	-	-	2HCA	-	-	-
3HBA	-	-	-	3HCA	-	-	-
4HBA	-	-	-	4HCA	-	-	-
2HBzAl	-	-	-	Methyl phenyl ketone	-	-	-
3HBzAl	-	-	-	Tyrosine	-	-	-
4HBzAl	-	-	-				
VICINAL DIHYDROXY							
2,3DHBA	1	1	1	3CQ potassium	1	1	1
3,4DHBA	1	1	1	3CQ caffeine salt	1	1	1
3,4DHBzAl	1	1	1	5CQ	1	1	1
3,4DHCA	1	1	1	1,4DCQ	1	1	1
3,4DHBz	1	1	1	3,5DCQ	1	1	1
DOPA	1	1	1	4,5DCQ	1	1	1
VICINAL TRIHYDROXY							
3,4,5THBz	1	1	1	3,4,5THBA	1	1	1
PARA-DIHYDROXY							
1,4DHBz	1	1	1	2,5DHBA	1	1	1
2,5DHBA	1	1	1				
META-DIHYDROXY + META-TRIHYDROXY							
2,4DHBA	-	-	-	2,4DHBzAl	-	-	-
2,6DHBA	-	-	-	2,4DHBz	-	-	-
3,5DHBA	-	-	-	2,4,6THBz	-	-	-
VICINAL HYDROXY-METHOXY							
3H, 4MBA	1	1	5	3H, 4MBzAl	1	1	5
3M, 4HBA	1	1	1	3M, 4HBzAl	1	1	1
3,4DM, 4HBA	1	1	1	3,5DM 4HBzAl	1	1	1
3H, 4MCA	1	1	1	3M, 4HCA	1	1	1
3,5DM 4HCA	1	1	1	3M, 4HBz	1	1	1
3,5DM 4HBz	1	1	1	Methyl vanilloyl ketone	1	1	5
3FQ	1	1	1	Methyl syrinyoyl ketone	1	1	1
MONOMETHOXY							
3MBzAl	-	-	-	4MBzAl	-	-	-
2MCAl	-	-	-				
DIMETHOXY + METHYLENEDIOXY							
3,4DMBA	-	-	-	3,4DMBzAl	-	-	-
3,4DMCA	-	-	-	3,4MDBzAl	-	-	-
				3,4MDCAl	-	-	-
OTHERS							
BzAl	-	-	-	CAl	-	-	-
4 isopropyl BzAl	-	-	-	3,4,5TMBzAl	-	-	-
Phenyl propional	-	-	-	Phenyl ethanal	-	-	-

responded, and the presence of other substituents such as carboxyl, aldehyde or propenoic acid did not interfere. Phenolic compounds with other substitution patterns gave no responses. The colour was stable indefinitely. The 5% periodate reagent easily detected all the sensitive compounds at the 1 μg level. The saturated reagent behaved identically, but was less useful because it tended to precipitate and block the spray gun nozzle, and to etch the glass vessels in which it was stored. It was also expensive to prepare. The 0.5% periodate reagent would not detect vanillin, vanillic acid or vanilloyl methyl ketone at the 1 μg level, but would just do so at the 5 μg level. It was thought that this reagent might produce false negative results with low concentrations of the phenol.

All the monomethyl ethers were slightly slower to respond than the corresponding dihydroxy compound, even with the saturated reagent, and this would be accounted for by the intermediate hydroxylation postulated by Adler and Magnusson (175). The greater the quantity of phenol applied, the deeper was the colour produced, and the colour produced at the 10 μg level of the methyl ether was noticeably redder than with the corresponding dihydroxy phenol. Adler reported (174) that an increased yield of the red and violet naphthoquinones was obtained at the expense of the yellow benzoquinones when relatively high levels of phenolic compound were oxidised.

It was immediately apparent that the structure-specific properties of these periodate reagents would be very useful, particularly so because these properties were distinctly different from, and complementary to, the properties of the established diagnostic reagents eg:-

1. Diazotised Sulphanilic Acid (DSA) (162, 167, 169). Detects any compound with one or more phenolic hydroxyl groups.

2. Sodium Molybdate Reagent (170). Detects vicinal di- and tri-hydroxy phenols.

   Vanillin (in concentrated acid) (162). Reported to detect most phenols with a 2,4-dihydroxy arrangement.

3. Wiesner Reagent (168). Reported to detect benzaldehydes and cinnamaldehydes, but not aromatic ketones. (It was found during the course of this work that this reagent also located certain furans. (See chapter 2, Section 10, page 113).

Conclusions.

Sodium metaperiodate (5%) is a valuable locating reagent which detects ortho and para-dihydroxy phenols and their monomethyl ethers. It can detect microgram quantities.

It is a valuable reagent for deducing the structure of unknown phenolic compounds, especially when used with other substituent - specific reagents.

The results suggest that reaction with periodate could be developed into a colorimetric technique that would give equal emphasis to CQ acids and FQ acids - a facility absent from established quantitative technieques.

CHAPTER TWO

SECTION FOUR

THE PERIODATE REAGENT

ESTABLISHMENT OF A QUANTITATIVE TECHNIQUE USING A PERIODATE REAGENT.

Introduction.

If the reaction sequence proposed by Adler (173) is correct, (See page 42), an ortho-dihydroxy phenol and its monomethyl ethers will be oxidised by periodate to virtually identical products. Therefore the molar extinction coefficients (MEC) of, for example, caffeic acid and ferulic acid, or CQ acids and FQ acids, should be virtually identical. The development of a colorimetric technique with this property would allow accurate quantitation of the depsides present in the green coffee bean.

Determination of the optimum conditions for quantitation, by periodate oxidation, of caffeic acid, ferulic acid and their depsides.

Materials.

Caffeic acid, ferulic acid, isoferulic acid and 3CQ were dissolved in 70% propan-2-ol at three concentrations. Due to a shortage of material the following were prepared at one concentration:- 3FQ; 3CQ caffeine potassium salt; 5CQ; 1,4 DCQ.

Periodate reagents were prepared at eight concentrations:- 0.05%; 0.10%; 0.15%; 0.25%; 0.50%; 1.0%; 2.5% and 5.0% $^{w}/_{v}$.

1. Determination of the absorption maxima of the periodate oxidation products.

Procedure.

An aliquot of 0.05% periodate (10.00ml) was pipetted into a glass-stoppered test tube, 1.00ml of a standard added, and the solutions thoroughly mixed. The coloured solution was examined in a Unicam SP700 double beam recording spectrophotometer between the wavelengths 200 nm and 500 nm. Silica cells (1cm path length) and a periodate blank were used. The absorption maxima were noted.

Results and discussion.

For each standard two maxima were observed, the weaker of which was just in the visible part of the spectrum. The three cinnamic acids gave a visible peak at 423 nm, and all the depsides gave a common peak at 406 nm. Since it was important to give equal emphasis to all components in a mixture the common peak in the visible region was chosen for all subsequent work.

Table 8 Wavelengths of maximum absorption (nm) for the periodate oxidation products of caffeic acid, ferulic acid and related depsides.

Phenolic Compound	Wavelength of Maximum Absorption 1^o	 2^o
Caffeic acid	323	423
Ferulic acid	323	423
Isoferulic acid	323	423
3CQ	320	406
3CQ Caffeine potassium salt	320	406
5CQ	320	406
3FQ	320	406
1,4 DCQ	312	406

2. Determination of the optimum concentration of periodate for stable colour production with caffeic acid, ferulic acid and 3CQ.

Procedure.

An aliquot (1.00ml) of caffeic acid solution was added to 10.00ml of each strength of periodate. The reagents were mixed and the absorption of the solution noted at 423 nm, one minute and ten minutes after mixing. The procedure was repeated for ferulic acid and 3CQ.

Results and discussion.

The results set out in Table 9 show that the more concentrated the periodate solution, the more rapidly is the colour produced and destroyed. Ferulic acid was the slowest to respond, presumably due to the time required for the intermediate methanolysis. Periodate (0.25%) was powerful enough to produce maximum colour, in less than ten minutes. Under these conditions the colour was stable for a further five minutes at least. Therefore the 0.25% periodate reagent in conjunction with a ten minute colour production period were considered to be the optimum conditions for stable colour production.

3. Determination of the molar extinction coefficient of caffeic acid, ferulic acid, isoferulic acid and related depsides after treatment with 0.25% periodate.

Procedure.

To a 10.00ml aliquot of 0.25% periodate was added 1.00 ml of

Table 9 The optical density (at the wavelength of maximum absorption) of periodate oxidised caffeic acid, ferulic acid and 3CQ.

Caffeic Acid

Periodate Strength % $^{w}/_{v}$	ϵ 423 after one minute	ϵ 423 after ten minutes
0.05	0.237	0.227
0.10	0.225	0.220
0.15	0.230	0.230
0.25	0.237	0.237
0.50	0.235	0.235
1.00	0.235	0.215
2.50	0.230	0.213
5.00	0.225	0.180

Ferulic Acid

Periodate Strength % $^{w}/_{v}$	ϵ 423 after one minute	ϵ 423 after ten minutes
0.05	0.010	0.050
0.10	0.015	0.080
0.15	0.035	0.100
0.25	0.040	0.125
0.50	0.080	0.125
1.00	0.125	0.125
2.50	0.122	0.122
5.00	0.120	0.070

Table 9 continued

3CQ

Periodate Strength $\% \, ^{w}/_{v}$	ϵ 406 after one minute	ϵ 406 after ten minutes
0.05	0.287	0.277
0.10	0.295	0.280
0.15	0.290	0.280
0.25	0.288	0.280
0.50	0.275	0.233
1.00	0.280	0.205
2.50	0.240	0.090
5.00	0.230	0.010

standard solution. The reagents were mixed and allowed to stand for ten minutes to attain maximum colour intensity. Optical density was measured at the appropriate wavelength as previously described. Determinations were made in quadruplicate.

Results and discussion.

Before carrying out this stage of the work, all the phenolic compounds were extensively examined by TLC for the presence of impurities, particularly other depsides or cinnamic acids. Full details are given elsewhere (See Chapter 2, Section 7, Page 67), but no impurities were detected in caffeic acid, ferulic acid, isoferulic acid, 3FQ, 5CQ or 1,4DCQ. A trace of a second CQ acid, presumably 4CQ since it was not 5CQ, was found in the 3CQ. Quite a large amount of caffeic acid was found in the 3CQ caffeine potassium salt. Since the quantitative technique is based on a reaction with the phenolic component of a depside, any adventitious caffeic or ferulic acid will cause an artificially high result. Since a DCQ has two residues of caffeic acid it will give a correspondingly larger response.

The results set out in Table 10 show that the values for the MEC of caffeic acid, ferulic acid and isoferulic acid fell in the range 2102-2201. The results for 3CQ, 5CQ and 3FQ were also in a narrow range (1565-1614), and the result for 1,4DCQ was slightly more than double this (3333).

The narrowness of these two ranges demonstrated that the periodate reagent gave equal emphasis not only to the ortho-dihydroxy compounds and their monomethyl ethers, but on the basis of their

Table 10 Molar Extinction Coefficients with the Periodate Reagent.

Standard Compound	Molar Concentration μm/ml	Absorption	Molar Extinction Coefficient	
			Range	Mean
Caffeic acid	0.014	0.030	2142	2142
		0.030	2142	
		0.030	2142	
		0.030	2142	
	0.032	0.070	2187	2187
		0.070	2187	
		0.070	2187	
		0.070	2187	
	0.101	0.210	2079	2102
		0.212	2099	
		0.215	2129	
		0.212	2099	
Ferulic acid	0.012	0.026	2167	2141
		0.026	2167	
		0.025	2083	
		0.027	2250	
	0.054	0.118	2185	2176
		0.118	2185	
		0.116	2148	
		0.118	2185	
	0.076	0.172	2205	2201
		0.174	2217	
		0.170	2179	
		0.172	2205	
Isoferulic acid	0.061	0.132	2164	2148
		0.130	2131	
		0.132	2164	
		0.132	2164	
3CQ	0.130	0.204	1571	1614
		0.213	1639	
		0.213	1639	
		0.209	1605	
	0.342	0.539	1576	1591
		0.543	1554	
		0.545	1599	
		0.543	1594	
	0.071	0.115	1616	1604
		0.115	1616	
		0.113	1592	
		0.113	1592	

Table 10 continued

Standard Compound	Molar Concentration μm/ml	Absorption	Molar Extinction Coefficient Range	Molar Extinction Coefficient Mean
3CQ Caffeine potassium salt	0.058	0.120	2069	
		0.122	2103	2094
		0.118	2172	
		0.120	2034	
5CQ	0.044	0.072	1636	
		0.068	1543	1608
		0.072	1662	
		0.070	1591	
3FQ	0.046	0.072	1568	
		0.070	1522	1565
		0.073	1587	
		0.073	1587	
1,4DCQ	0.021	0.067	3190	
		0.070	3333	3333
		0.073	3476	
		0.070	3333	

cinnamic acid content, also to the individual isomers of CQ acid that were examined. From this it can reasonably be assumed that equal emphasis will be given to all isomers of CQ, FQ (and IsoFQ) and DCQ on the basis of their cinnamic acid content.

On a weight for weight basis DCQ (or DFQ) gave a stronger response with this reagent than CQ (or FQ) - 550g DCQ are equivalent to 742g CQ. Since there is no easy way of determining the proportions of DCQ and CQ, results will be expressed as CQ, although this result will be slightly high if any DCQ is present.

Two calibration curves were prepared from the data obtained in this experiment, one for the free cinnamic acids (Fig 1) and the other for the depsides (Fig 2). From these it can be seen that concentrations as low as 1µg/ml should be detectable.

Swain and Hillis (170) reported that the Molybdate Reagent exhibited a similar sensitivity to the ortho-dihydroxy compounds. It was thought therefore that the Molybdate Reagent and Periodate Reagent could be used in association to allow the molybdate-sensitive and 'total' periodate-sensitive compounds to be determined separately, and a measure of the monomethyl ethers to be obtained by difference. Such a differential quantitative technique could be usefully applied to coffee beans where both types of depsides are present. To investigate the feasability of such a technique several experiments were performed.

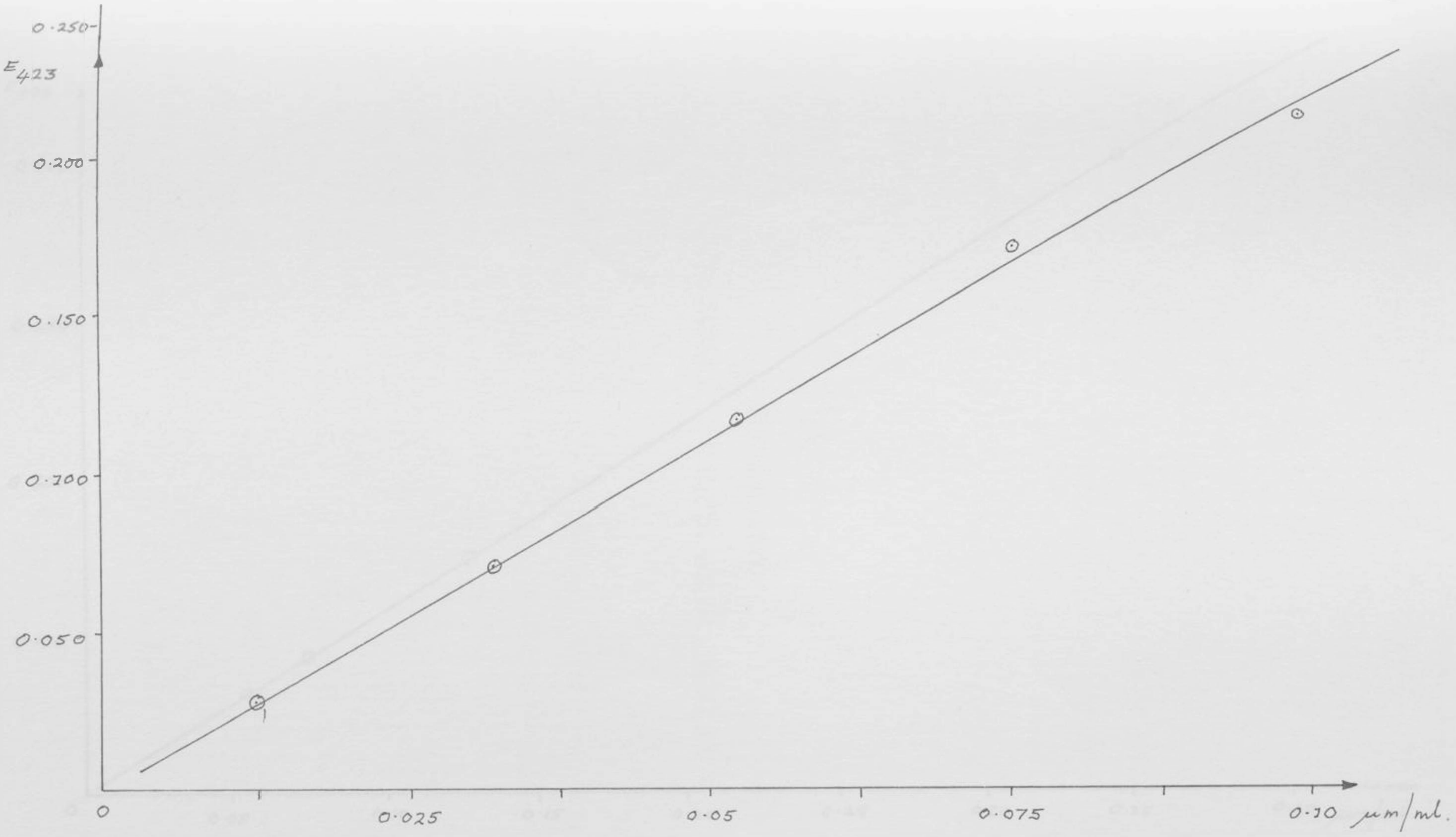


FIG 1 PERIODATE REAGENT CALIBRATION CURVE FOR CINNAMIC ACIDS.

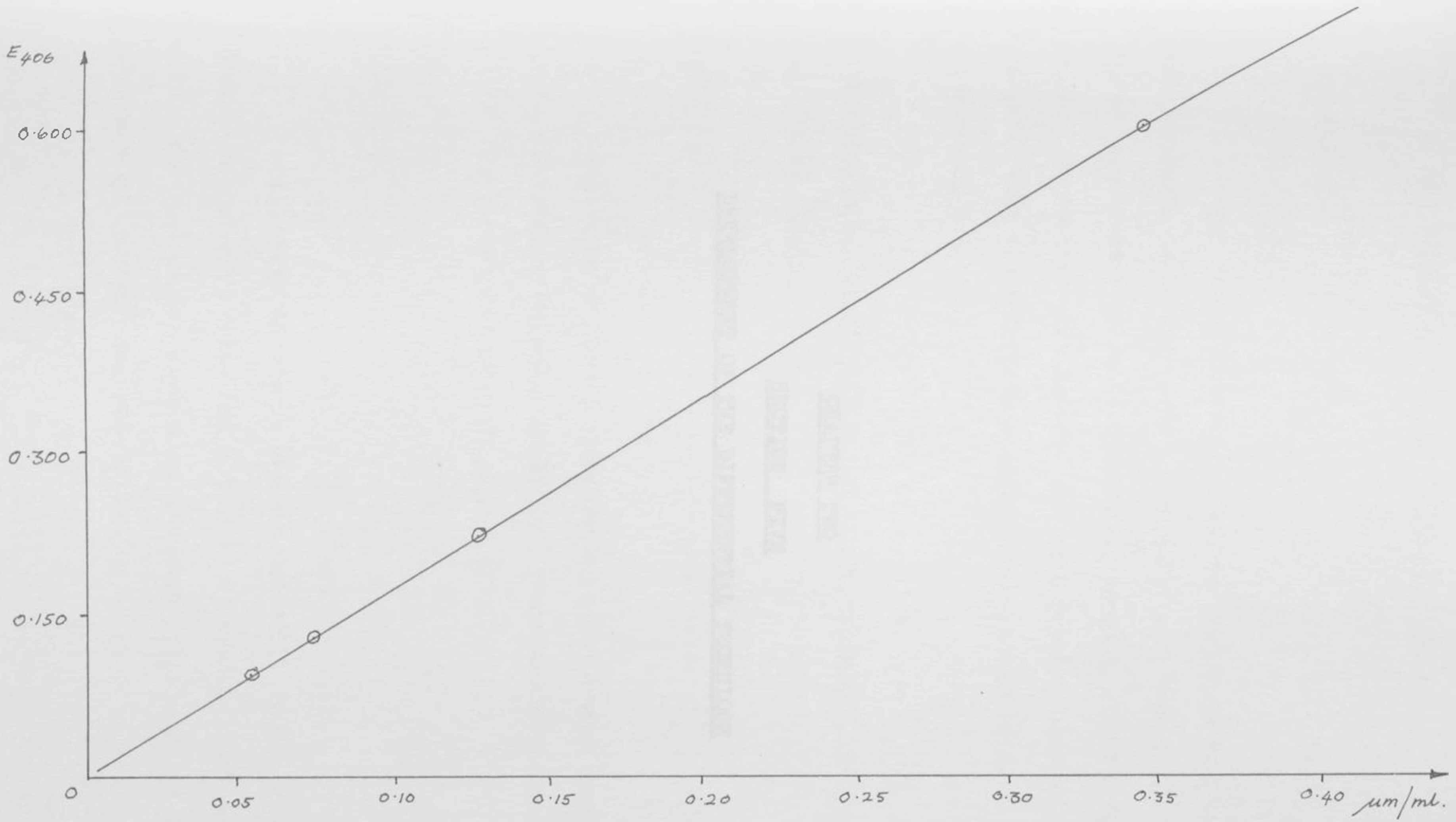


FIG 2 PERIODATE REAGENT CALIBRATION CURVE FOR CQ AND FQ ACIDS.

CHAPTER TWO

SECTION FIVE

DEVELOPMENT OF THE DIFFERENTIAL TECHNIQUE

DEVELOPMENT OF THE DIFFERENTIAL TECHNIQUE.

Introduction.

It has been demonstrated that the Periodate Reagent gives equal emphasis to caffeic acid and ferulic acid depsides. If the Molybdate Reagent were to be used in conjunction with the Periodate Reagent, to specifically measure the caffeic acid depsides, it was essential that the Molybdate Reagent gave equal emphasis to all the caffeic acid depsides.

Determination of the MEC of the molybdate complex of caffeic acid and related depsides.

Materials.

Solutions of phenolic compounds as previously prepared.

Molybdate Reagent. Analytical grade sodium molybdate ($5\%\ ^{w}/_{v}$) in phosphate buffer at pH 6.5 (170).

Procedure.

Aliquots of Molybdate Reagent (10.00ml) were pipetted into glass-stoppered test tubes and 0.10ml of a phenolic solution was added. The mixture was shaken and the absorption noted at the wavelength of maximum absorption. Silica cells and a Molybdate Reagent blank were used.

A control was prepared by adding 0.10ml of the phenolic compound to 10.00ml deionised water. Absorption of this mixture was read, against a water blank, at the wavelength used for the molybdate complex. This value was subtracted from that obtained for the molybdate complex.

Results and discussion.

The range of values obtained for the MEC of caffeic acid was 12064-12100; for 3CQ 16500-16700; for 5CQ 16724 and for 1,4DCQ 33390. These results showed that the Molybdate Reagent gave approximately equal emphasis to 3CQ, 5CQ and 1,4DCQ on the basis of their caffeic acid content. It was assumed that other caffeic acid depsides would behave in the same manner. Calibration curves were prepared for caffeic acid (Fig 3) and for depsides (Fig 4).

From these results it was deduced that the Molybdate Reagent and the Periodate Reagent could be used in conjunction to allow the monomethyl ethers to be measured by difference. Mixtures of caffeic acid and ferulic acid, 3CQ and 3FQ were prepared to test the deduction.

Determination of a monomethyl ether in standard solutions by the Differential Technique.

Materials.

Standard solutions.

Two solutions containing 3CQ and 3FQ were prepared by

Table 11 Molar Extinction Coefficients with the Molybdate Reagent.

Standard Compound	Molar Concentration µm/ml	Absorption	Molar Extinction Coefficient Range	Mean
Caffeic Acid	0.015	0.181	12066	12100
		0.180	12000	
		0.180	12000	
		0.185	12333	
	0.035	0.427	12200	12064
		0.420	12000	
		0.422	12057	
		0.420	12000	
	0.055	0.665	12090	12099
		0.670	12181	
		0.662	12036	
		0.665	12090	
3CQ	0.0145	0.236	16700	16700
		0.236	16700	
		0.236	16700	
		0.236	16700	
	0.0189	0.354	16500	16500
		0.354	16500	
		0.354	16500	
		0.354	16500	
5CQ	0.0119	0.196	16570	16724
		0.205	17230	
		0.195	16390	
		0.200	16801	
1,4DCQ	0.058	0.195	33620	33190
		0.195	33620	
		0.190	32760	
		0.190	32760	

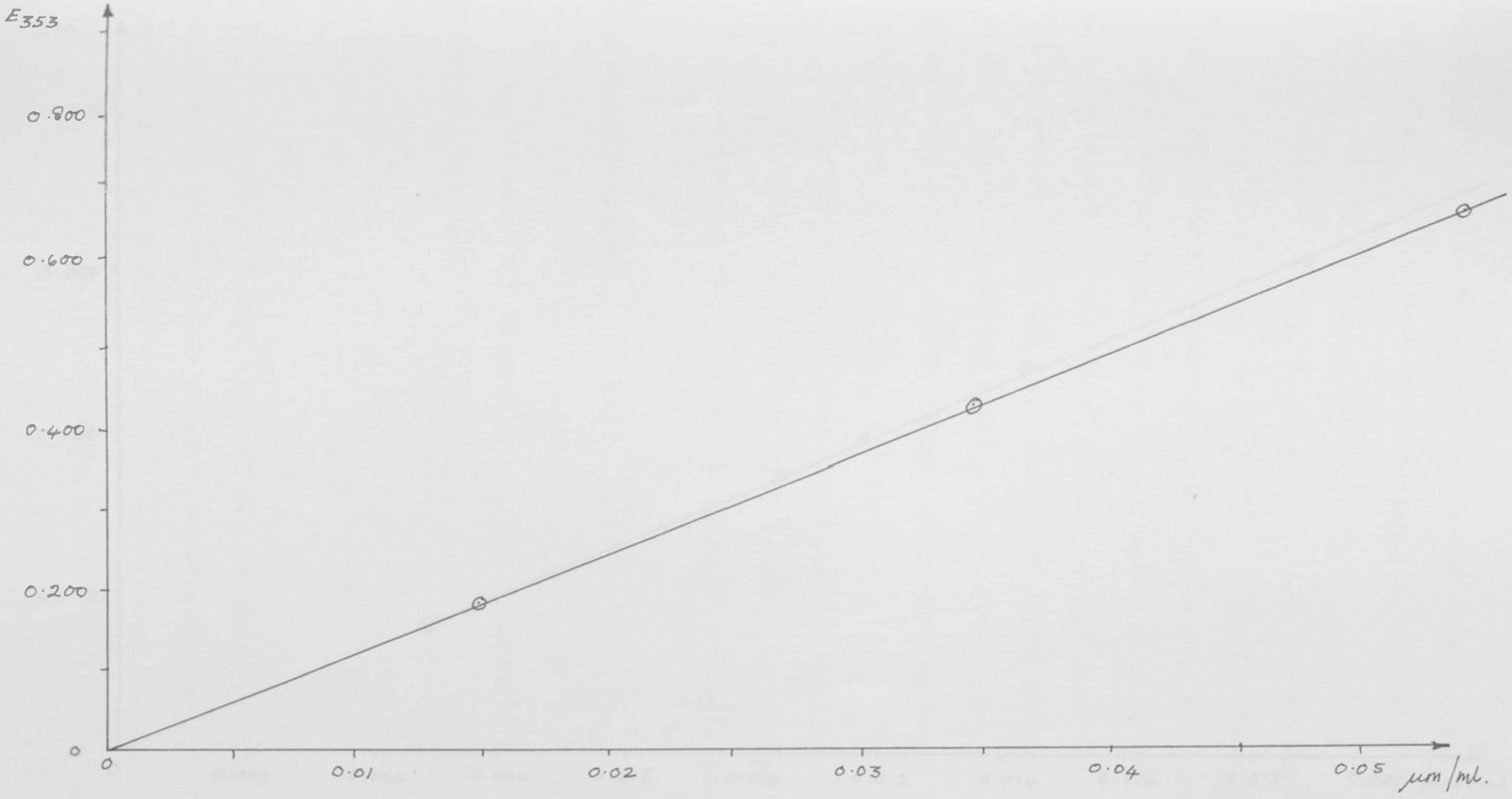


FIG 3 MOLYBDATE REAGENT CALIBRATION CURVE FOR CAFFEIC ACID.

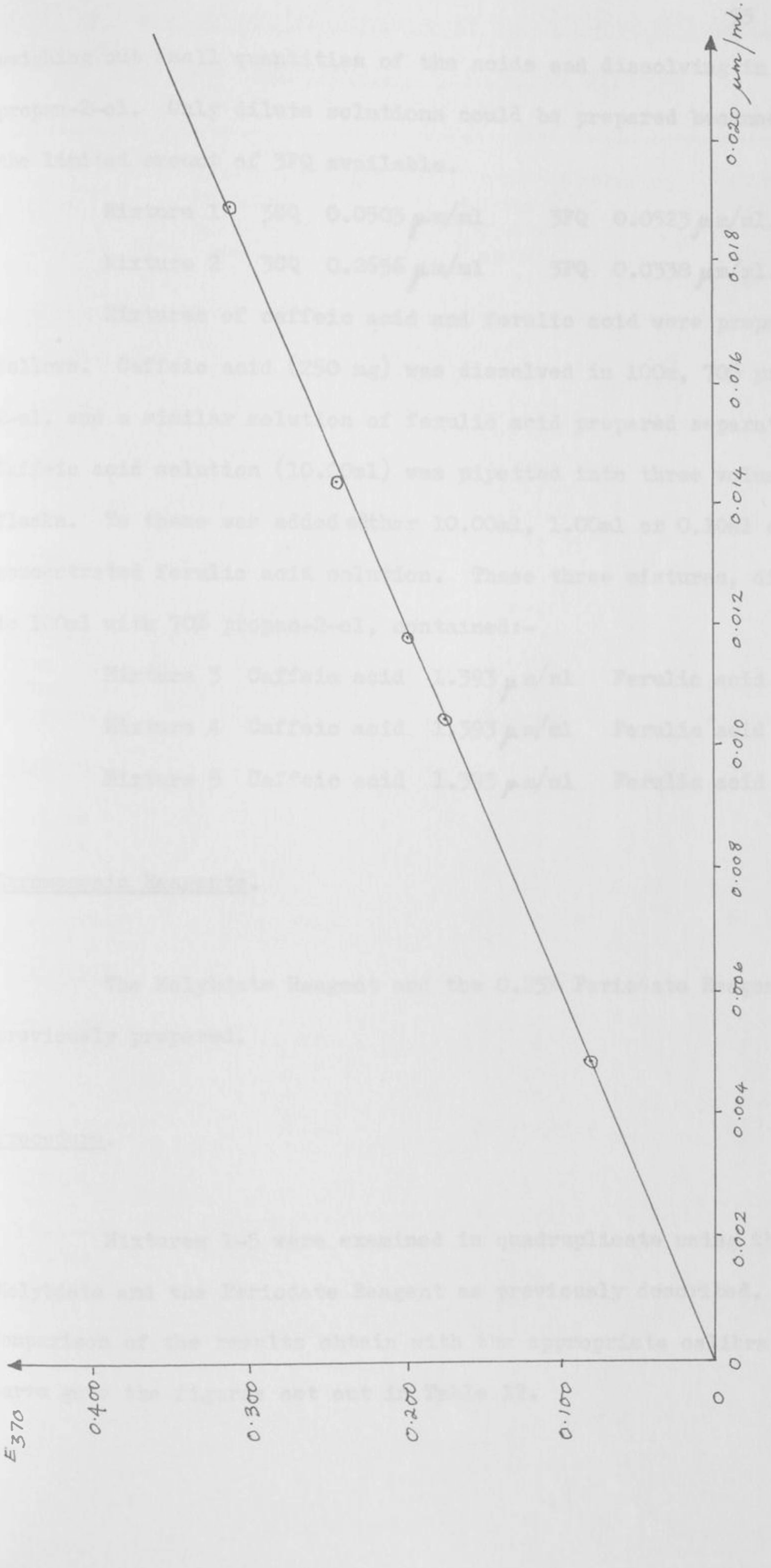


FIG 4 MOLYBDATE REAGENT CALIBRATION CURVE FOR CQ ACIDS.

weighing out small quantities of the acids and dissolving in 70% propan-2-ol. Only dilute solutions could be prepared because of the limited amount of 3FQ available.

Mixture 1 3CQ 0.0503 μm/ml 3FQ 0.0523 μm/ml.
Mixture 2 3CQ 0.2656 μm/ml 3FQ 0.0338 μm/ml.

Mixtures of caffeic acid and ferulic acid were prepared as follows. Caffeic acid (250 mg) was dissolved in 100m, 70% propan-2-ol, and a similar solution of ferulic acid prepared separately. Caffeic acid solution (10.00ml) was pipetted into three volumetric flasks. To these was added either 10.00ml, 1.00ml or 0.10ml of the concentrated ferulic acid solution. These three mixtures, diluted to 100ml with 70% propan-2-ol, contained:-

Mixture 3 Caffeic acid 1.393 μm/ml Ferulic acid 1.290 μm/ml.
Mixture 4 Caffeic acid 1.393 μm/ml Ferulic acid 0.129 μm/ml.
Mixture 5 Caffeic acid 1.393 μm/ml Ferulic acid 0.013 μm/ml.

Chromogenic Reagents.

The Molybdate Reagent and the 0.25% Periodate Reagent, as previously prepared.

Procedure.

Mixtures 1-5 were examined in quadruplicate using the Molybdate and the Periodate Reagent as previously described. Comparison of the results obtain with the appropriate calibration curve gave the figures set out in Table 12.

Table 12 The recoveries obtained by the Differential Technique when applied to standard mixtures.

	Dihydroxyphenol detected by Molybdate (μm/ml)	'Total' phenol detected by Periodate (μm/ml)	Monomethyl Ethers calculated by difference (μm/ml)
Mixture 1			
Maximum	0.0589	0.1140	0.0579
Minimum	0.0561	0.1065	0.0476
Mean	0.0570	0.1105	0.0535
Actual	0.0593	0.1116	0.0523
% Recovery	94.6-99.3	95.5-102.2	79.3-110.8
Mixture 2			
Maximum	0.2585	0.3015	0.0441
Minimum	0.2574	0.2970	0.0385
Mean	0.2578	0.2981	0.0403
Actual	0.2656	0.2994	0.0338
% Recovery	97.0-97.3	99.2-100.7	114.0-130.5
Mixture 3			
Maximum	1.381	2.667	1.243
Minimum	1.360	2.624	1.307
Mean	1.369	2.645	1.276
Actual	1.393	2.683	1.290
% Recovery	97.6-99.1	97.8-99.4	96.3-101.3
Mixture 4			
Maximum	1.374	1.538	0.0185
Minimum	1.353	1.507	0.0133
Mean	1.365	1.519	0.0154
Actual	1.393	1.522	0.0129
% Recovery	97.1-98.6	99.0-102.0	110.3-143.2
Mixture 5			
Maximum	1.381	1.375	0.0029
Minimum	1.346	1.342	-0.0039
Mean	1.351	1.354	0.0003
Actual	1.393	1.406	0.0130
% Recovery	96.6-99.1	95.5-97.9	0 - 223

Results and discussion.

Table 12 compares the range of values obtained for each component of each mixture with the quantities known to be present. The spread of the observations did not exceed ± 2½% for any of the direct observations. Recovery of the ortho-dihydroxy compounds by the Molybdate Reagent was between 94 and 100% of theoretical, and for the 'total' phenols by the Periodate Reagent, was 95% - 103%. Recoveries of this accuracy were considered to be satisfactory; however, the calculated content of monomethyl ethers was subject to the accumulated errors of the two techniques.

When the monomethyl ethers were present as 50% of the total phenols, recovery was comparable with that for the other dihydroxy phenols ie 96 - 102% for Mixture 3. A reduction in the monomethyl ether content from using a more dilute solution (Mixtures 1 and 2) or by lowering the molar proportion (Mixtures 4 and 5) led to a less reliable recovery of the minor component. When the monomethyl ethers were present at about 9% of total, a moderately accurate mean recovery was possible although the range of values was quite large. When the monomethyl ethers were responsible for only 1% of the total phenols, the mean recovery was only a rough estimate of the amount known to be present. However, the result obtained for this was quite distinguishable from that obtained for the 1:10 mixture.

Conclusions.

The Differential Technique allows accurate measurement of 'total' phenols, and the ortho-dihydroxy phenols since these are measured by a selective reagent. When the monomethyl ethers are 10% or more of the 'total' phenols these are measured with moderate accuracy. When the monomethyl ethers are less than 10% of the mixture the accuracy of recovery falls rapidly until only an estimate can be obtained. For comparative purposes this is still of value, and the technique is considered suitable for examination of the phenolic compounds of green and roasted coffee beans.

CHAPTER TWO

SECTION SIX

APPLICATION OF THE DIFFERENTIAL TECHNIQUE

APPLICATION OF THE DIFFERENTIAL TECHNIQUE.

Quantitation of the CQ and FQ acids of green coffee beans.

Materials.

The following green beans were examined:-

Coffee arabica	Santos
	Sao Paulo
Coffee canephora var. robusta	Ghana
	Uganda

The beans were ground, and the extracts prepared by the method described on page 31.

Procedure.

The Differential Technique was used as previously described. Each extract was examined in quadruplicate.

Results and discussion.

It is generally accepted that Arabica beans contain less chlorogenic acids than Robusta beans. Clark (6) reported about 6% (dmb) and 10% (dmb) respectively. A more thorough examination of the literature revealed that Arabicas have been reported to contain 4.15 - 8.05% (dmb) (68, 72, 91, 94, 100, 177) and Robustas 7.20 - 10.30% (dmb) (178). However, these results are not directly comparable since different techniques had been used to obtain them and different errors had been incurred. The lowest Arabica values

(72, 177) of 4.15% (dmb) and 4.25% (dmb) for Santos beans were obtained gravimetrically after extraction of the phenolic compounds and extensive purification by countercurrent distribution and recrystallisation. It can be assumed that these values will be low due to the loss of material at each of the many purification stages. The highest values of 7.0 - 8.05% (dmb) were obtained by Merritt and Proctor (68) who used the direct UV spectrophotometric technique of Waiss (109). These results could easily be inflated by the interference of non-phenolic compounds for which no blank could be taken. The value for Santos beans obtained by these workers was 6.86% (dmb). A GLC examination of an aqueous extract from Santos beans, by Kung, Ryder and Feldman, (100) gave a result of 7.25% (dmb). This aqueous extract was very impure and nearly 40 components were separated, of which only three were considered to be CQ acids. It is impossible to be certain that impurities did not give an inflated result. Pictet and Brandenburger (94) examined a Columbian Arabica bean, and obtained a result of 6.25% (dmb). They separated and partially purified the chlorogenic acids by paper chromatography and quantitated the eluates by their UV absorption. A correction was applied for losses during chromatography and elution. Merritt and Proctor (68) obtained a result of 7.57% (dmb) for this variety of bean by the same method when no purification of the extract was made.

The only published results for Robusta beans were obtained by Chassevent et al (178) who purified extracts by polyamide column chromatography before quantitating by direct UV spectrophotometry. Their results were 7.20 - 10.30% (dmb).

Since no worker had examined Arabica and Robusta beans by the same technique no genuine comparison had been made. Differences of technique allied to an unquantifiable variation in natural materials made the published results unreliable for comparative purposes.

The results set out in Table 13 agree with the generalisation made by Clark (6) and confirm that there is a substantial difference (about 40%) between the Arabicas and Robustas. Of the four types of bean examined, there was information in the literature relating only to the Santos. It was particularly interesting to note that in this investigation the total content of chlorogenic acids was found to be 6.42% (dmb). This value falls neatly between the low values of Corse and co-workers (72, 177) and the probably inflated values of Merritt and Proctor (68) and Kung et al (100).

The results from the Molybdate Technique showed that the CQ acids are responsible for 96 - 97% of the Arabica chlorogenic acids, but only 85 - 92% in the case of the Robustas.

According to the results obtained for the standard mixtures, the FQ content of the Robustas can be accepted as approximately accurate or perhaps a little high. The result for the Arabicas is less accurate, but can be fairly taken as indicating a considerably lower content of FQ acids than is present in the Robustas.

Very little information has been published concerning the content of FQ acids in coffee or other plant material. Three simple FQ acids (3FQ; 4FQ; 5FQ) have been reported present in green coffee beans on the basis of TLC and NMR characterisation of the components (172, 74, 94, 95), and also one or two of the monomethyl ethers of 3,5DCQ (99). Corse, Sondheimer and Lundin (95) isolated

Table 13 The content of CQ acids and FQ acids in green coffee beans determined by the Differential Technique.

Component	Green Coffee Bean			
	Arabica		Robusta	
	Santos	Sao Paulo	Ghana	Uganda
CQ Acids (% dmb).				
Range	6.02-6.29	5.41-5.83	7.48-8.21	7.47-7.96
Mean	6.17	5.61	7.97	7.80
FQ Acids (% dmb).				
Range	0-0.47	0-0.86	0.79-2.08	0.36-1.43
Mean	0.25	0.23	1.35	0.77
Total phenolic acids (% dmb).				
Range	6.25-6.49	5.56-6.29	9.00-9.56	8.32-8.90
Mean	6.42	5.84	9.32	8.57
Mean Molar Ratio CQ:FQ	26:1	25:1	6:1	10.5:1
FQ acids (as % total phenolic acids)	3.7	3.8	14.3	8.7

3FQ from a green Robusta coffee by countercurrent distribution and purified it by repeated recrystallisation. The yield was about 0.1% (dmb) and an Arabica was found to contain much less. During the purification stages the 4FQ and 5FQ and 3,5DCQ monomethyl ethers were deliberately removed, and inevitably some 3FQ would also have been lost. Therefore, it is reasonable to assume that their yield was low.

It was decided that confirmation of the considerably higher FQ content in the Robustas was desirable and the results of the TLC examination appear later. (See page76). It was also decided to follow the destruction of the chlorogenic acids during roasting by means of the Differential Technique.

Quantitation of the CQ and FQ acids of roasted coffee beans.

Materials and methods.

Green beans (500g) were roasted as described in the Appendix. The four types of bean available were each subjected to four degrees of roast which were measured by their weight loss. The extracts were prepared exactly as for their green beans, and the same reagents were employed in the quantitative procedures.

Results and discussion.

Lentner and Detherage (74) and Merritt and Proctor (68) reported respectively that 32 - 52% and 24 - 51% of the green bean chlorogenic acids were destroyed on roasting. The exact loss was dependent on the type of bean, and became greater as the roast proceeded. When the final roast temperature was standardised at 205° six Arabicas showed losses of 31-37% (28). These results were all obtained by direct spectrophotometric techniques and the results could have been subject to interference by non-phenolic compounds.

In 1961 Kraseman (110) examined the changes which occurred in green bean phenolic substances and pure 3CQ when roasted. The beans and model system gave similar results. The green bean showed an 81% loss of chlorogenic acids at 17.8% total roasting loss. The loss of chlorogenic acids at intermediate stages was 40% at 13.1% roast loss, and 23% at 10.2% roast loss. Kung et al (100) using GLC reported a 56% loss of chlorogenic acids from a Santos coffee bean subjected to a 15% roast loss.

The results summarised here were all obtained for Arabica type beans. Clark (6) suggested that considerably greater loss of chlorogenic acids occurred with Robusta beans, but no data have been found in the literature to confirm this. There was no information relating directly to the behaviour of the FQ acids, but previous workers had established that during the roasting of Arabica beans a progressive loss of total chlorogenic acids occurred.

The results obtained in the current investigation are set out in graphical form (Fig 5-8). The loss of the molybdate-sensitive CQ acids was very similar in the four beans. The two Arabicas and Robusta Ghana lost 75% to 80% of the initial CQ acid content at a total roasting loss of 18%. Robusta Uganda lost 66% at a 15% total roasting loss. Roasting this bean more severely resulted in complete carbonisation. In absolute terms the Robusta beans showed a greater weight loss of CQ acids than the Arabica beans. The loss of 'total' phenols showed a similar overall trend to the loss of CQ acids and was in general agreement with the previous reports.

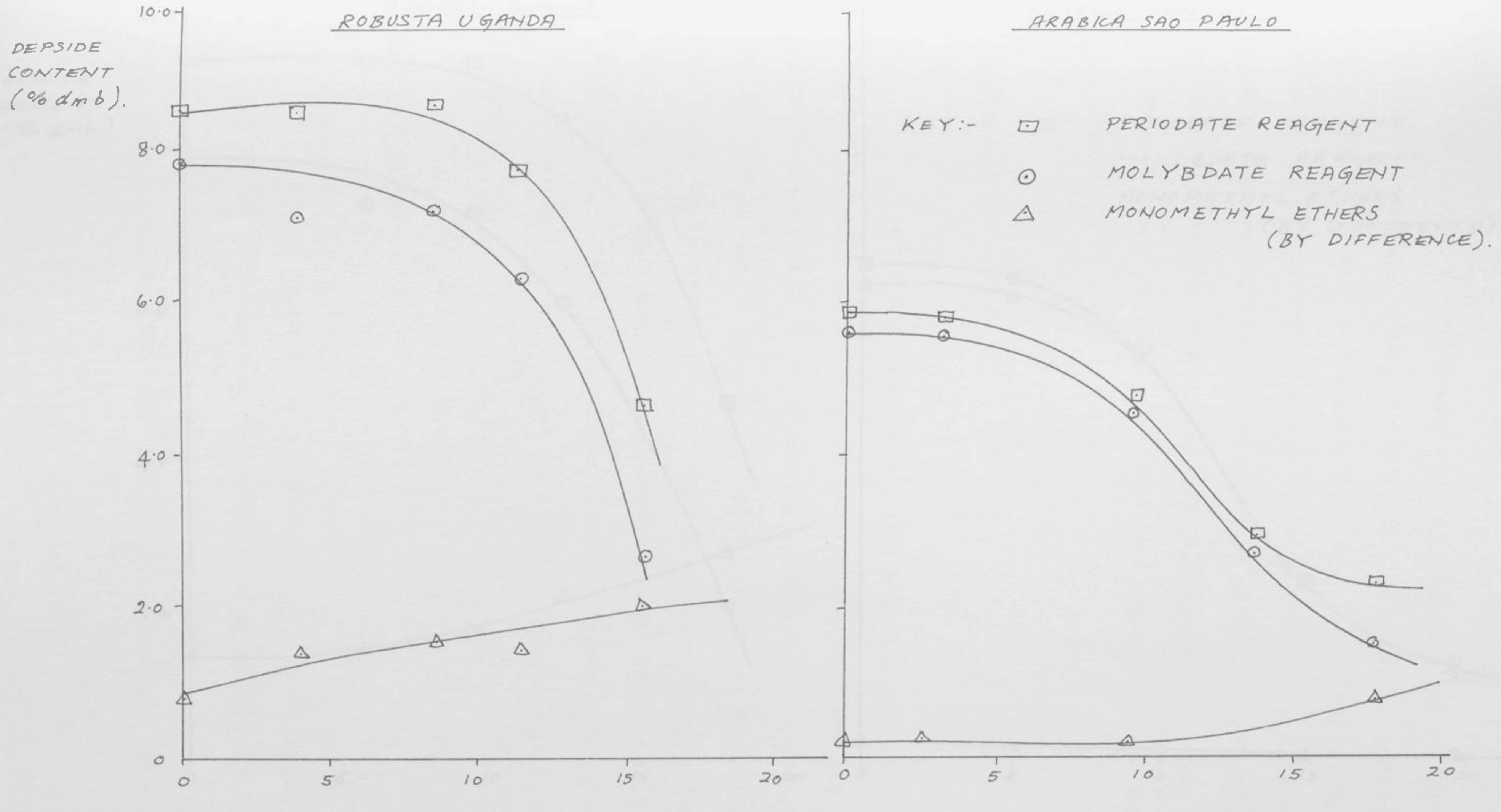


FIG 5 AND 6 THE EFFECT OF ROASTING ON THE DEPSIDE CONTENT OF ROBUSTA UGANDA AND ARABICA SAO PAULO COFFEE BEANS.

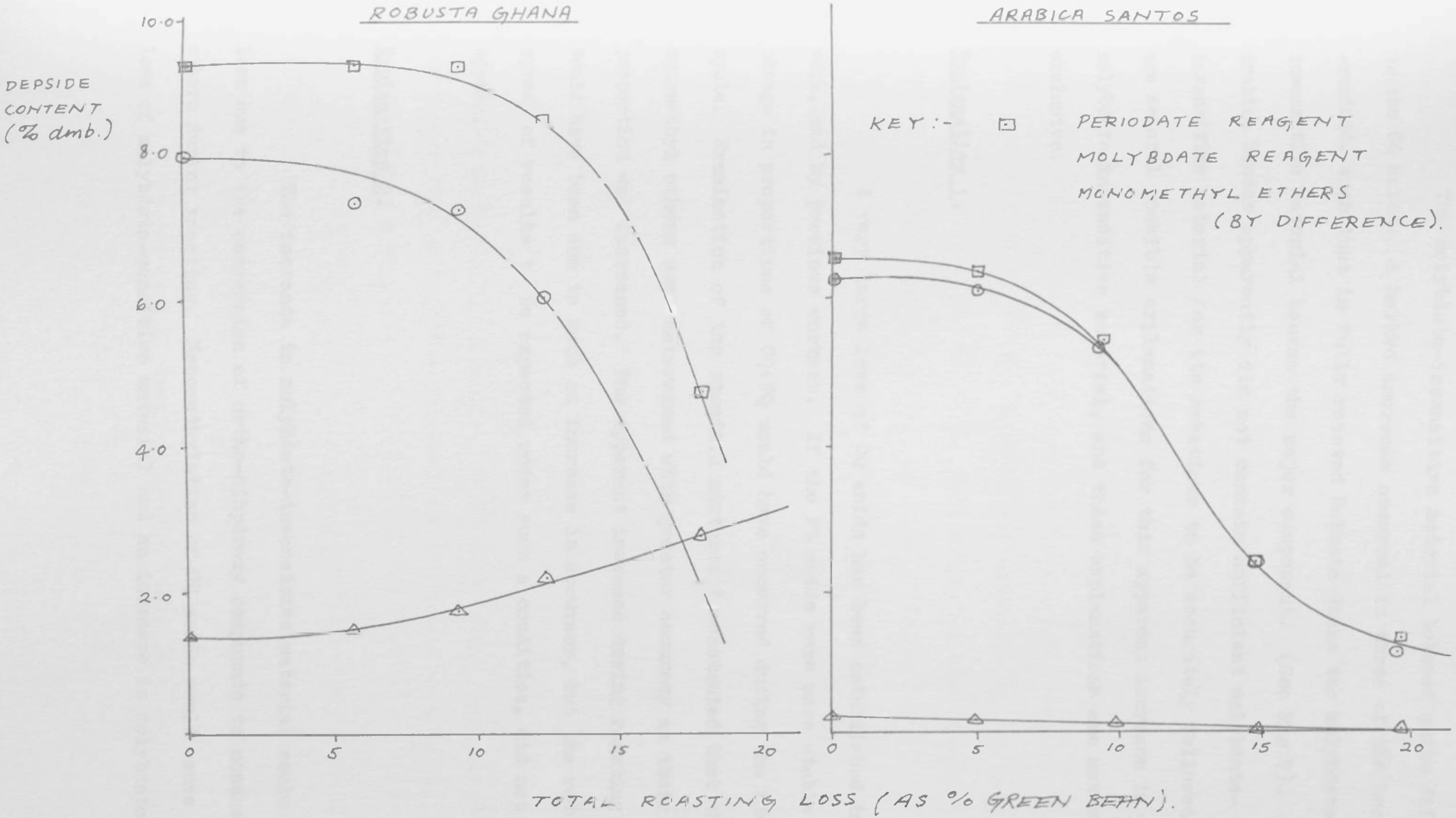


FIG 7 AND 8 THE EFFECT OF ROASTING ON THE DEPSIDE CONTENT OF ROBUSTA GHANA AND ARABICA SANTOS COFFEE BEANS.

The molybdate-insensitive material behaved quite differently to the CQ acids. A marked increase occurred in three of the beans examined, such that in fully roasted Robusta Ghana the molybdate-insensitive material became the major component. (See Fig 7). Arabica Santos apparently did not contain sufficient molybdate-insensitive material for its behaviour to be accurately followed. There are several possible explanations for this apparent increase in molybdate-insensitive material, and these explanations are not mutually exclusive.

Explanation 1.

A very large loss of CQ acids has been established in this work, and by previous workers. If the FQ acids were more stable a change in proportions of CQ:FQ would have occurred during the roasting cycle. Examination of the standard mixtures demonstrated that the monomethyl ethers were determined with greater accuracy as their proportion was increased. The apparent increase during roasting would have been due to such an increase in accuracy, but the reduced spread of results to be expected under such a condition, did not appear.

Explanation 2.

The increase in molybdate-insensitive material could have been due to the conversion of ortho-dihydroxy compounds to monomethyl ethers during roasting. Monomethylation of CQ acids would cause a loss of molybdate-sensitive material and an increase in molybdate-insensitive material. Pausen (179) suggested that a quaternary amine could carry out the methylation. It is possible that trigonelline has this property. Trigonelline has been reported to be in green coffee beans at levels varying from 0.6-1.9% (dmb), and to demethylate during roasting with up to 80% loss. (6, 53, 65, 68).

Methionine is also a possible methylating agent since it has been reported to demethylate when heated in model systems at 300-320° (144). Merrit, Robertson and MacAdoo (180) reported that amino acids bound in protein behaved very similarly to free amino acids when subjected to roasting. Methionine has been reported present in green beans at a level of about 1% (dmb). (53).

Explanation 3.

Degradation products that are phenolic, whatever their origin, could have caused interference in four ways, of which two would result in an apparent increase in monomethyl ethers, and two in an apparent decrease.

A Molybdate-sensitive products. An increase would have occurred if these were less molybdate-sensitive, but relatively more periodate-sensitive than a CQ acid.

B Molybdate-insensitive products. An increase would have occurred if these were relatively more sensitive to periodate than a CQ acid.

There are several possible sources for such degradation products.

Traces of phenolic compounds have been reported amongst the degradation products of heated sugars and polysaccharides (139, 146) and from the reaction of phenolic amino acids (free or protein based) and lipid (181). Heating of lignin, quinic acid, caffeic acid and ferulic acid under conditions similar to roasting coffee beans has given rise to a range of phenolic compounds (29, 111, 145, 148, 182, 183). Some of these products are molybdate-and/or periodate-sensitive under the conditions employed for the Differential Technique. Release of propan-2-ol-soluble degradation products from lignin could have made a very significant contribution to the monomethyl ethers.

Since many of the reactions that occur during roasting are oxidative, it was thought that some of the roasting degradation products of the chlorogenic acids might also be intermediates in the periodate oxidation of chlorogenic acids. Some of these intermediates may have lost their molybdate-chelating ability but may have fully or partially retained their periodate sensitivity.

The correct explanation is probably a complex of the factors suggested. However, an increase of the order observed required a considerable quantity of phenolic material to produce it, and this probably ruled out carbohydrates and phenolic amino acids as sources since their yield could only have been minute. To elucidate the situation the following experiments were carried out:-

1. Examinations were made by TLC of phenolic compounds extracted from green and roasted coffee beans.

2. A series of model systems were prepared, containing caffeic acid, ferulic acid, quinic acid or 3CQ, and after roasting these were examined gravimetrically, by TLC, and by the Differential Technique. The effect of including sugars, organic acids, methionine or trigonelline was also studied.
3. Phenolic compounds which may be produced during roasting were examined for their relative sensitivity to molybdate and periodate.
4. Green and roasted coffee beans were analysed for lignin content. (See Appendix D).

Conclusions.

1. Green Robusta beans have a higher content of CQ acids and FQ acids than green Arabica beans.
2. The CQ acids are extensively destroyed under conditions that are typical of commercial roasting. (Medium roast = 16 to 18% total roasting loss).
3. The behaviour of the FQ acids appears to be quite different from the CQ acids but further investigation is required to confirm this.

CHAPTER TWO

SECTION SEVEN

THIN LAYER CHROMATOGRAPHY

THIN LAYER CHROMATOGRAPHY OF CHLOROGENIC ACIDS.

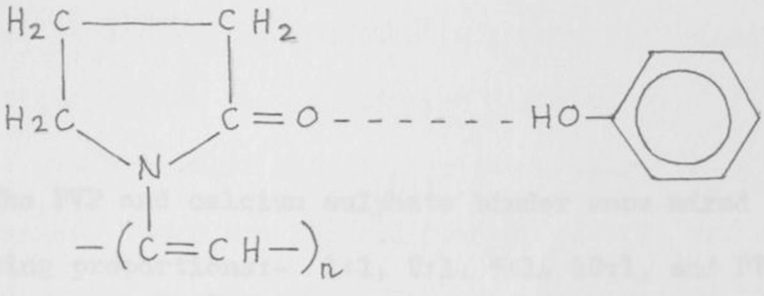
Introduction.

TLC was chosen in preference to PC for three reasons. Chromatograms can be developed rapidly, a particular advantage if two developments or two dimensional developments are necessary. Thick layers may be used, allowing separation of larger quantities than is possible with PC. The range of absorbants is wider and this allows separations to be based on methods other than partition chromatography.

Few systems have been reported that separate the CQ isomers (73, 184) and only one was found which gave mention of an FQ isomer (185). These three systems (73, 184, 185) were based on Silica gel G adsorption chromatography.

One of the objects of this experiment was to follow changes in the FQ acids during the roasting cycles. It was felt that no TLC system would be able to resolve the nine depsides that have been reported in green coffee beans, since their structures and properties are very similar. However, the three classes of depsides (feruloyl, caffeoyl, dicaffeoyl) have one very important difference - their content of phenolic hydroxyl groups.

Sanderson and Perera (186) in 1966 reported that insoluble poly-N-vinyl pyrrolidone (PVP) bound phenolic compounds and could be used for removing these phenols from solutions where they caused interference. Anderson and Somers (187) reported that the optimum conditions for binding caffeic acid to PVP occurred at pH 3.5 in the presence of dilute alcohol. They postulated that binding was due to hydrogen bonds between the carbonyl group and the phenolic hydroxyl.



Quarmby (188) tried to develop a TLC system using a PVP layer. He reported that the layer was difficult to spread evenly and was prone to crack on drying, and that only highly polar solvents were suitable, and mixed solvents tended to separate and cause diffusion and disruption of the spots. However, it was felt that a system based on PVP would allow a separation to be made through differences of hydrogen bonding which would be directly related to the number of phenolic hydroxyl groups present in the molecule.

POLY-N-VINYL PYRROLIDONE TLC.

Development of a PVP TLC System.

Materials.

Insoluble PVP (Polyclar AT supplied by GAF Ltd. Manchester).

Calcium sulphate ($CaSO_4 . \frac{1}{2}H_2O$)

Solutions of caffeic acid, ferulic acid and 4-hydroxycinnamic acid in 70% propan-2-ol (5 mg/ml).

Solvent Systems:-

1. Butan-2-one, methyl phenyl ketone, 50% acetic acid 5:5:4 $^{v}/_{v}$.

2. Urea, water 1:1 $^{w}/_{v}$.

3. Propan-2-ol, water 70:30 $^{v}/_{v}$.

Procedure.

The PVP and calcium sulphate binder were mixed thoroughly in the following proportions:- 1:1, 2:1, 5:1, 10:1, and PVP alone. Deionised water was added gradually and the slurry homogenised until a smooth paste was obtained. This slurry was transferred to a conventional spreader, and trial plates prepared in the normal manner. The correct amount of water was determined initially by inspection.

Plates were allowed to dry either at room temperature, or at 105° for thirty minutes, and allowed to cool to room temperature before use.

Results and discussion.

The slurry of pure PVP was prone to settle out of suspension and was difficult to spread. Uneven layers were obtained and drying caused the layer to crack and disintegrate. Addition of calcium sulphate to the slurry improved the spreading of the layer, but a network of fine cracks developed on drying. Quarmby (188) had used calcium sulphate and PVP in the ratio 1:7, and reported that very severe cracking occurred on drying the plates at room temperature. However, it was felt that these plates with fine cracks could be used, and samples of caffeic acid, ferulic acid and 4-hydroxycinnamic acid were applied and the three solvent systems examined.

Solvent 1 was based on the system of Grodzinska-Nachweija *et al* (184) and was known to be suitable for moving phenolic acids on silica gel layers. Solvent 2 was chosen because it was reported to reduce the hydrogen bonding between the phenols and PVP (188), and Solvent 3 because it is a good solvent for phenolic compounds and would have little effect on the hydrogen bonding.

All the air dried plates gave poor separation and the components tailed. When the solvent containing urea was used the oven dried plates also gave poor separation with a tendency to tail. Solvents 1 and 3 gave good separation with distinct spots, but Solvent 3 was inconvenient because it required five hours for a 15cm development. Solvent 1 required only seventyfive minutes, but tended to form two solvent fronts moving about 2cm apart after 15cm development.

The quantity of calcium sulphate only marginally affected the Rf values, the greater the content the higher the Rf and larger the spot. This diffusion was probably caused by a reduction in PVP content rather than an increase in calcium sulphate.

The best layer was found to be prepared by using calcium sulphate and PVP in the proportions 1:5. Trial and error showed that 8ml of water were required for 1g of PVP. This system was used for all subsequent work.

Table 14 Rf values of Standard Phenolic Compounds on PVP layer.

Standard	Rf x 100	
	Solvent 1	Solvent 3
Caffeic Acid	25	20
Ferulic Acid	59	54
4-hydroxycinnamic acid	46	33

The results in Table 14 show that caffeic acid had an appreciably lower Rf value than the two monohydroxycinnamic acids. It was considered to be unlikely that differences of solubility in the solvents had more than a minor effect on the Rf values, since both solvents are known to be good solvents for these acids. Thus it was reasonable to assume that the extent of hydrogen bonding was principly responsible for the separation. To investigate this hypothesis in detail, all available phenolic compounds were chromatographed on PVP.

Conclusions.

PVP can be used successfully as a TLC adsorbant. The most satisfactory method of preparation was:-

Thoroughly mix 12.0g PVP and 2.4g $CaSO_4.\frac{1}{2}H_2O$. Place in a homogeniser and add 96ml deionised water, and homogenise at high speed for one minute. Spread immediately - slurry is sufficient for 5 plates 20cm x 20cm x 500nm. Oven dry at 105° for thirty minutes and allow to cool before use.

An investigation of the properties of a PVP layer.

Materials.

Solutions of all available phenolic compounds were prepared in 70% propan-2-ol (5mg/ml).

Plates were prepared as described above.

Locating Reagents:- (See Appendix A).

Periodate Reagent 5.0%.

Diazotised Sulphanilic Acid.

Wiesner Reagent.

Ninhydrin.

UV fluorescence.

Procedure.

The chromatograms were prepared in the usual manner. Loadings were 5 μl and the development was 15cm. Location was initially by UV fluorescence, and then by the locating reagent appropriate to the class of compound being examined. The Rf values shown in Table 15 were obtained from three separate chromatograms.

Results and discussion.

The components within any particular class separate according to the number of phenolic hydroxyls (Table 15). The more phenolic hydroxyls, the lower the Rf value, eg the DCQ, CQ and FQ acids, 10, 16-27 and 44-46 respectively. This supports the hypothesis that the degree of hydrogen bonding is responsible for the separation. Very discrete spots were obtained in all cases.

Comparison of the Rf values for groups 1 and 2 with group 3 (See Table 15) demonstrated that the presence of a carboxyl group reduced the Rf values by the same amount as an extra hydroxyl group. This effect was also noticed by Anderson and Somers (187). The amino

Table 15 Rf values for a range of phenolic compounds separated on PVP using the solvent system butan-2-one. methyl phenyl ketone, 50% acetic acid 5:5:4 $^{v}/_{v}$.

Class of Compound	Hydroxylation Pattern and Rf x100									
	No hydroxyl group		1 hydroxyl group		2 hydroxyl groups		3 hydroxyl groups		4 hydroxyl groups	
1. Phenols			3M4H	88	1,4DH	50-54	3,4,5TH	29-32		
			2,6DM,1H	88-92	2,4DH	38-42	2,4,6TH	10-11		
					3,4DH	54-57				
2. Benzaldehydes and Cinnamaldehydes	3M	98-100	2H	67-73	2,4DH	55				
	4M	98-100	3H	65-68	3,4DH	48-54				
	3,4DM	98-100	4H	65-68						
	3,4,5TM	98-100	3M4H	70-74						
	BzAl	98-100	4M3H	68-72						
			3,5DM,4H	78-80						
	CAl	95-98								
	2MCAl	95-98								
	3,4MDCAl	95-98								
3. Benzoic Acids			2H	52-56	2,3DH	31-35	3,4,5TH	10-12		
			3H	52-56	2,4DH	28-32				
			4H	52-56	2,5DH	37-40				
			3M4H	58-60	2,6DH	31-35				
			3,5DM, 4H	57-62	3,4DH	33-35				
					3,4DH	28-32				
4. Cinnamic Acids and Depsides	CA	70	2H	46	3,4DH	25-27			1,4DCQ	10
	3,4DM	70	3H	46	3CQ	20-22				
			4H	46	5CQ	16-18				
			3M4H	59	3CQ caffeine potassium salt	20				
			3FQ	44-46						
			4M3H	59						
			3,5DM,4H	70						
5. Amino Acids	PHE	42-46	TYR	26-30	DOPA	15-19				

acids have Rf values considerably lower than the benzoic acids or cinnamic acids, probably because they were relatively insoluble in the solvent employed. After this work was completed there were publications reporting the use of PVP column chromatography to separate phenylalanine, tyrosine and tryptophan (189) and nucleotides, pyrimidines and purines (190), and for isolating flavanoid pigments (191). Hydrogen bonding mechanisms were reported to be responsible for these separations.

The TLC system developed by this investigation has a certain diagnostic value when applied to unknown compounds. If the class of compound can be determined by other means, eg structure-specific locating reagents, the number of hydroxyl groups can be determined by the Rf value.

Conclusion.

This TLC system separates phenolic compounds according to their degree of hydroxylation.

Examination of the phenolic extracts of green and roasted coffee beans by PVP TLC.

Materials.

The PVP plates and solvent system employed were as previously developed.

The concentrated phenolic extracts were prepared by evaporating 50.0ml the original 70% propan-2-ol extract to dryness at reduced pressure, and redissolving the residue in 2.0 ml 70% propan-2-ol. These extracts were stored in stoppered glass vials, in the dark at -10°.

Standard solutions of the available depsides (5mg/ml) were used for controls.

Locating Reagents:- (See Appendix A).

Periodate Reagent 0.25%.

Molybdate Reagent.

DNPH Reagent.

Procedure.

Loadings of 1 μl were used for the standard solutions and initially of 2μl, 4μl, and 6μl for the concentrated extracts. A 15cm development was used. The solvent was thoroughly removed by a stream of warm air before the spots were located, initially by UV fluorescence followed by spraying with Molybdate Reagent. After noting these results the plates were sprayed with the Periodate Reagent, and the appearance of any additional spots recorded.

The DNPH reagent was used on plates run specifically for the separation of carbonyl compounds.

Results and discussion.

The most useful loading of the concentrated extracts was found to be 4μl. Figure 9 shows a drawing of a typical chromatogram

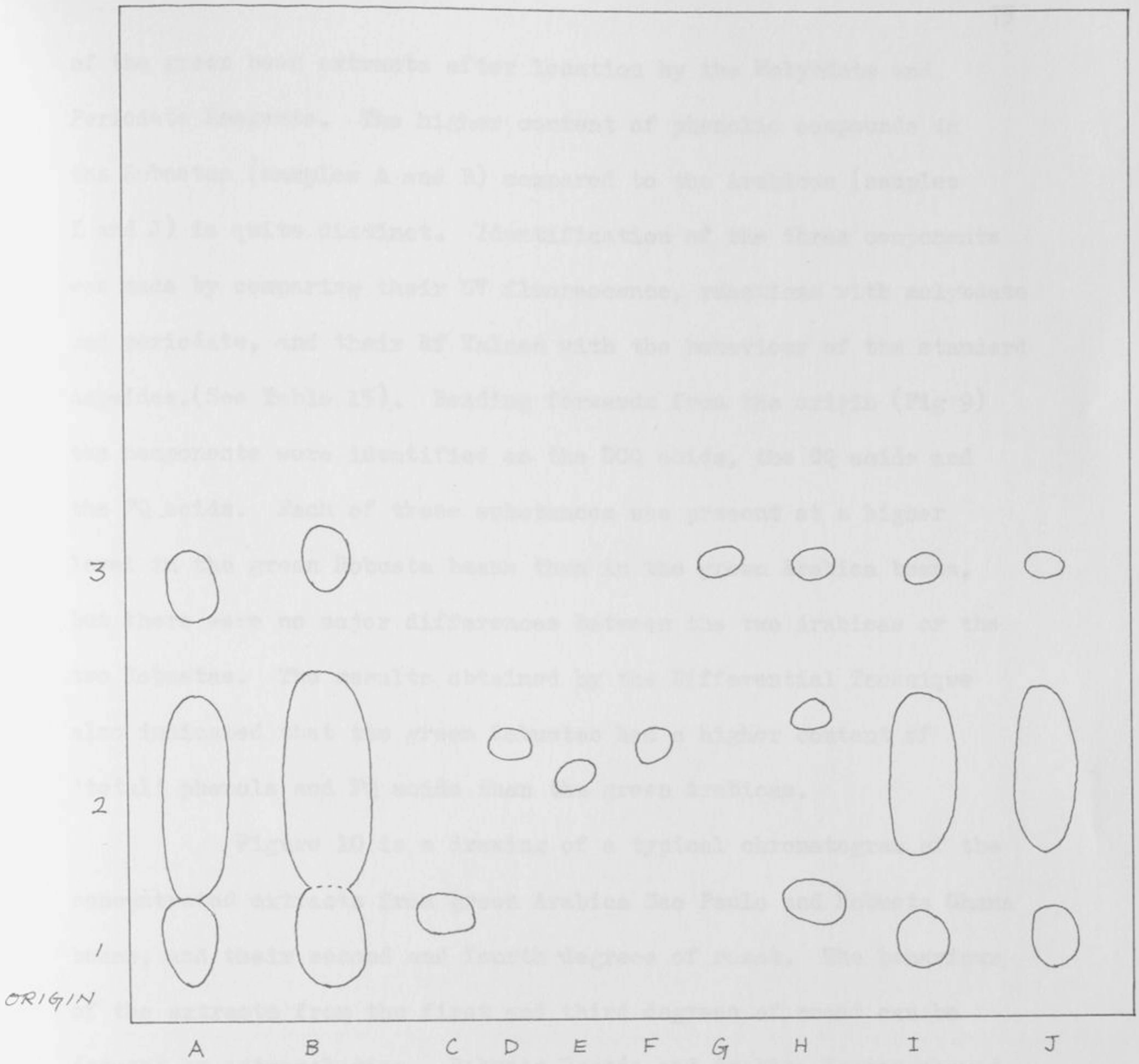


FIG 9 THIN LAYER CHROMATOGRAM OF CONCENTRATED PHENOLIC EXTRACTS FROM GREEN COFFEE BEANS.

LAYER – PVP

SOLVENT – BUTAN-2-ONE, METHYL PHENYL KETONE, 50% ACETIC ACID.
5:5:4 V/V

SAMPLE KEY:-

A	ROBUSTA UGANDA	F	3CQ CAFFEINE POTASSIUM SALT.
B	ROBUSTA GHANA	G	3FQ
C	1,4 DCQ	H	MIXTURE OF C, D AND G
D	3CQ	I	ARABICA SANTOS
E	5CQ	J	ARABICA SAO PAULO.

of the green bean extracts after location by the Molybdate and Periodate Reagents. The higher content of phenolic compounds in the Robustas (samples A and B) compared to the Arabicas (samples I and J) is quite distinct. Identification of the three components was made by comparing their UV fluorescence, reactions with molybdate and periodate, and their Rf Values with the behaviour of the standard depsides.(See Table 15). Reading forwards from the origin (Fig 9) the components were identified as the DCQ acids, the CQ acids and the FQ acids. Each of these substances was present at a higher level in the green Robusta beans than in the green Arabica beans, but there were no major differences between the two Arabicas or the two Robustas. The results obtained by the Differential Technique also indicated that the green Robustas had a higher content of 'total' phenols and FQ acids than the green Arabicas.

Figure 10 is a drawing of a typical chromatogram of the concentrated extracts from green Arabica Sao Paulo and Robusta Ghana beans, and their second and fourth degrees of roast. The behaviour of the extracts from the first and third degrees of roast can be deduced by extrapolation. Robusta Uganda and Arabica Santos showed almost identical behaviour to Ghana and Sao Paulo respectively.

Components of the roasted bean extracts did not separate quite as well as the components of the green bean extracts, but the tendency to tail was not sufficient to mask the components. The DCQ acids and CQ acids (components 1 and 2) decreased considerably during the roasting cycle, but the FQ acids (component 3) did not. Slight quantitative changes would not have been demonstrated by TLC, but it is quite clear that the FQ acids behaved differently to the DCQ and CQ acids.

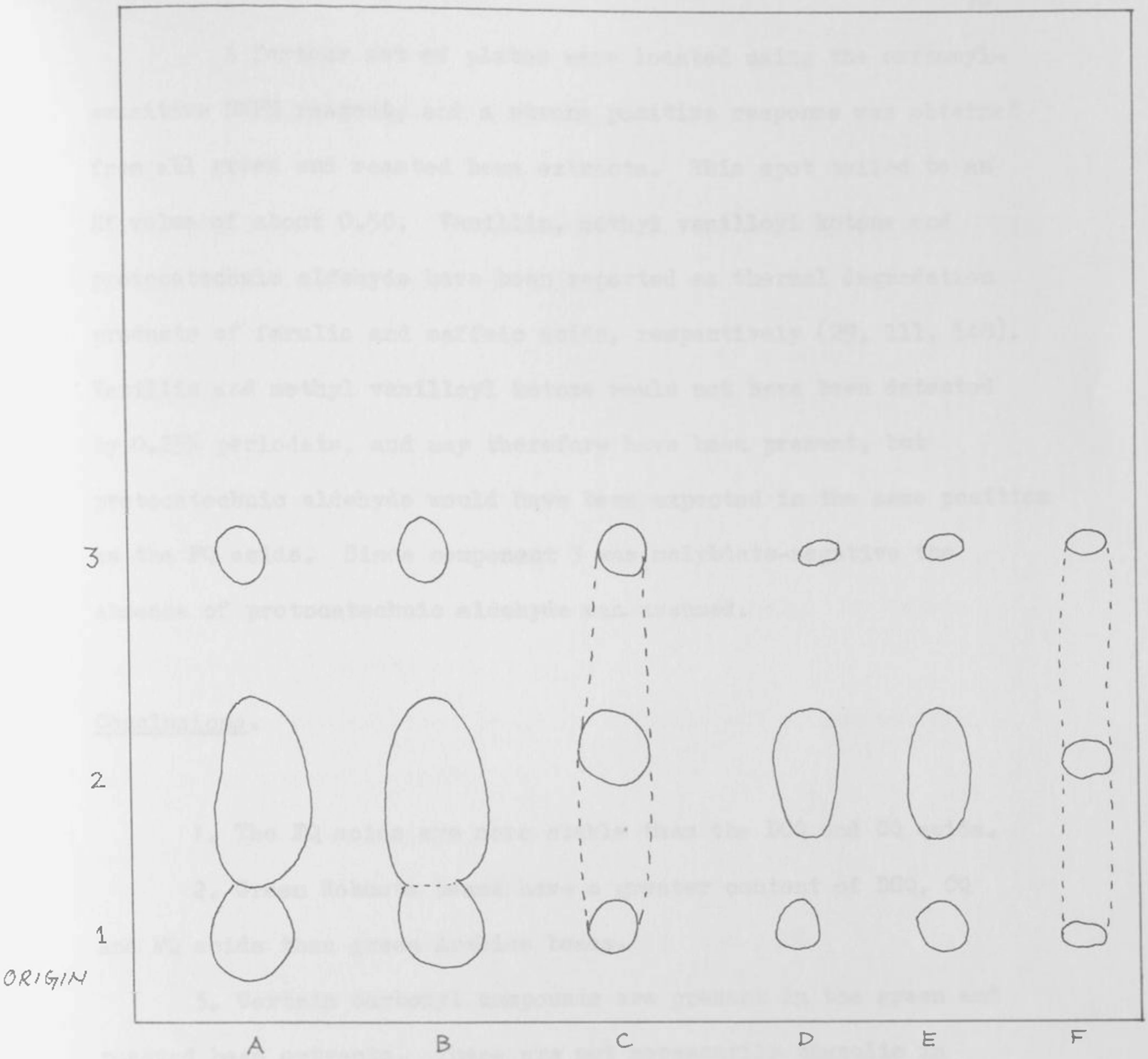


FIG 10 THIN LAYER CHROMATOGRAM OF CONCENTRATED PHENOLIC EXTRACTS FROM GREEN AND ROASTED COFFEE BEANS.

LAYER – PVF

SOLVENT – BUTAN-2-ONE, METHYL PHENYL KETONE, 50% ACETIC ACID. 5:5:4 V/V.

SAMPLE KEY:–

A, B AND C	ROBUSTA GHANA – GREEN, SECOND AND FOURTH DEGREES OF ROAST RESPECTIVELY.
D, E AND F	ARABICA SAO PAULO – GREEN, SECOND AND FOURTH DEGREES OF ROAST RESPECTIVELY.

A further set of plates were located using the carbonyl-sensitive DNPH reagent, and a strong positive response was obtained from all green and roasted bean extracts. This spot tailed to an Rf value of about 0.50. Vanillin, methyl vanilloyl ketone and protocatechuic aldehyde have been reported as thermal degradation products of ferulic and caffeic acids, respectively (29, 111, 148). Vanillin and methyl vanilloyl ketone would not have been detected by 0.25% periodate, and may therefore have been present, but protocatechuic aldehyde would have been expected in the same position as the FQ acids. Since component 3 was molybdate-negative the absence of protocatechuic aldehyde was assumed.

Conclusions.

1. The FQ acids are more stable than the DCQ and CQ acids.

2. Green Robusta beans have a greater content of DCQ, CQ and FQ acids than green Arabica beans.

3. Certain carbonyl compounds are present in the green and roasted bean extracts. These are not necessarily phenolic in nature.

CONVENTIONAL SILICA GEL TLC.

Determination of the relative proportions of the individual depsides present in the green and roasted bean.

Materials.

Silica gel G TLC plates, prepared conventionally. (See Appendix B).

The solvent system toluene, ethyl formate, 97% formic acid (5:5:1 $^{v}/_{v}$) of Van Sumere et al (185).

The concentrated phenolic extracts and standards as used in the preceding work.

Locating Reagents (See Appendix A).

Molybdate Reagent.
Periodate Reagent 0.5%; and 5.0%.
DNPH Reagent.
Wiesner Reagent for aromatic aldehydes.
Cartwright and Roberts' Reagent for quinic acid.

Procedure.

The sample loads were 1µl for the standard solutions, and 4µl for the concentrated extracts. Two developments to 15cm were used since the depsides were reported to have low Rf values, and compounds of lower molecular weight to move much faster (185). Location of spots was initially by UV fluorescence, and the Molybdate and Periodate Reagents were then used sequentially. The

DNPH Reagent, Wiesner Reagent or Cartwright and Roberts' Reagent were used as appropriate.

Results and discussion.

A drawing of a typical chromatogram is shown in Fig 11. Spot identification was based on colour of UV fluorescence, colour with locating reagents and Rf values in comparison with standard phenolic compounds. (This data is shown in Table 29).

There are marked differences between the Arabicas and the Robustas, but little difference between the two members of each group. Spot 1 was probably a mixture of DCQ acids, Spot 2 was 3CQ and/or its caffeine complex, Spot 4 was 5CQ and Spot 5 by a process of elimination was probably 4CQ. Spot 3 certainly contained 3FQ and perhaps a little of the other FQ acids. Although these spots are almost certainly impure, the use of the structure-specific Molybdate and Periodate Reagents made it possible to say that the CQ and DCQ acids were present at higher levels than the FQ acids.

Spots 6-10 were minor components of doubtful purity. Spots 6 and 8 were thought to be caffeic acid and ferulic acid respectively, and they may have been artefacts released by hydrolysis of the depsides during storage of the extracts. However, Spots 6-10 were also sensitive to the DNPH Reagent and Wiesner Reagent, which suggested that they were aromatic aldehydes. Since the Arabica beans had a larger and more varied content of these substances than the Robustas, these components were examined in more detail. (See Chapter 2, Section 10).

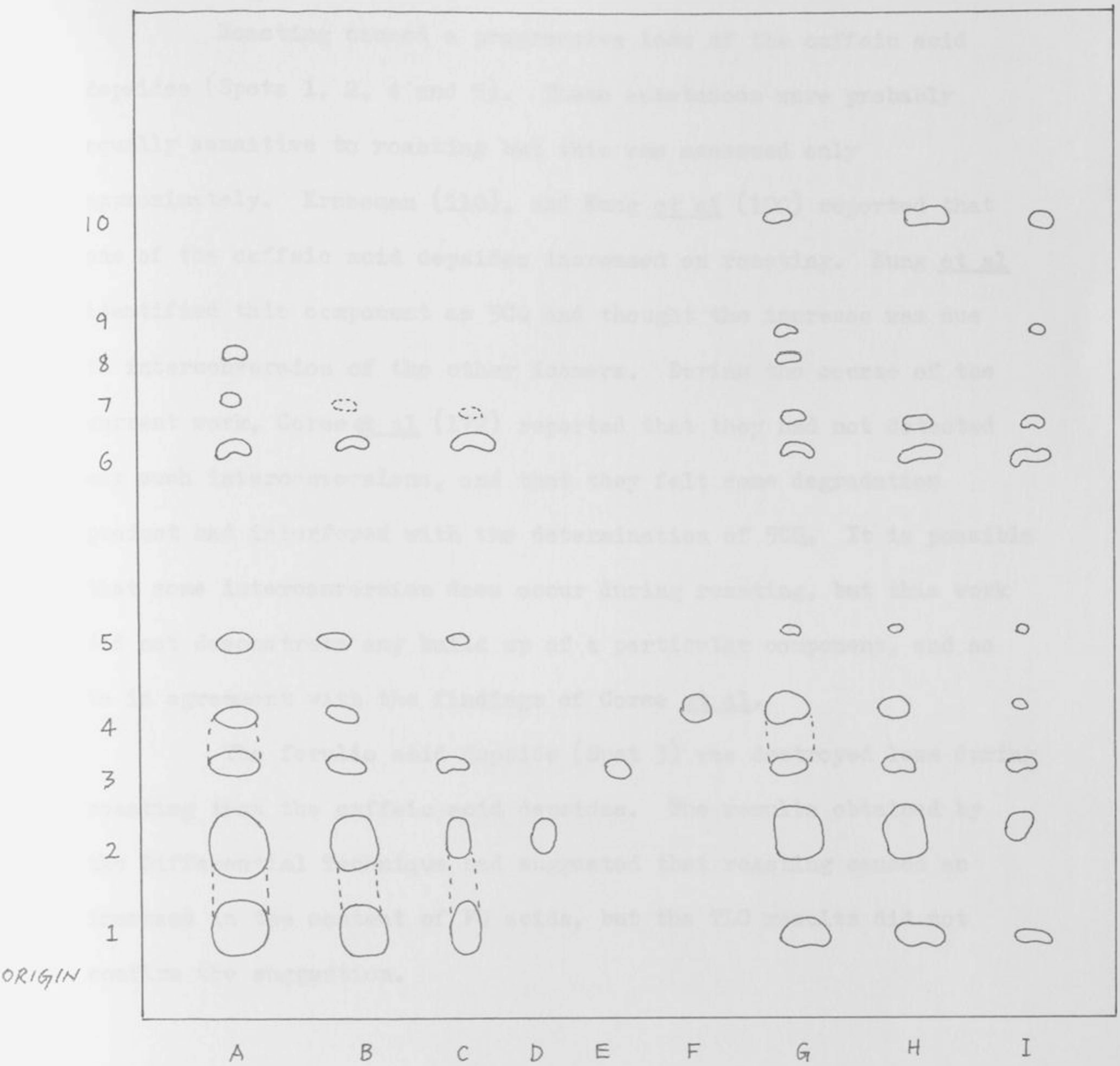


FIG 11 THIN LAYER CHROMATOGRAM OF CONCENTRATED PHENOLIC EXTRACTS FROM GREEN AND ROASTED COFFEE BEANS.

LAYER - PVP

SOLVENT - TOLUENE, ETHYL FORMATE, FORMIC ACID, 5:4:1 V/V

SAMPLE KEY:-

A, B AND C	ROBUSTA GHANA GREEN, SECOND, AND FOURTH DEGREES OF ROAST RESPECTIVELY.
D	STANDARD 3CQ
E	STANDARD 3FQ
F	STANDARD 5CQ
G, H AND I	ARABICA SAO PAULO GREEN, SECOND AND FOURTH DEGREES OF ROAST RESPECTIVELY.

Roasting caused a progressive loss of the caffeic acid depsides (Spots 1, 2, 4 and 5). These substances were probably equally sensitive to roasting but this was assessed only approximately. Kraseman (110), and Kung et al (100) reported that one of the caffeic acid depsides increased on roasting. Kung et al identified this component as 5CQ and thought the increase was due to interconversion of the other isomers. During the course of the current work, Corse et al (172) reported that they had not detected any such interconversions, and that they felt some degradation product had interfered with the determination of 5CQ. It is possible that some interconversion does occur during roasting, but this work did not demonstrate any build up of a particular component, and so is in agreement with the findings of Corse et al.

The ferulic acid depside (Spot 3) was destroyed less during roasting than the caffeic acid depsides. The results obtained by the Differential Technique had suggested that roasting caused an increase in the content of FQ acids, but the TLC results did not confirm the suggestion.

Conclusions.

1. The CQ isomers decrease progressively during roasting of Arabica and Robusta beans.

2. The FQ acids are much more stable than the CQ isomers during roasting of Arabica and Robusta beans.

3. Robusta beans have a higher content of each depside than do Arabicas.

4. Green coffee beans contain a range of aromatic aldehydes, and Arabicas contain a wider range and a greater total quantity than Robustas. These components require a more detailed investigation.

CHAPTER TWO

SECTION EIGHT

MODEL SYSTEMS

THE BEHAVIOUR OF GREEN COFFEE BEAN PHENOLIC COMPOUNDS IN MODEL SYSTEMS.

Introduction.

Coffee roasting is essentially a high-temperature short-time pyrolysis at low initial moisture content. The temperatures involved may be as high as 230°, and this temperature was chosen by Kraseman (110), and Corse *et al* (177) for their model systems.

To obtain more detailed information about the effects of roasting on phenolic compounds in model systems a series of experiments was carried out. It was desired to investigate individually each depside which is present in the green bean, but because these are not commercially available, and because facilities for preparing or isolating them on a large scale were also lacking, a compromise was inevitable. It was decided to investigate caffeic acid, ferulic acid and 3CQ thoroughly, and then make whatever comparisons were possible with 3CQ caffeine potassium salt and 5CQ.

Facilities for roasting model systems at 230° were not available so a temperature of 180° with a maximum time period of twenty minutes, with sampling at five minute intervals, was chosen. It was thought that this range would be coincident at some period with the conditions existing during typical coffee roasting. Samples were placed in small glass vials and were roasted in a thermostatically controlled hot air oven.

Each model system was prepared in triplicate, one sample being examined by TLC for degradation products, and the other two quantitatively by the Differential Technique. The overall weight change was recorded for each sample. In multicomponent systems a 1:1 molar ratio was used for the individual components, and the quantity of phenolic compound was adjusted to ensure an adequate zero time response to the Differential Technique. It was intended that examination of the model systems by the Differential Technique would not only yield information about the model systems, but also about the response of the technique to roasted phenolic compounds, so that it would facilitate interpretation of the results obtained for roasted coffee beans.

The TLC examination was by means of established systems, one for the likely phenolic degradation products (185) and the other for the amino acids which were used in the model systems.

1. The behaviour of ferulic acid, caffeic acid and 3CQ when heated alone.

Materials.

Triplicate samples of caffeic acid (20mg), ferulic acid (20mg) and 3CQ (40mg) were prepared in preweighed glass vials for heating at 180° for 5, 10, 15 and 20 minutes.

Conventional Silica Gel G TLC plates were used. The solvent (52) was prepared by mixing chloroform, glacial acetic acid and water in the proportions 4:1:1 $^{v}/_{v}$ and shaking for thirty minutes. The organic phase was recovered and employed as the solvent.

The following locating reagents were used as required in this and the following sections, and details of preparation are given in Appendix A:- Periodate Reagent 0.25% and 5.0%, Molybdate Reagent, Diazotised Sulphanilic Acid (DSA), Wiesner Reagent, DNPH Reagent, Vanillin/concentrated sulphuric acid, Vanillin/12% Hydrochloric acid, and Ninhydrin Reagent.

Procedure.

Each sample was spread evenly over the bottom of the vial, and all vials to receive a particular treatment were placed on a small metal tray. The trays were placed in a hot air oven that was steady at 180°, and each tray removed after the appropriate period of heating. The heated samples were allowed to cool to room temperature in a desiccator before weighing. The weight change was recorded. Two replicates were dissolved in 70% propan-2-ol, the contents transferred to volumetric flasks (100.00ml) and diluted to volume. Duplicate aliquots of the standard solutions were examined by the Differential Technique. The residue from heating the third replicate was dissolved in a volume (0.25ml) of n-butyl acetate and examined by TLC. Zero time controls containing the appropriate quantity of phenolic compound in 0.25ml n-butyl acetate were chromatographed alongside the heated systems.

Results and discussion.

The results are shown in Figures 12, 13 and 14, and Tables 16 and 17. The graphs show that the heating at 180° caused mild progressive degradation, and that heat sensitivity was in the order

Table 16 The behaviour of cinnamic acids and depsides when heated alone 180° for 20 minutes.

Sample	Percentage loss as measured by			Appearance	Aroma
	Weight loss	Molybdate Reagent	Periodate Reagent		
Caffeic acid	6.0	3.5	11.0	No visible change.	No detectable aroma.
Ferulic acid	13.0	-	46.0	Fused, white.	
3CQ	4.0	30.0	34.0	Particulate brown.	Faint non-descript aroma.

Table 17 Rf values x100, and colour with locating reagents for cinnamic acid and depside model systems on Silica Gel G chromatograms in chloroform acetic acid, water 4:1:1 organic phase.

		Colour with			
Sample	Rf x100	Molybdate Reagent	Periodate Reagent	DSA	UV
Caffeic acid 5,10,15,20 minutes at 180°	24-32 tailed.	Yellow	Yellow	Brown	Bright Blue
Caffeic standard	28-32	Yellow	Yellow	Brown	Bright Blue
Ferulic acid 5,10,15,20 minutes at 180°	94-95	-	Yellow	Red	Purple
Ferulic standard	95	-	Yellow	Red	Purple
3CQ 5,10,15,20 minutes at 180°	02	Yellow	Yellow	Brown	Bright Blue
3CQ standard	02	Yellow	Yellow	Brown	Bright Blue

FIG 12 A DRAWING PREPARED FROM SEVERAL THIN LAYER CHROMATOGRAMS OF THE PHENOLIC MODEL SYSTEMS AFTER ROASTING.

LAYER SILICA GEL G

SOLVENT - CHLOROFORM, GLACIAL ACETIC ACID, WATER
4 : 4 : 1 v/v ORGANIC PHASE.

SAMPLE KEY:-

A, B, C AND D - CAFFEIC ACID + CITRIC ACID
5, 10, 15 AND 20 MINUTES AT 180°

E, F, G AND H - FERULIC ACID + CITRIC ACID
5, 10, 15 AND 20 MINUTES AT 180°

I 5CQ + CITRIC ACID, 20 MINUTES AT 180°
AFTER 3 DEVELOPMENTS.

J CAFFEIC ACID + CITRIC ACID + METHIONINE
20 MINUTES AT 180°

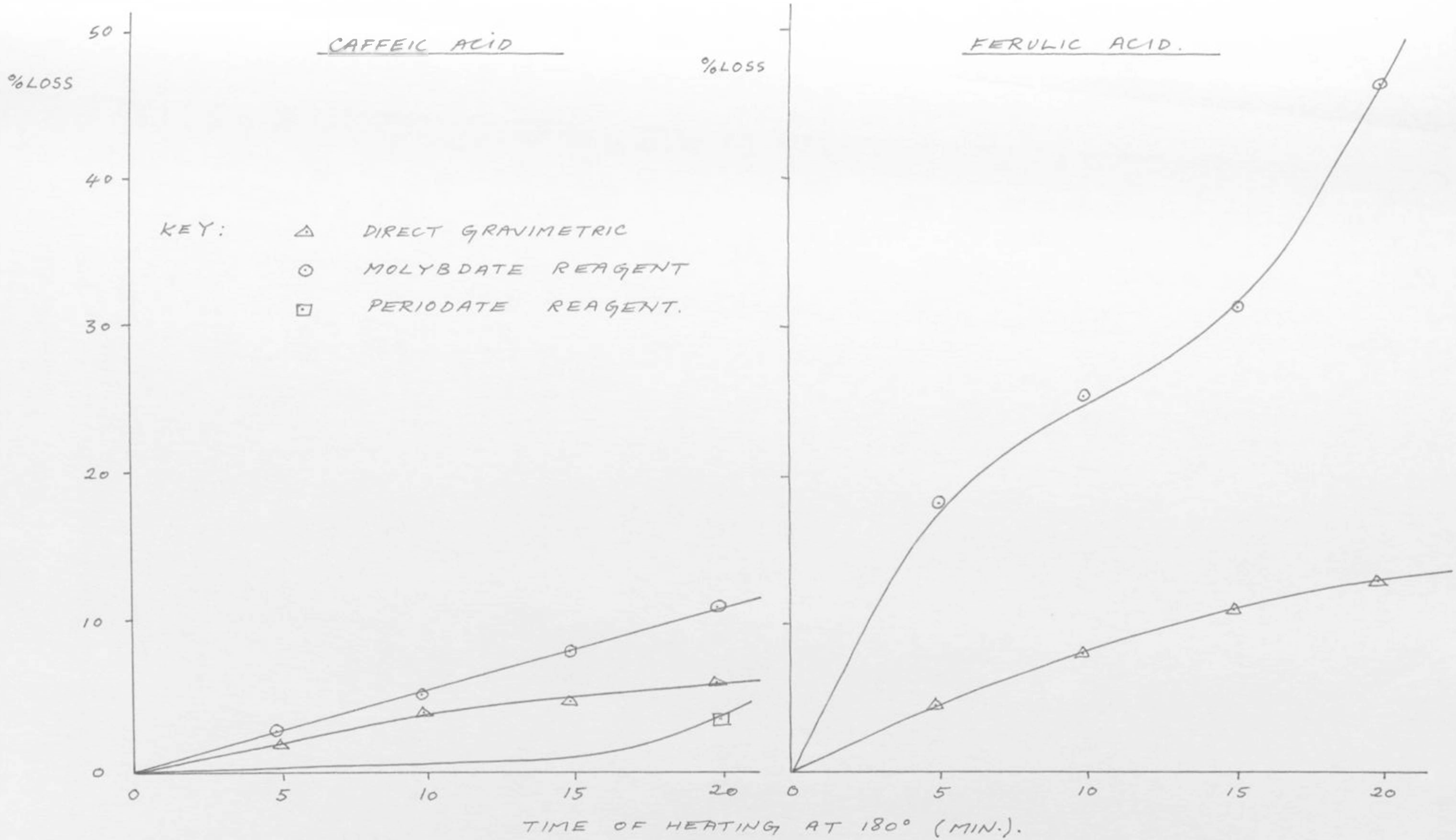


FIG 13 THE EFFECT OF HEAT ON CAFFEIC ACID AND FERULIC ACID

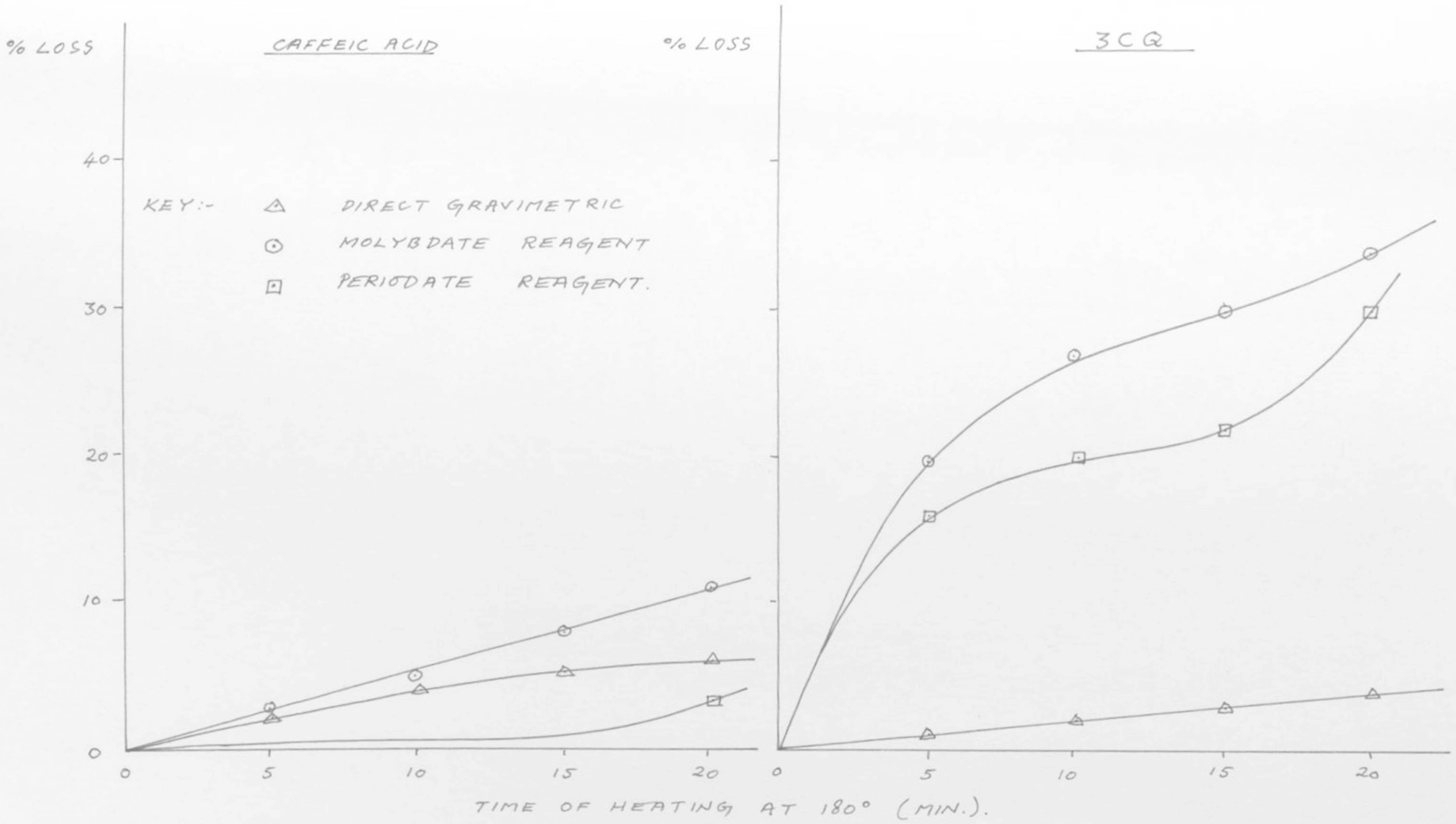


FIG 14 THE EFFECT OF HEAT ON CAFFEIC ACID AND 3CQ

ferulic > 3CQ > caffeic. This order parallels their reported melting points of 170°, 208° and 226° (95) respectively. The progressive loss of ferulic and 3CQ was confirmed by reduction in spot intensity on the chromatograms, but the small loss of caffeic acid could not be detected by this technique. No degradation products were detected, indicating that they were either present at too low a concentration, or non-phenolic, or had been lost as volatiles.

Krasemann (110) quantitating by a direct spectrophotometric technique reported a 78% loss of 3CQ heated at 230°/30 seconds. Assuming a Q_{10} value of 2.0, generally accepted for most chemical reactions 180°/20 minutes would be equivalent to 230°/37½ seconds, but the loss recorded was far less than Krasemann reported. It is possible that the Q_{10} value for this reaction is greater than 2.0, or that heat sensitivity increases considerably at temperatures in excess of the melting point (208°). Loss of 3CQ during the roasting of coffee beans has been reported as 65% by Kung et al (100) and 87% by Corse et al (177). The influence of higher temperatures and/or interactions of 3CQ with other green bean components was indicated.

The results obtained with the Periodate Reagent indicated a slightly greater loss than those obtained with the Molybdate Reagent. This could have been due to interference by degradation products that were not detected by TLC. The weight loss indicated that some contribution to the volatiles of roasted coffee can be expected from these phenolic compounds.

2. The influence of organic acids on the thermal degradation of phenolic compounds.

Materials.

The following model systems were prepared in triplicate for examination at 180° 5, 10, 15 and 20 minutes.

Caffeic acid 20 mg, citric acid 22 mg (1:1 Molar).
Ferulic acid 20 mg, citric acid 22 mg (1:1 Molar).
3CQ 40 mg, citric acid 22 mg (1:1 Molar).

The following systems were prepared in triplicate for examination only at 180° 20 minutes.

Caffeic acid 20 mg, tartaric acid 16 mg. (1:1 Molar).
Caffeic acid 20 mg, quinic acid 20 mg. (1:1 Molar).
Caffeic acid 20 mg, glycine 8 mg. (1:1 Molar).
5CQ 40 mg, citric acid 22 mg. (1:1 Molar).
3CQ caffeine potassium salt 60 mg,
citric acid 22 mg. (1:1 Molar).
Quinic acid 20 mg.)
) (1:1 Molar) controls
Quinic acid 20 mg, citric acid 20 mg.)

Procedure.

As described in the preceding section.

Results and discussion.

Comparison of these results with those of the previous section showed that an organic acid had a pronounced effect on the thermal degradation of the phenolic compounds examined. (Table 16 and 18). The Molybdate and Periodate Reagents indicated nearly 100% loss of caffeic acid from the caffeic acid - citric acid system (180°/20 min.), about ten times the loss recorded for caffeic acid alone. The relative heat sensitivities of ferulic acid, 3CQ and caffeic acid were reversed, suggesting that the ortho-dihydroxy group renders the molecule more sensitive. However TLC demonstrated that the ortho-dihydroxy group remained intact in all degradation products that were detected. Since under these conditions ferulic acid was less heat sensitive than caffeic acid, it was thought that 3FQ would be less heat sensitive than 3CQ. TLC examination of green and roasted bean phenolic extracts (See Page 75) also suggested that 3FQ was less heat sensitive than the CQ acids.

The usual chromatogram did not separate any degradation products from the depside - containing systems, all the material remaining at the origin, although in the case of 3CQ a progressive decrease occurred during the roasting cycle. Four degradation products (Spots 1, 2, 4 and 5 in Fig 12) were separated from the caffeic acid - containing systems. They were all sensitive to the Molybdate Reagent, 0.25% Periodate Reagent and DSA. Spot 3 was residual caffeic acid and a progressive decrease left just a trace after twenty minutes. Spot 1 reached a maximum after fifteen minutes and then decreased at twenty minutes and Spot 2 decreased throughout the roasting period. Spots 4 and 5 were detected only in the fifteen minute sample and were present at such a low level that it was difficult to determine their response to the locating reagents. It is possible that they were protocatechuic acid and aldehyde

Table 18 The degradation products from cinnamic acids and depsides heated with organic acids.

Spot No. or Standard	Rf x100	Responses to Locating Reagents								Probable Identity
		Self locating	Molybdate	Periodate 0.25%	Periodate 5.0%	DSA	DNPH	Wiesner	Vanillin	
1	05-07	+	+	+	+	Red	-	-	Purple	Pyrogallol
2	10-12	+	+	+	+	Red	-	-	Purple	Pyrogallic acid
3	28-32	-	+	+	+	Red	-	-	-	Caffeic acid
4	52-56	-	Faint	+	+	Yellow	-	-	-	Protocatechuic acid
5	63-66	-	Faint	+	+	Yellow	?	?	-	Protocatechuic aldehyde
6	74-80	-	-	-	-	Brown	-	-	-	A monohydroxy phenol
7	86-88	-	-	-	-	Yellow	-	-	-	A monohydroxy phenol
8	90-92	-	-	-	-	Yellow	-	-	-	4-hydroxy benzoic acid
9	93-95	-	-	+	+	Brown	-	-	-	Ferulic acid
10	96-100	-	-	-	+	Red	+	-	-	Methyl vanilloyl ketone and vanillic acid?
X	03-05	-	-	-	+	Yellow	-	-	-	?
Y	06-08	-	-	-	+	Yellow	-	-	-	?
A	03*	+	+	+	+	Red	-	-	Purple)	(vicinal
B	06*	+	+	+	+	Red	-	-	Purple)	(trihydroxy
C	11-12*	+	+	+	+	Red	-	-	Purple)	(compounds, polymers?
Caffeic acid	28-32	-	+	+	+	Red	-	-	-	
Protocatechuic acid	48-50	-	+	+	+	Red	-	-	-	
Protocatechuic aldehyde	60-63	-	+	+	+	Red	+	+	-	
Pyrogallol	05-06	+	+	+	+	Red	-	-	Purple	
Pyrogallic acid	12-14	+	+	+	+	Red	-	-	Purple	
4-hydroxy benzoic acid	88-91	-	-	-	-	Yellow	-	-	-	
Ferulic acid	93-95	-	-	+	+	Brown	-	-	-	
Vanillic acid	96-98	-	-	-	+		-	-	-	
Vanillin	86-89	-	-	-	+		+	+	-	
Methyl vanilloyl ketone	96-98	-	-	-	+		+	-	-	
Guaiacol	88-90	-	-	+	+		-	-	-	
Catechol	86	-	+	+	+		-	-	-	
Quinol	83	-	-	+	+		-	-	-	
3CQ	0	-	+	+	+	Red	-	-	-	
3CQ caffeine potassium salt	0	-	+	+	+	Red	-	-	-	
5CQ	0	-	+	+	+	Red	-	-	-	

*Rf value obtained after triple development of the chromatogram.

respectively. Spots 1 and 2 were noticed to be self locating if the developed chromatogram was left exposed to the atmosphere for about ten minutes, before using a locating reagent. These spots became blue, a phenomenon first noticed with pyrogallol (3,4,5THBz) while investigating the new PVP TLC system. When the available trihydroxy compounds were chromatographed alongside the caffeic acid degradation products, pyrogallol and pyrogallic acid (3,4,5THBz and 3,4,5THBA) were found to correspond with Spots 1 and 2. For confirmation those compounds were examined by the Vanillin Reagent (in concentrated sulphuric acid) which Swain and Goldstein (162) reported to detect meta (in this case 3, 5) hydroxyl groups. This reagent was found to be non-selective and to locate many phenolic compounds due to an effect of the concentrated acid. Since it was known that a meta-dihydroxy compound (Phloroglucinol 2,4,6THBz) in 12% hydrochloric acid would locate aromatic aldehydes (Wiesner Reagent) it was deduced that an aromatic aldehyde in 12% hydrochloric acid would detect phloroglucinol and other meta-dihydroxy phenols. The available meta-dihydroxy phenols were not of equal sensitivity to this reagent (See Appendix A), but Spots 1 and 2, and pyrogallol and pyrogallic acid gave identical responses. This similarity coupled with the evidence of the other locating reagents was considered to confirm the presence of a vicinal trihydroxy group in compounds 1 and 2. Pyrogallol has been reported as a chlorogenic acid degradation product, with the suggestion that it was formed from the quinic acid moiety. (29, 111). However, no phenolic compounds were formed in the quinic acid or quinic acid and citric acid model systems.

The six DSA - sensitive degradation products separated from the ferulic acid - citric acid model system were quite different from those produced by caffeic acid. Only Spot 9 was located by 0.25% periodate and this was residual ferulic acid. In agreement with the quantitative results a progressive decrease in content paralleled the roasting period. Periodate (5%) additionally located Spots X, Y and 10. Spot 10 was found to be sensitive to the DNPH Reagent, but not to the Wiesner Reagent, and behaved identically with methyl vanilloyl ketone. This substance has been reported as a degradation product of ferulic acid (148) and could be responsible for the vanillin-like aroma of this model system after roasting. It must be pointed out that if vanillic acid (3M,4HBA)(the monomethyl ether of protocatechuic acid) were also produced, it would be masked by the ketone since they both have the same Rf value in this solvent. Since Spots 6, 7 and 8 were located only by DSA they were considered to be monohydroxy compounds, and Spot 7 behaved similarly to 4-hydroxybenzoic acid. However, this identification was based essentially on negative evidence and, therefore, is not so reliable as one based on positive evidence. The nature of minor components X and Y is uncertain. According to their reactions to locating reagents they were quite distinct from compounds 1 and 2 produced in the caffeic acid systems, and could not be their methyl ether derivatives. All degradation products increased during the roasting period.

Since heating the caffeic acid depsides with citric acid did not give rise to the degradation products typical of caffeic acid, it was assumed that hydrolytic release of caffeic acid did not occur. The high temperature and low initial water content in the model system would not favour hydrolysis. Such a lack of degradation products was not in agreement with the results of the quantitative

Table 19 The behaviour of cinnamic acids and depsides when heated with organic acids at 180°/20 minutes.

Sample	Percentage Loss Determined by			Appearance after heating	Aroma after heating
	Weight Loss	Molybdate Reagent	Periodate Reagent		
Caffeic acid + citric acid	38.2	98.0	92.4	Fused, dark brown, effervescent.	
Ferulic acid + citric acid	28.2	-	55.0	Fused, dark brown, effervescent.	Reminiscent of vanillin or methyl vanilloyl ketone.
3CQ and citric acid	12.5	78.0	68.0	Fused, dark brown, effervescent.	
3CQ caffeic potassium salt + citric acid	26.0	43.0	44.0	Fused, yellow, relatively insolubale effervescent.	
5CQ and citric acid	26.0	76.0	59.0	Fused, dark brown, effervescent.	
Caffeic acid + tartaric acid	41.0	94.0	63.0	Fused, reddish-brown, effervescent.	
Caffeic acid + quinic acid	9.5	56.0	24.0	Fused, dark brown, effervescent.	
Caffeic acid + glycine	22.0	90.0	53.0	Fused, dark brown.	
Quinic acid	11.0	-	-	Fused, green.	
Quinic acid and citric acid	16.5	-	-	Fused, green.	

techniques (Table 19) and it was felt that some degradation products must have remained at the origin along with the depside. Accordingly, several chromatograms were subjected to thrice - repeated developments in the usual solvent. The 5CQ citric acid mixture gave three distinct spots (A, B and C), and 3CQ and 3CQ caffeine potassium salt gave traces of the same. Spot B had the same Rf value as the original depside, but all three spots had the self-locating property noted for pyrogallol etc., and responded to the new Vanillin Reagent. This indicated that Spot B was impure since such depsides did not normally have those properties. One of these products could be the 5CQ lactone since Corse et al reported that 5CQ lactonized when heated in acid conditions (177). It was felt that these compounds probably arose by a mechanism similar to that which produced compounds 1 and 2 from caffeic acid.

Tartaric acid, glycine and quinic acid exerted on caffeic acid similar though not identical effects to citric acid. Glycine did not yield any phenolic, or ninhydrin-sensitive degradation products, or residual caffeic acid after heating for twenty minutes. When compared with citric acid, only Spot 4 was missing from the tartaric acid and quinic acid degradation pattern, but the transitory nature of this substance had been noted in the case of citric acid. Hassan (192) believed that in an amino acid - sugar model system, the amino acid operated in two ways; initially as an organic acid it caused the decomposition of the sugar, then the interaction of the amino group with the degradation products modified the initial products. Similar behaviour in the glycine - caffeic acid system would account for the loss of the expected degradation products.

Rinderknecht and Jurd (193) Burton, McWeeny and Pandhi (194) have reported non-enzymic browning reactions between glycine and phloroglucinol, and catechol and orthobenzoquinone.

In most of the organic acid model systems the Periodate Reagent detected a smaller loss of material than the Molybdate Reagent. The graphs (Fig 15 and 16), show that this difference changed as roasting proceeded. Differences in the proportions of the degradation products could have been responsible, since, for example, a monomethyl ether of caffeic acid or 3CQ would be molybdate-insensitive, periodate- and DSA-positive. The absence of molybdate-insensitive spots from the caffeic acid and 3CQ model systems tends to rule out the possibility that monomethylation was responsible for the hysteresis. Different amounts of hysteresis were detected in each model system demonstrating that, while there was a common pattern, changing a component caused some modification of the reactions involved. Monomethylation of caffeic acid degradation products during the roasting of coffee beans was suggested as a possible explanation of the hysteresis detected by the Differential Technique. The failure to detect monomethyl ethers in the products of these model systems suggested that other mechanisms might have been responsible for the hysteresis.

A comparison of the results for the depsides revealed interesting behaviour. The mixture containing the 3CQ caffeine potassium salt showed twice the weight loss of free 3CQ, but with the Molybdate and Periodate Reagents the complex showed only half the loss compared to the free acid. (See Table 19). Smith (29, 90), reported that the caffeine complexes were the most stable form of green bean CQ acids. Sublimation of the caffeine could have accounted for the extra weight loss, and it is possible that this loss of

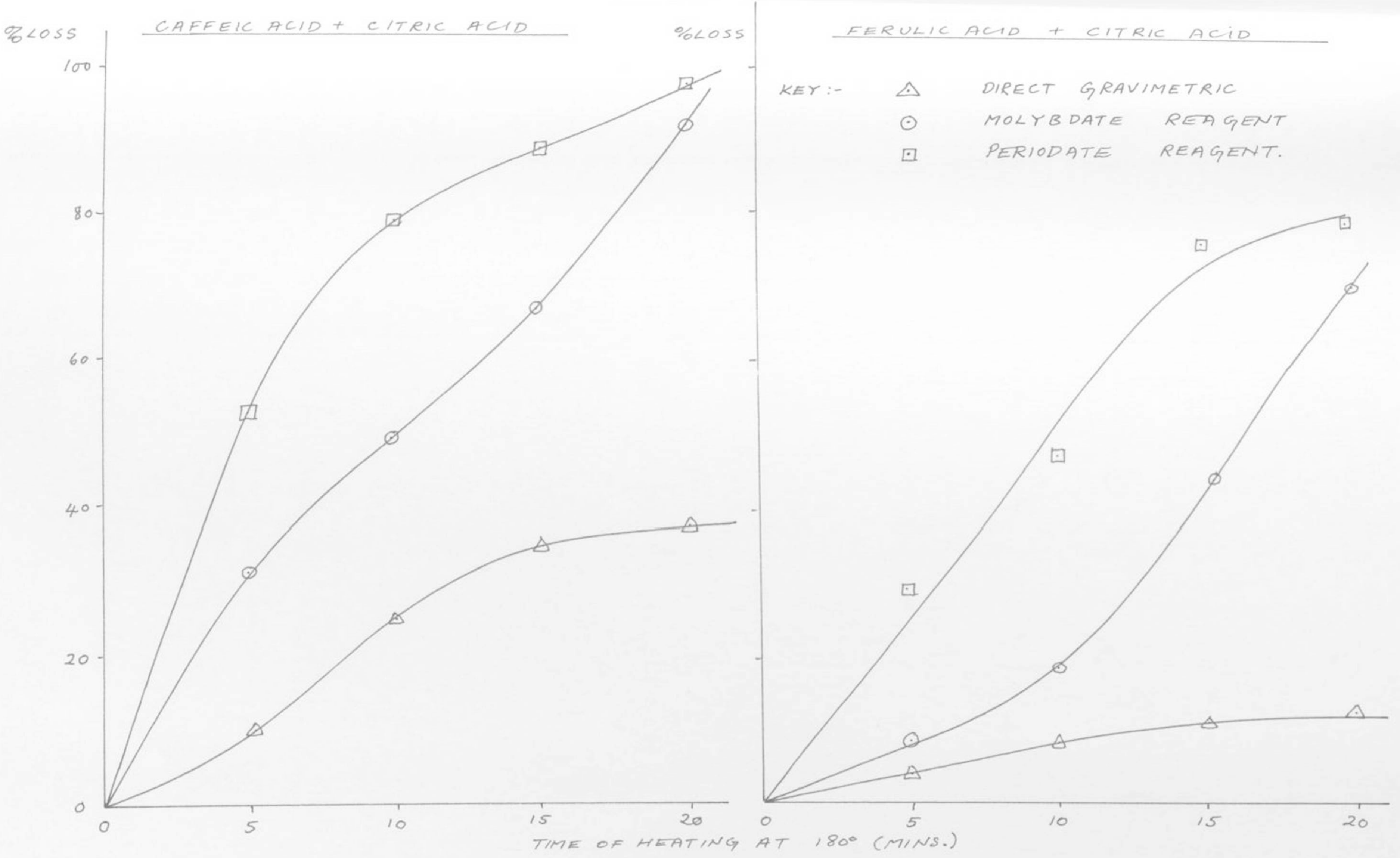


FIG 15 THE EFFECT OF HEAT ON MIXTURES OF CAFFEIC ACID AND CITRIC ACID (1:1 MOLAR), AND 3CQ AND CITRIC ACID (1:1 MOLAR)

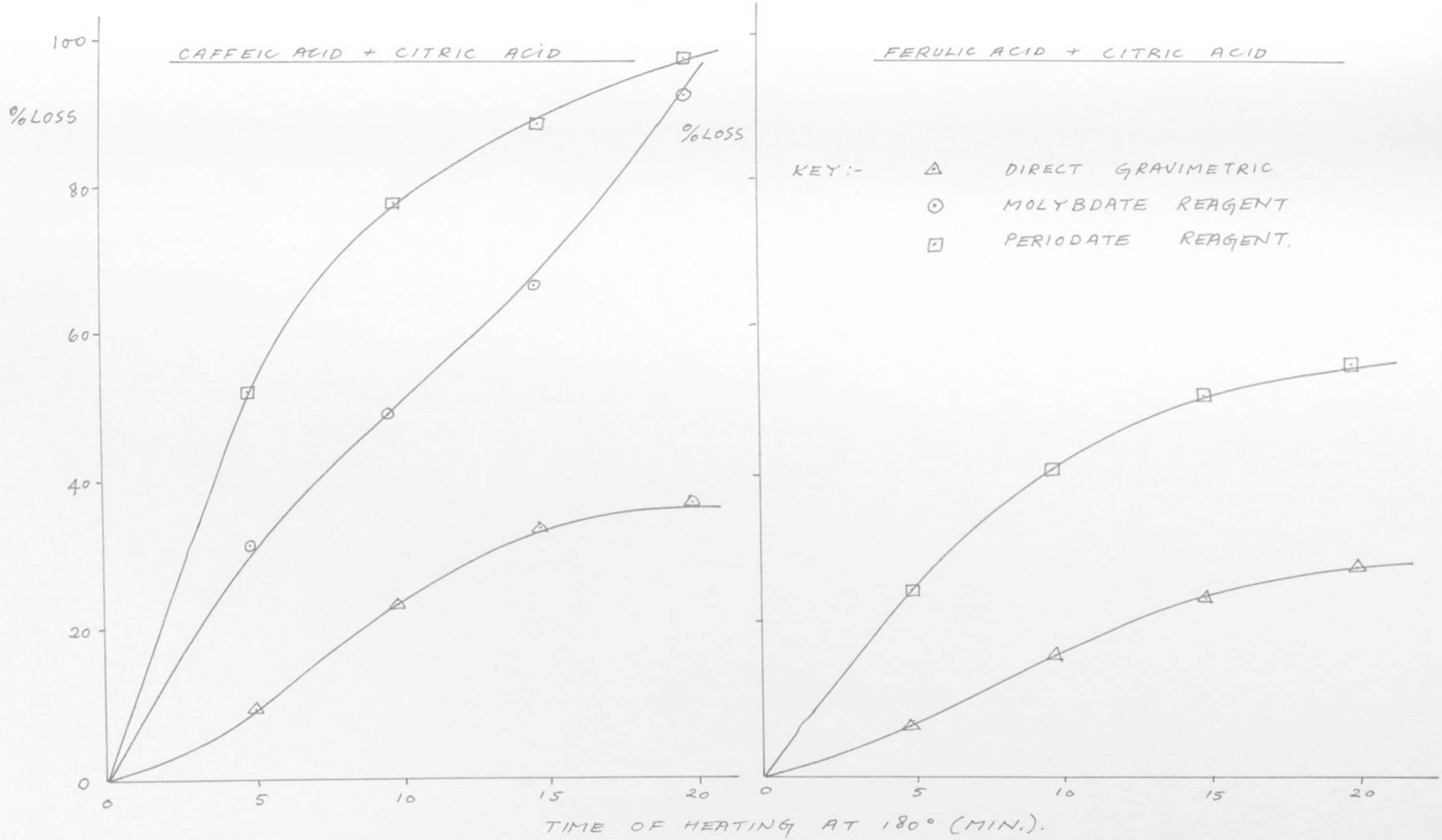


FIG 16 EFFECT OF HEAT ON MIXTURES OF CAFFEIC ACID AND CITRIC ACID (1:1 MOLAR) AND FERULIC ACID AND CITRIC ACID (1:1 MOLAR).

caffeine affords some protection to the phenolic component.

The results showed that free 3CQ and 5CQ behaved very similarly in these model systems, and this was in agreement with their behaviour in the roasted bean as demonstrated by TLC.

3. The influence of sugars on the thermal degradation of phenolic compounds.

Materials.

The following model systems were prepared in triplicate for examination at 180° 5, 10, 15 and 20 minutes.

Caffeic acid 20 mg, glucose 20 mg (1:1 Molar).
Ferulic acid 20 mg, glucose 20 mg (1:1 Molar).

The following model systems were prepared in triplicate for examination only at 180° 20 minutes.

Caffeic acid 20 mg, fructose 20 mg (1:1 Molar).
Caffeic acid 20 mg, fructose 20 mg, glycine 8 mg (1:1:1 Molar).
Caffeic acid 20 mg, glucose 20 mg, glycine 8 mg (1:1:1 Molar).
Ferulic acid 20 mg, fructose 20 mg (1:1 Molar).
3CQ 40 mg, glucose 20 mg (1:1 Molar).
Quinic acid 20 mg, glucose 20 mg (1:1 Molar), control.

Procedure.

The model systems were treated as described in the first section.

Results and discussion.

The quantitative and chromatographic results (Figures 12 and 17) fall roughly midway between the results for the phenolic compounds alone and the organic acid-containing model systems. The relative sensitivity to heating with glucose was ferulic > 3CQ > caffeic, and to heating with fructose, ferulic > caffeic. This was the order of sensitivity found for the phenolic compounds heated alone, and the reverse of the order with the organic acid-containing model systems.

In the absence of glycine both sugars produced a small progressive loss of caffeic acid and a somewhat more pronounced loss of ferulic acid. Caffeic acid yielded one degradation product - Spot 5 (Fig 12) after 180° 20 minutes, and ferulic acid yielded methyl vanilloyl ketone in the 15 and 20 minute samples. No degradation products were detected from the 3CQ model system. Since the degradation products detected in these sugar-containing model systems were not the first to appear in the organic acid-containing model systems, it was deduced that a different balance had been struck for the various reactions. It is possible that a phenolic acid and a sugar have a mutually catalytic action; initially the phenolic acid catalyses degradation of the sugar, then the sugar degradation products, perhaps including organic acids, catalyse destruction of the phenolic compound. Hassan (192) reported that citric acid or 3CQ increased the production of volatiles from sugars heated at temperatures in the range 125° to 175°, and a range of organic aliphatic acids have been reported to form during the pyrolysis of carbohydrates (136).

Table 20 The influence of sugars (with and without glycine) on the thermal degradation of cinnamic acids and depsides.

Sample	Percentage Loss Determined by			Appearance after heating	Aroma after heating
	Weight Loss	Molybdate Reagent	Periodate Reagent		
Caffeic acid and glucose	11.6	29.0	17.0	Fused, brown, effervescent.	Caramel.
Ferulic acid and glucose	18.9	-	28.0	Fused, brown, effervescent.	Caramel.
3CQ and glucose	16.0	30.0	20.6	Fused, brown, effervescent.	Caramel.
Caffeic acid and fructose	20.0	30.0	6.0	Fused, brown, effervescent, darker than glucose.	Caramel.
Ferulic acid and fructose	22.0	-	11.0	Fused, brown, effervescent.	Caramel.
Caffeic acid, glycine and glucose	25.0	96.0	75.0	Fused, black, effervescent.	Harsh, caramel.
Caffeic acid, glycine and fructose	17.5	92.0	51.0	Fused, black, effervescent.	Harsh, caramel.
Quinic acid and glucose.	7.0	-	-	Fused, brown.	Antiseptic.

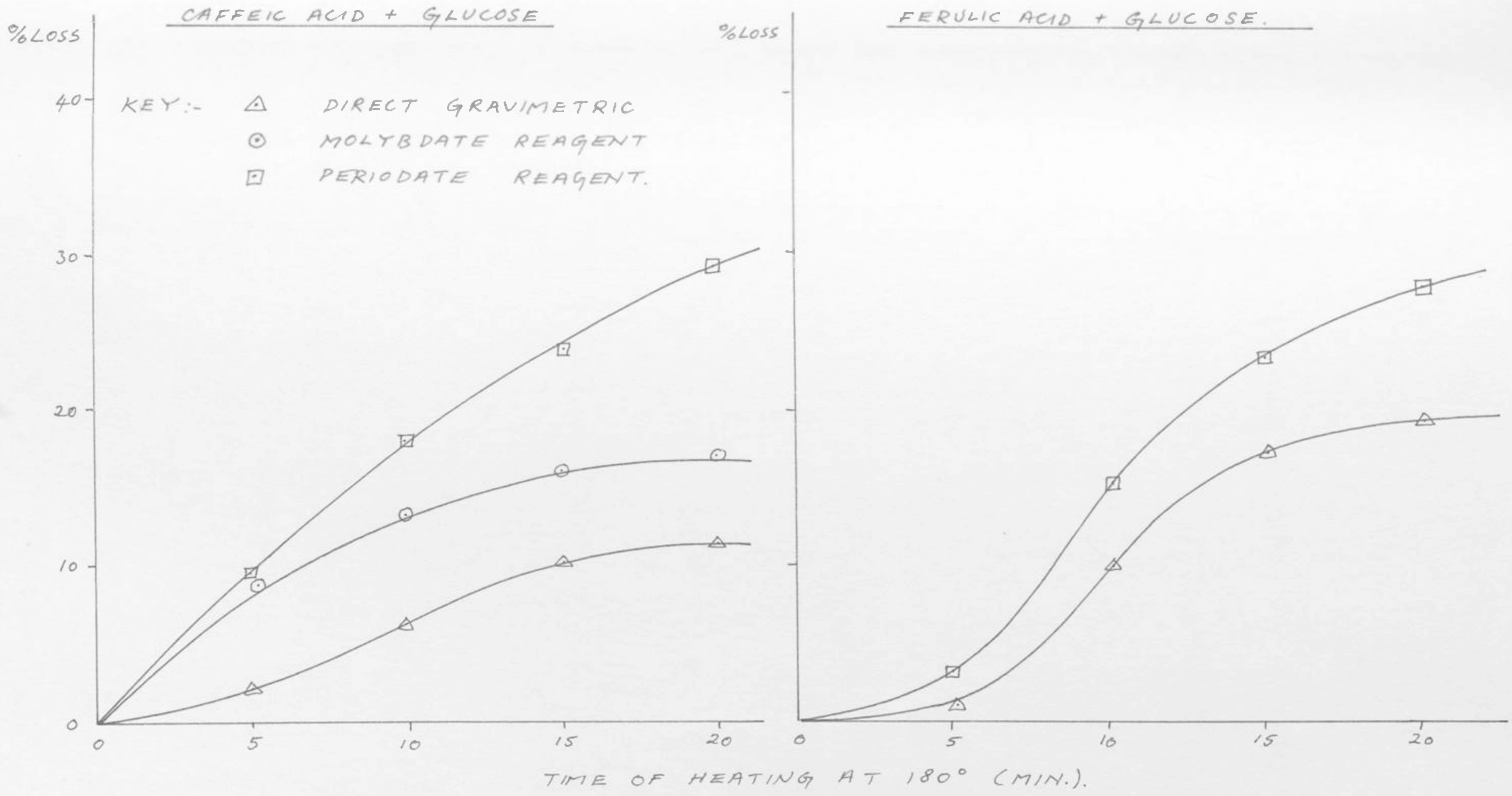


FIG 17 EFFECT OF HEAT ON MIXTURES OF CAFFEIC ACID AND GLUCOSE (1:1 MOLAR), AND FERULIC ACID AND GLUCOSE (1:1 MOLAR).

The glycine-sugar-phenol model systems behaved very similarly to the glycine-caffeic acid system which was examined in the previous section. No phenolic, or ninhydrin-sensitive degradation products were separated by TLC. Since these model systems were examined only after roasting for twenty minutes, it is not possible to decide whether inclusion of the sugars had had any additional effect. This type of model system meritted a more detailed examination.

Fructose caused less destruction of caffeic acid or ferulic acid than did glucose, but the fructose-containing model systems suffered a greater total weight loss. This may have been due to the tendency for fructose to decompose at a lower temperature than glucose (103° compared to 147°). Hassan (192), El Ode (195), and Casey et al (196) reported that fructose in model systems produced a greater quantity of volatiles (ie suffered greater degradation) than did glucose.

The hystereses were again encountered, and fructose-containing model systems showed a larger hysteresis than glucose-containing systems. In the case of the caffeic acid or ferulic acid-sugar systems the hysteresis increased markedly at the same time as the degradation products became detectable on the chromatograms. However, the amino acid-containing model systems showed the largest hysteresis, but only traces of degradation products.

4. The influence of methionine and trigonelline on the thermal degradation of caffeic acid.

Materials.

The following model systems were prepared in triplicate for examination at 180° 5, 10, 15 and 20 minutes.

Caffeic acid 20 mg, citric acid 22mg, methionine 14 mg (1:1:1 Molar).

Caffeic acid 20 mg, citric acid 22mg, trigonelline 15 mg (1:1:1 Molar).

Procedure.

As described previously.

Results and discussion.

These experiments were designed to examine the ability of methionine and/or trigonelline to methylate caffeic acid, since it was thought that a similar reaction might occur during coffee bean roasting. Citric acid was included in the model systems because it has been shown to form a highly reactive system with caffeic acid, and such a system could be more accurately compared with a green coffee bean.

The results are set out in Figures 18 and 19, and Table 21. These two model systems behaved very similarly in their responses to the Molybdate and Periodate Reagents, but the hystereses were

Table 21 The influence of Methionine and Trigonelline on the degradation of caffeic acid.

Sample	Percentage Loss Determined by			Appearance after roasting	Aroma after roasting
	Weight Loss	Molybdate Reagent	Periodate Reagent		
Caffeic acid + citric acid + trigonelline	52.0	96.0	76.0	Fused, brown, effervescent.	Objectionable, reminiscent of pyridine.
Caffeic acid + citric acid + methionine	22.0	90.0	66.0	Fused, brown, effervescent.	Meaty, reminiscent of methionine.

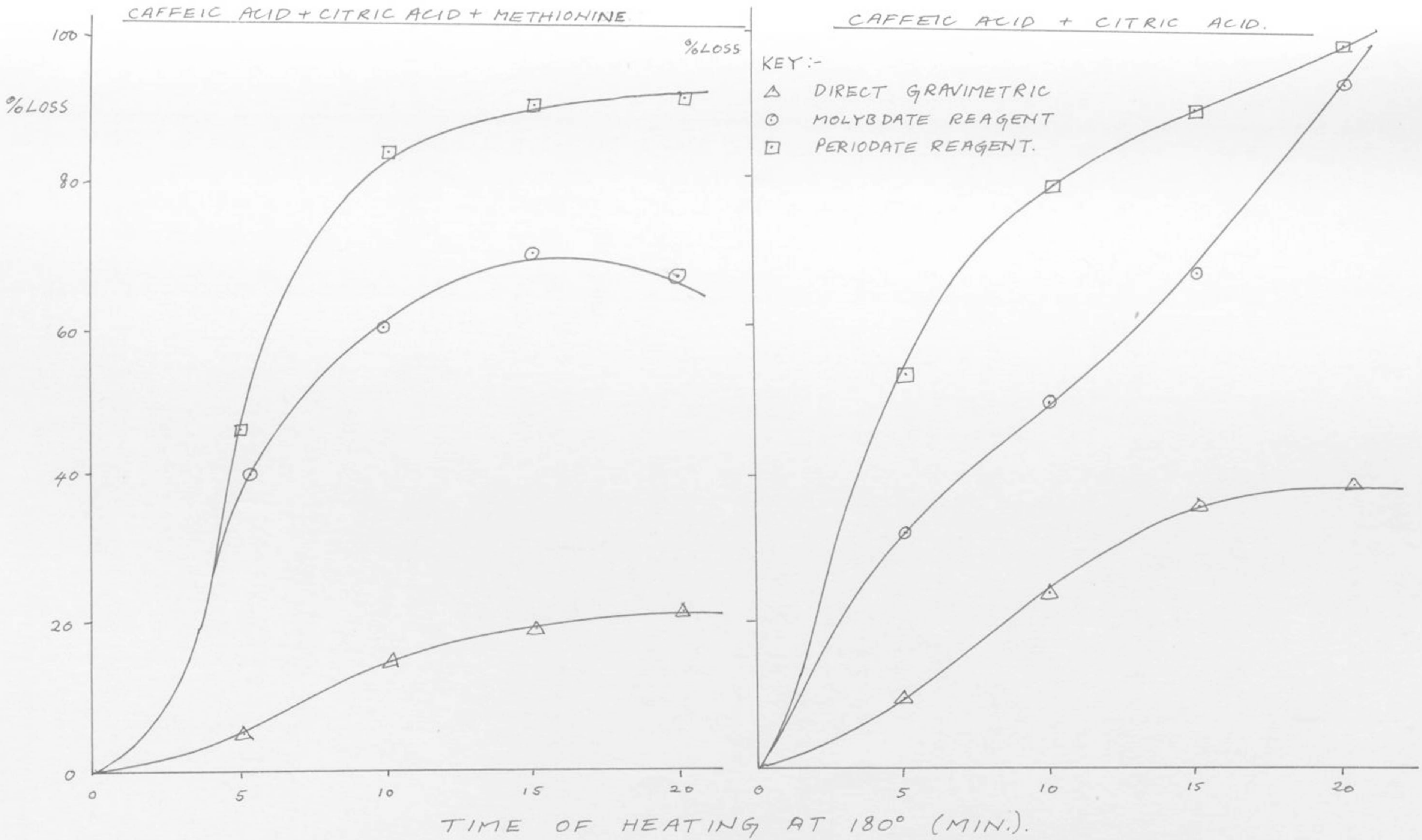


FIG 18 EFFECT OF METHIONINE ON THE THERMAL DEGRADATION OF A MIXTURE OF CAFFEIC ACID AND CITRIC ACID (1:1 MOLAR)

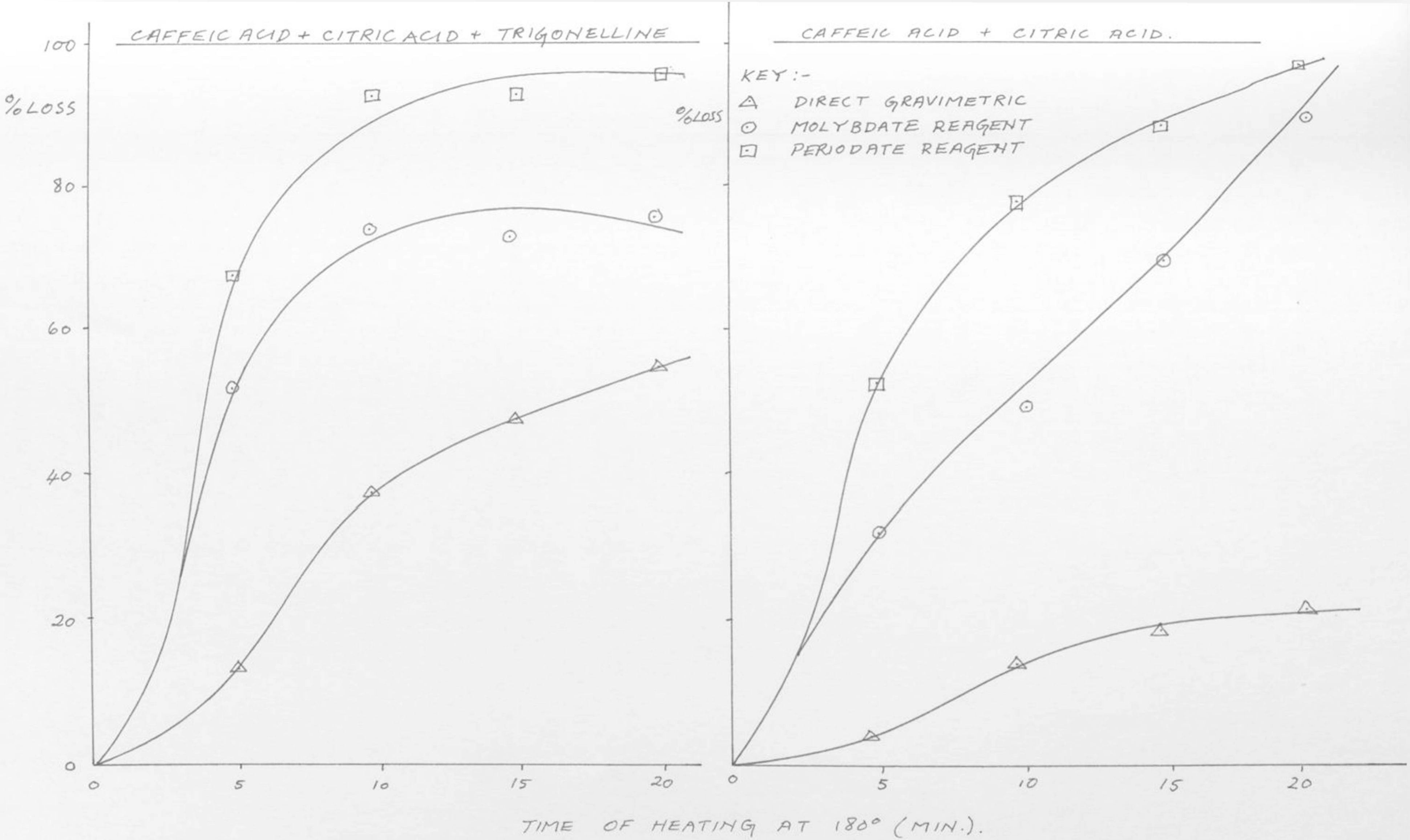


FIG 19 EFFECT OF TRIGONELLINE ON THE THERMAL DEGRADATION OF A MIXTURE OF CAFFEIC ACID AND CITRIC ACID (1:1 MOLAR).

markedly different to that produced by a caffeic acid-citric acid model system. Chromatographic examination of the trigonelline-containing model system showed a small residue of caffeic acid in the sample that was heated for five minutes, and more thereafter. No degradation products were separated by TLC.

In the methionine-containing model system were formed Spots 1-5, as in the caffeic acid-citric acid model system, and two new products (2a and 3a in Fig 12). Products 2a and 3a were positive to the Molybdate Reagent, Periodate Reagent and DSA. No degradation products were located in the five minute sample, but the ten minute sample suffered almost complete destruction of the caffeic acid. Spots 1, 2, 2a and 3a appeared in this sample and increased in concentration during further roasting to reach a much higher level than was recorded for the caffeic acid-citric acid model system. Chromatographic examination for amino compounds showed a progressive and almost complete loss of methionine, but no other ninhydrin-sensitive compounds. The increase in degradation products clearly paralleled the increase in hysteresis, and in the light of this observation it was possible to see a similar coincidence in the organic acid and sugar-containing model systems. However, a hysteresis occurred in some samples which apparently did not contain degradation products, eg trigonelline-containing model system, glycine-sugar model systems.

No molybdate-negative, periodate-positive degradation products were detected, and since this type of compound would have been produced by monomethylation of an ortho-dihydroxy compound, it was assumed that monomethylation had not occurred in either the methionine or trigonelline-containing model systems. These findings do not entirely rule out the possibility of such a reaction during coffee bean roasting, but they do render it less probable. These results, and those of the previous model system sections, suggest that the hystereses arose from interference by degradation products that made a larger response to periodate than to molybdate under the conditions employed.

To investigate this hypothesis the identified degradation products were examined for the ability to cause such interference, and roasted coffee beans were examined for the presence of the same or similar degradation products.

5. Examination of roasted coffee beans for phenolic degradation products.

Materials.

Samples of the following ground, roasted beans:-

Variety	Degree of Roast 1	2 (weight loss %)	3	4
Arabica Santos	5.0	9.3	15.0	20.0
Arabica Sao Paulo	3.0	9.6	13.8	17.8
Robusta Ghana	5.8	9.4	12.6	18.0
Robusta Uganda	4.0	8.6	11.4	15.5

Procedure.

Samples of the roast beans (10g) were extracted by shaking for two hours, with n-butyl acetate (25ml). These extracts were allowed to settle and filtered through a Whatman No. 1 filter paper. Each extract was collected in a beaker, and placed in an operating fume cupboard to remove the solvent. The brown residue was dissolved with agitation in 2ml n-butyl acetate and the concentrated extracts were examined by TLC using the method established for examining the model systems.

Results and discussion.

All samples which had been subjected to greater than a 10% roast contained brown material which streaked to the solvent front, masking any minor components which might have been present. However, it was possible to detect a component that corresponded to compound 1 from roasted caffeic acid model systems (See Fig 12). All the third and fourth degree roasts contained approximately the same quantity of this material. It was possible that the Robustas also contained a little of compound 1 in their second degree roasts, and a little of compound 2 in their third and fourth degree roasts. Spots 1 and 2 are thoght to be pyrogallol and pyrogallic acid respectively. (See Table 18).

When chromatograms were triple-developed to separate depside degradation products, very heavy streaking completely obliterated the chromatogram. The residue of apparently unchanged depside was too great to allow the presence or absence of depside degradation products to be ascertained.

THE INFLUENCE OF PHENOLIC DEGRADATION PRODUCTS ON THE ACCURATE DETERMINATION, BY THE DIFFERENTIAL TECHNIQUE, OF CAFFEIC ACID AND CQ MONOMETHYL ETHERS.

Introduction.

Pyrogallol and pyrogallic acid, which are found in roasted coffee bean and roasted phenolic compound model systems, are thought to be two of the degradation products of heated caffeic acid or CQ acids. Protocatechuic acid and aldehyde (found in caffeic acid-containing model systems but not roasted coffee beans) are thought to be two other caffeic acid degradation products. It was suggested in Chapter 2, Section 6, Page 64, that phenolic degradation products might interfere with the accuracy of the Differential Technique, particularly the determination of monomethyl ethers. The following experiment was designed to investigate the possibility.

Materials.

Standard solutions of pyrogallol, pyrogallic acid, protocatechuic acid and aldehyde in 70% propan-2-ol (100-120 μg/ml).

Periodate Reagent)
) As used in the Differential Technique.
Molybdate Reagent)

Procedure.

Each standard solution was examined in duplicate by the

Differential Technique, and with both reagents the absorption was measured at the wavelengths used for the quantitation of caffeic acid and 3CQ (353 and 370 nm; 406 and 423 nm). The molar extinction coefficient (MEC) was calculated for each standard solution at each wavelength.

Results and discussion.

Table 22 compares the MEC of caffeic acid, ferulic acid, 3CQ and the suspected degradation products at the several wavelengths used for the Differential Technique. The results showed that when measuring caffeic acid or ferulic acid (eg in the roasted model systems) the presence of pyrogallol, pyrogallic acid, or protocatechuic aldehyde causes a higher periodate-reading than molybdate-reading. In contrast the presence of protocatechuic acid causes a higher response to the Molybdate Reagent than to the Periodate Reagent. When measuring the depsides the interference was much greater. Pyrogallol, pyrogallic acid and protocatechuic aldehyde give responses to the Periodate Reagent that are approximately x7, x9 and x8 their responses to the Molybdate Reagent.

These results demonstrated how seriously a degradation product can interfere in the Differential Technique, particularly when calculating the content of monomethyl ethers. It was assumed that any hysteresis in the Differential Technique results from the model systems, were due to traces of degradation products, even when TLC failed to demonstrate their presence. It was also assumed that the small quantity of pyrogallol found in roasted coffee beans, perhaps with traces of other degradation products, was responsible

Table 22 Molar extinction coefficients of caffeic acid, 3CQ and associated degradation products.

1. Molybdate Reagent.

Compound	Molar Extinction Coefficients				
	At wavelength of maximum absorption	At 353nm	Caffeic acid equivalent %	At 370nm	CQ acid** equivalent %
Caffeic acid	12088	12088	100	-	-
3CQ	16600	-	-	16600	100
Pyrogallol	4438	4161	34	3051	18
Pyrogallic acid	2714	2364	20	1751	11
Protocatechuic acid	14381	13715	114	11468	70
Protocatechuic aldehyde	3316	2630	20	1658	10

2. Periodate Reagent.

Compound	Molar Extinction Coefficients				
	At wavelength of maximum absorption	At 423nm	Caffeic acid* equivalent %	At 406nm	CQ acid** equivalent %
Caffeic acid	2144	2144	100	-	-
3CQ	1603	-	-	1603	100
Pyrogallol	1942	1456	68	1803	113
Pyrogallic acid	1664	1489	62	1664	104
Protocatechuic acid	1119	559	26	839	53
Protocatechuic aldehyde	4082	829	39	1275	80

* Or ferulic acid equivalent %

** Or FQ acid equivalent %

for the apparent increase in monomethyl ethers during roasting. This assumption does not contradict the observation that the FQ acids are considerably more heat stable than the CQ acids, since this was confirmed by TLC.

None of the TLC-separated degradation products from ferulic acid-containing model systems were sensitive to 0.25% periodate, therefore, the periodate figure for these systems may be taken as accurate. However, the degradation products from the caffeic acid-containing model systems have been shown to interfere with the results obtained with both reagents. In the case of caffeic acid heated alone the nett effect of the degradation products is molybdate-enhancing. Since there was only a small weight loss (See Table 16), and no degradation products were detected by TLC, only traces of degradation products could have been present. Therefore, the loss of caffeic acid could not have been greatly enhanced, and there is no reason to revise the conclusion that ferulic acid is more heat sensitive than caffeic acid. The greater weight loss from ferulic acid supports this conclusion.

All the other caffeic acid-containing model systems showed either considerable hysteresis, or a considerable quantity of degradation products by TLC, or some combination of the two. Since the nett effect of the degradation products was periodate-enhancing, less caffeic acid remained than the results appeared to show. In the presence of organic acids it was concluded that caffeic acid was more heat sensitive than ferulic acid, and any allowance made for interference accentuates this caffeic acid sensitivity.

If such an allowance is made for the sugar-containing model systems it would appear that caffeic acid is at least as sensitive as ferulic acid, and probably more so, in contrast to the original conclusion. Such a situation would be more in keeping with the general trend of the results, and with the suggestion that phenolic acids convert sugars to organic acids which in turn destroy the phenolic acids.

The lack of hysteresis observed for 3CQ caffeine potassium salt compared with 3CQ suggests the absence of phenolic degradation products and supports the conclusion that the caffeine complex is more stable.

Conclusion.

Hysteresis is due mainly to the interference of phenolic degradation products. In the case of periodate-enhancement a little monomethylation cannot be completely ruled out.

CHAPTER TWO

SECTION NINE

THE HIGH MOLECULAR WEIGHT COMPOUNDS

THE HIGH MOLECULAR WEIGHT (HMW) PHENOLIC SUBSTANCES OF ROASTED COFFEE BEANS.

Introduction.

Maier et al (47) reported the presence of hydrolysable phenolic compounds in two substances of HMW (5,000-10,000) that they had isolated from roasted coffee. Klocking et al (55) reported that "humic substances" were produced at a level of 15% roasted bean soluble substances, by polymerisation of phenolic substances during roasting. Obenaus (197) reported that the "humic acid" content of coffee beans rose from 1.4% to 16.1% during roasting, and that a portion of these "humic acids" formed about 15% of roast bean soluble substances (15% roast bean solubles = 5% roast bean approximately).

The techniques used by these workers had certain limitations. A positive response to Turnbull's Blue was considered to be proof of the phenolic nature of a HMW substance (Maier) (47). Turnbull's Blue detects substances that reduce Fe^{3+} to Fe^{2+}, a property not restricted to phenolic compounds. The HMW materials were obtained from aqueous extracts, or lead acetate precipitates of aqueous extracts, from roasted coffee. The HMW components were isolated by gel filtration chromatography; Maier et al (47) also used a preliminary separation on polyamide. Streuli (198) reported that chlorogenic acid did not behave in the manner to be expected of a substance with a molecular weight of 360 when subjected to gel filtration chromatography, and could therefore be expected in fractions of higher molecular weight.

Thus there is a possibility that the substances examined by previous workers either were not phenolic, or contained low molecular weight phenolic compounds.

To avoid these limitations it was decided to use prolonged dialysis against an organic solvent to remove low molecular weight compounds, particularly the low molecular weight phenols. To facilitate this procedure a continuous, thin film, countercurrent dialyser was designed (See Fig 25). The dialysate and retentate were propelled in opposite directions by a multichannel peristaltic pump. Up to five independent dialyses could be made at one time with a minimum of supervision. Full details of the construction, assembly and operation of the dialyser are given in appendix C.

The isolation of HMW compounds from green and roasted coffee beans.

Materials.

Ground green, and roasted Arabica Sao Paulo.
Ground green, and roasted Robusta Ghana.

Procedure.

Duplicate samples (15g) of each green and roasted bean were extracted by shaking with 100ml aliquots of boiling deionised water until no further extraction of colour occurred. Usually five extractions, each by shaking for thirty minutes, were sufficient. It was intended that this procedure be similar to domestic coffee brewing. The extracts were bulked and dialysed against 70% propan-2-ol.

Dialysis was continued until no more colour was removed into the solvent (about 7 days). Full details of this operation are given in appendix C.

When dialysis was complete the retentate was diluted with deionised water and pumped into a suitable receptacle, and all tubing thoroughly washed to ensure a high recovery. The retentate was transferred to pre-weighed dishes, freeze dried, and the yield of HMW recorded.

A quantity of this material (500mg) was added to 25.0ml 97% ethanol contained in a stoppered flask and shaken for thirty minutes. It was allowed to stand overnight to fully solvate. The suspension was centrifuged and the supernatant decanted. The precipitate was re-extracted twice and the supernatants bulked. The ethanol was removed from the solutions by evaporation at reduced pressure, and the residue was dissolved in a minimal quantity of deionised water, transferred to preweighed containers and freeze dried. The alcohol-insoluble material was similarly freeze dried.

Results and discussion.

The water-soluble HMW compounds of green and roasted coffee beans have been reported to be proteins and a range of polysaccharides. These proteins have been reported to be insoluble in 80% ethanol (49), and polysaccharides are classically considered to be alcohol-insoluble. It was therefore assumed, that treatment of the crude HMW material with 97% ethanol would precipitate the proteins and polysaccharides, and the absence of alcohol-soluble HMW material from the green beans

Table 23 The yields of non-dialysable material isolated from green and roasted coffee beans.

Variety of coffee bean and the degree of roast		Yield % (green bean dmb)		
		Total water-soluble material	Alcohol-insoluble material	Alcohol-soluble material
Arabica, Sao Paulo	Green	7.8 - 9.2	6.3 - 7.6	Trace
	1	15.6 - 17.0	12.7 - 14.2	Trace
	2	19.0 - 19.7	12.7 - 15.0	0.7 - 0.9
	3	14.3 - 17.8	9.5 - 10.4	3.2 - 3.7
	4	16.6 - 16.8	10.9 - 11.9	1.3 - 1.5
Robusta, Ghana	Green	5.8 - 6.3	4.9 - 5.2	Trace
	1	3.2 - 6.7	1.9 - 4.7	Trace
	2	11.9 - 14.8	8.0 - 8.4	1.2 - 1.3
	3	12.0 - 17.9	10.7 - 13.5	2.9 - 3.8
	4	15.7 - 16.9	14.1 - 14.7	1.0 - 1.4

and the first degree roasts supports this assumption. The yield of water-soluble HMW material (See Table 23) from the green beans can be accounted for by water-soluble protein (about 3% dmb) (49, 50), and water-soluble polysaccharide (up to 4% dmb) (42, 99). The decrease in water-soluble HMW material from Robusta beans after the first degree roast was probably due to the insolubilisation of protein. The large increase in water-soluble, alcohol-insoluble material during the early stages of roasting could have been due to degradation and solubilisation of water-insoluble, structural polysaccharides.

The material isolated from the green beans was white and flaky after freeze drying, but that from the roasted bean was darker and much more powdery.

HYDROLYSIS OF THE HMW SUBSTANCES.

1. Development of a hydrolysis system to release phenolic compounds.

Introduction.

Klocking et al (55) employed 5N NaOH, 170°, 24 hours to release phenolic compounds from coffee "humic acids." Coulson et al (200) used 6N HCl, 100°, 24 hours during investigation of "humic acids" from soils. Model system experiments have shown that heating caffeic acid with an organic acid at 180° for five minutes causes extensive degradation of caffeic acid. It was assumed that the hydrolysis systems used by Klocking et al and Coulson et al would be too severe and likely to produce misleading artefacts. The following milder hydrolysis systems were devised and investigated.

Hydrolysis systems.

1. 2N HCl, 110°, 1 hour.
2. 2N HCl, 110°, 2 hours.
3. 2N HCl, 110°, 6 hours.
4. 2N HCl, 110°, 24 hours.
5. 6N HCl, 110°, 15 minutes.
6. 2N NaOH, 110°, 1 hour.
7. 2N NaOH, 110°, 2 hours.
8. 2N NaOH, 110°, 6 hours.
9. 2N NaOH, 110°, 24 hours.
10. 5N NaOH, 110°, 15 minutes.

Procedures.

The hydrolyses were performed in screw-capped test tubes using 10mg samples of caffeic acid or ferulic acid, and 2ml of the appropriate acid or alkali. After hydrolysis the phenolic compounds were extracted into 0.25ml n-butyl acetate, and this solution was examined for the presence degradation products by using the TLC system chloroform, acetic acid, water 4:1:1 $^{v}/_{v}$ and silica gel plates. The more successful hydrolysis systems were applied to 3CQ.

Results and discussion.

From all caffeic acid hydrolysis systems it was possible to separate at least four degradation products, which corresponded to Spots 1, 2, 4 and 5 obtained from several caffeic acid model systems. (See Fig 12). The 24 hour hydrolysis systems contained no caffeic acid, and the 6 hour hydrolysis systems contained only a trace. Alkali produced essentially the same results. Ferulic acid yielded the degradation products that were found in several of the ferulic acid-containing model systems. Hydrolysis systems 1, 2, 6 and 7 were repeated using an atmosphere of nitrogen, but the degradation products were still produced. Hydrolysis of 3CQ by systems 1, 2, 6 and 7 gave the range of products obtained from caffeic acid, and thus suggested that 3CQ was hydrolysed to caffeic acid and quinic acid.

Systems 1, 2, 6 and 7 were tested on a sample of the alcohol-soluble HMW material. Systems 2 and 7 yielded the range of degradation products typical of caffeic acid or 3CQ. A similar pattern, but lower yields was obtained with systems 1 and 6. Since these relatively mild hydrolysis systems produced artefacts it was concluded that the hydrolysis systems of Coulson *et al* (200) and Klocking *et al* (55) were far too severe for a satisfactory investigation of the HMW material isolated from roasted coffee beans.

Hydrolysis system 2 was chosen for further work since it was known that any polysaccharides that were present would be hydrolysed at the same time as the phenols, and any sugars released would remain in the aqueous phase after extraction of the phenolic compounds into n-butyl acetate.

2. Hydrolysis of the HMW material isolated from green and roasted coffee beans.

Materials.

Samples of alcohol-soluble and alcohol-insoluble HMW material isolated as previously described from each sample of beans.

Silica gel G TLC plates. Those for examination of sugars were prepared with 0.1M sodium metabisulphite solution. (See Appendix B).

Solvent systems.

1. Chloroform, acetic acid, water 4:1:1 $^v/_v$ for phenolic compounds.
2. Chloroform, methanol, 17% ammonia 2:2:1 $^v/_v$ as used by Hassan for amino acids.
3. Butan-2-one, acetic acid, water 60:10:15 $^v/_v$ as used by Hassan for sugars.

Locating Reagents.

DSA for phenols.

Ninhydrin Reagent for amino compounds.

Alpha-naphthol in 66% sulphuric acid for sugars.

Standard Solutions.

Arabinose, galactose, glucose, mannose, rhamnose 5mg/ml aqueous.

Procedure.

The hydrolysis system developed in the preceding section was used with 10mg samples of each alcohol-soluble and alcohol-insoluble HMW material. The released phenolic compounds extracted into 0.25ml n-butyl acetate. Samples of caffeic acid (10mg), ferulic acid (10mg) and 3CQ (20mg) were hydrolysed as controls. The organic phase was removed by a Pasteur pipette and examined by TLC.

Each aqueous phase was evaporated to dryness at reduced pressure and the residue dissolved in a minimal quantity of deionised water. Up to 10 µl of these solutions was spotted onto a chromatogram and examined with the Alpha-naphthol Reagent. Those solutions that gave a positive response with this reagent were examined by TLC using bisulphite-impregnated silica gel plates and solvent system 3.

To release amino compounds a standard protein hydrolysis was used ie 10mg sample with 2ml 6N HCl, 110°, 24 hours, under a nitrogen atmosphere. The hydrolysate was evaporated to dryness under reduced pressure and the residue dissolved in a minimal quantity of deionised water. Those solutions that were positive to the Ninhydrin Reagent were examined by TLC on silica gel using solvent system 2.

Results and discussion.

Each protein hydrolysate yielded eight amino compounds, and it was apparent that several spots were impure. The yield of amino compounds was higher from the Arabica HMW material than from the Robusta. The alcohol-insoluble material showed a gradual decrease in yield of amino compounds during the roasting cycle, but the alcohol-soluble material showed a severe decrease from the second to third degree roasts and then a further small decrease in the fourth degree roasts. These amino compounds were not investigated further.

The sugar releasing hydrolyses of the alcohol-soluble material were negative to the α-naphthol reagent and were not investigated further. The alcohol-insoluble material yielded two sugars which corresponded with galactose and mannose. (See Table 24). Water-soluble polysaccharides containing these sugars have been reported by many workers (31, 34, 39, 40, 42, 44, 199, 201-203).

Table 24 Sugars released by hydrolysis (2N HCl, 110°, 2 hours) from alcohol-insoluble HMW material of green and roasted coffee beans. TLC on bisulphite-impregnated (0.1M) silica gel in solvent system butan-2-one, acetic acid, water 60:10:12 $^{V}/_{V}$ Located with naphthol sulphuric acid reagent.

Coffee bean Sample or Standard		Rf x100 and colour with the Alpha-naphthol Reagent.	
	green	20 purple	33 red
	1	21 purple	34 red
Arabica	2	23 purple	35 red
Sao Paulo	3	22 purple	35 red
	4	20 purple	35 red
	green	21 purple	32 red
	1	21 purple	35 red
Robusta	2	22 purple	36 red
Ghana	3	20 purple	35 red
	4	23 purple	35 red
Arabinose		44 blue	
Galactose		24 purple	
Glucose		28 deep purple	
Mannose		34 red	
Rhamnose		60 red	

Only a trace of phenolic compounds were released from the alcohol-insoluble material, but a high yield was obtained from the alcohol-soluble fraction, eleven components were separated by TLC, and nine of these were found in one or more of the control hydrolyses, (See Table 25), but Spots E and G were not. However, all eleven hydrolysate components were found amongst the degradation products of the various model systems, Spots E and G corresponding to Spots 3a and 5 (See Table 18 and Fig 12). The presence of Spots E and G in the hydrolysates of the alcohol-soluble HMW material and the roasted model systems, and their absence from the control hydrolyses, suggested that the two compounds were not hydrolysis artefacts. Therefore, it is possible that the other hydrolysis products were not necessarily hydrolysis artefacts, but had been bound in that form to the HMW material.

The intensity of the spots released from the alcohol-soluble HMW material were compared with the intensity of the spots from a range of loadings of the control hydrolyses. It was demonstrated that the third and fourth degree roast alcohol-soluble HMW material contained the equivalent of 50-60% CQ acids, and the second degree roast 25-30% CQ acids. The material from the Arabica Sao Paulo contained only a very faint trace of FQ acids, but the third and fourth degree Robusta Ghana roasts yielded 1-3%. These hydrolysates were complex mixtures, probably containing amino compounds and perhaps sugars in addition to the phenolic compounds. The model systems demonstrated that such compounds react together when heated resulting in a destruction of the phenolic component and it was assumed that the yield of phenolic material from the hydrolysates was low due to loss by interaction. Similarly the

Table 25 Rf values of phenolic compounds released by hydrolysis alcohol-soluble HMW material, and from the control hydrolyses. Hydrolysis conditions 2N HCl, 110°, 2 hours.

TLC on Silica gel using solvent system chloroform, acetic acid, water 4:1:1 $^{v}/_{v}$.

Spot Identification		Rf x100 of the products from				
This Expt.	Model* Systems	Alcohol-soluble HMW material	Caffeic control	3CQ control	Ferulic control	Colour of spot when located with DSA
A	1	05-07	06	06		Red
B	2	12-16	12	14		Red
C	2a	22-24		24		Orange
D	3	28-30	28	28		Red
E	3a	43-44				Yellow
F	4?	48-51	50	48		Red
G	5?	60-64				Red
H		67-69	67			Red
I	6	75-76	75			Yellow
J	7				86	Red
K	9	91-93			90	Yellow
L	10	94-98			94	Red

* See Table 18 and Fig. 12.

failure to detect sugars in the hydrolysate was not proof of the absence of polysaccharide in the HMW material.

Osmotic pressure measurement* showed that the alcohol-soluble HMW material had a molecular weight in excess of 20,000. Microanalysis* showed that the alcohol-soluble material from the second degree roasts contained about 20% nitrogen and that the third and fourth degree roasts contained about 5% nitrogen (See Table 26). This fall in nitrogen content paralleled the yields of hydrolysable amino compounds and was the reverse of the yields of hydrolysable phenolic compounds. Sivetz (204) used a Nitrogen to protein conversion factor of 6.25 for green coffee bean protein, indicating a protein-nitrogen content of about 16%. Using this conversion factor it was calculated that the alcohol-soluble HMW material from the second degree roasts contained 63-66% protein ie a protein: non-protein ratio of approximately 2:1. The HMW material from the third and fourth degree roasts contained 28-31% protein, a 1:2 protein: non-protein ratio. These deduced protein contents are in good agreement with the calculated phenol contents.

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* Osmotic pressure measurement and microanalyses by courtesy of the Microanalysis Laboratory.

Table 26 Microanalyses of the alcohol-soluble HMW material isolated from roasted Arabica and Robusta beans (corrected for ash content.)

Arabica Sao Paulo	%C	%N	%H	Robusta Ghana	%C	%N	%H
Roast 2	53.2	10.51	7.10	Roast 2	51.7	10.13	6.82
3	50.9	5.03	5.43	3	49.0	4.95	5.06
4	51.1	4.52	5.01	4	50.7	4.73	5.10

The applicability of this conversion ratio to "roasted protein" is debatable, and it is possible that some of the nitrogen determined by microanalysis is non-protein nitrogen. Therefore, the deduced protein: non-protein ratios are only approximate. However, the general trend of the results suggested that the alcohol-soluble HMW material was tanned protein(s). Forsyth et al (205) reported that tanned proteins were produced during cacao curing, and that as the phenolic content rose the water solubility of the tanned material fell. In the later stages of tanning they believed that phenol-phenol polymerisation occurred without involving additional protein. The HMW material was isolated from aqueous extracts of the coffee beans and it was assumed that severely tanned proteins would not have been extracted, thus explaining the reduced yield of alcohol-soluble HMW material from the fourth degree roasts.

CHAPTER TWO

SECTION TEN

THE WIESNER REAGENTS

THE COMPONENTS OF THE GREEN AND ROASTED COFFEE BEAN THAT REACT WITH THE WIESNER REAGENTS.

Introduction.

In Chapter 2, Section 7, it was reported that propanolic extracts of green and roasted coffee beans contained several components that reacted with the Wiesner Reagent. This reagent (1% phloroglucinol in 12% hydrochloric acid - in future called Wiesner Reagent A) has been reported to be specific for aromatic aldehydes (168), but a similar reagent (phloroglucinol in concentrated hydrochloric acid) reacts with the furanose form of ketoses to produce a red colour (206). Before investigating the Wiesner-sensitive compounds of the green bean it was necessary to investigate the specificity of these reagents.

The specificity of the Wiesner Reagents.

Materials.

Wiesner Reagent A	1% phloroglucinol in 12% hydrochloric acid.
Wiesner Reagent B	1% phloroglucinol in concentrated hydrochloric acid.

Standard Solutions.

Aromatic aldehydes and ketones 5mg/ml in 70% propan-2-ol.
Furans 5mg/ml in 70% propan-2-ol.
Fructose, sucrose, citric acid, DL-malic acid, maleic acid,

maleic anhydride, DL-tartaric acid, succinic acid

5mg/ml aqueous.

Procedure.

Each standard solution was spotted onto two silica gel chromatograms and one chromatogram located with Wiesner Reagent A, and the other with Wiesner Reagent B.

Results and discussion.

The results are shown in Table 27. Aromatic ketones, phenyl propanal, saturated (tetrahydro-) furans, furan-carboxylic acids, DL-malic acid, DL-tartaric acid, maleic acid and succinic acid did not react with either Wiesner Reagent even after heating the chromatograms. All the aromatic aldehydes (including phenyl ethanal), the furan aldehydes and maleic anhydride responded immediately with Wiesner Reagent A, and gave the same response with Wiesner Reagent B. Fructose, sucrose, citric acid, furan, 2-methyl furan, 5-hydroxymethyl furan responded after heating with Wiesner Reagent A or rapidly in the cold with Wiesner Reagent B.

It has been reported that heating fructose in acid conditions produces 5-hydroxymethyl-furan-2-aldehyde (207). This substance gave an orange colour with both Wiesner Reagents, and it was assumed that it was responsible for the positive response of fructose and sucrose.

The heating of citric acid has been reported to produce citraconic anhydride (3-methyl-furan-2,5-dione) (208). Since

Table 27 The specificity of the Wiesner Reagents.

Compound	Wiesner Reagent A Response and colour	Wiesner Reagent B Response and colour
2HBzAl	Immediate, red-brown	Immediate, red-brown
3HBzAl	Immediate, red-brown	Immediate, red-brown
4HBzAl	Immediate, red-brown	Immediate, red-brown
2,4DHBzAl	Immediate, red-brown	Immediate, red-brown
3,4DHBzAl	Immediate, red-brown	Immediate, red-brown
3MBzAl	Immediate, red-brown	Immediate, red-brown
4MBzAl	Immediate, red-brown	Immediate, red-brown
3,4DMBzAl	Immediate, red-brown	Immediate, red-brown
3,4,5TMBzAl	Immediate, red-brown	Immediate, red-brown
3M,4HBzAl	Immediate, red-brown	Immediate, red-brown
3H,4MBzAl	Immediate, red-brown	Immediate, red-brown
3,5DM,4HBzAl	Immediate, red-brown	Immediate, red-brown
4 isopropyl BzAl	Immediate, red-brown	Immediate, red-brown
3,4MDBzAl	Immediate, red-brown	Immediate, red-brown
Phenyl ethanal	Immediate, blue green	Immediate, blue-green
Phenyl propional	Negative	Negative
BzAl	Immediate, orange	Immediate, orange
CAl	Immediate, orange	Immediate, orange
2MCAl	Immediate, purple	Immediate, purple
3,4MDCAl	Immediate, purple	Immediate, purple
Methyl phenyl ketone	Negative	Negative
Methyl vanilloyl ketone	Negative	Negative
Methyl syringoyl ketone	Negative	Negative

Compound	Wiesner Reagent A Response and colour	Wiesner Reagent B Response and colour
Tetrahydrofuran	Negative	Negative
5-hydroxymethyl-tetrahydrofuran	Negative	Negative
Furan	Slow, green	Rapid, green
Furan-2-carboxylic acid	Negative	Negative
Furan-2-aldehyde	Rapid, blue	Immediate, blue
5-hydroxymethyl-furan-2-aldehyde	Rapid, orange	Immediate, orange
2-methyl furan	Slow, pink	Rapid, pink
5-hydroxymethyl-furan	Slow, green	Rapid, green
Furyl acrylic acid	Negative	Negative
Fructose	Negative	Immediate, red
Sucrose	Negative	Immediate, red
Citric acid	Negative	Orange
Malic acid	Negative	Negative
Maleic acid	Negative	Negative
Maleic anhydride	Rapid, orange	Immediate, orange
Tartaric acid	Negative	Negative
Succinic acid	Negative	Negative

maleic anhydride (furan-2,5-dione) responded readily with Wiesner Reagent A, it was assumed that citraconic anhydride reacted similarly, accounting for the positive response by citric acid.

Conclusions.

1. Wiesner Reagent A is not specific for aromatic aldehydes. It also gives a positive response with furanic aldehydes and ketones containing 3 conjugated double bonds. It must be assumed that other compounds (excepting aromatic ketones) which have this structure will also give a positive response.

2. Wiesner Reagent B or Wiesner Reagent A and heat, give a positive response with compounds that are not furans but which are converted to sensitive furans by the action of the Reagent.

3. Neither Wiesner Reagent reacts with aromatic ketones. Therefore, a positive response to the Wiesner Reagent and at least one other reliable phenol-specific reagent is indicative of an aromatic aldehyde. The colour of this response bears a relationship to the structures of the aldehyde which may be of diagnostic value. (See Table 27).

4. Comparison of the structures of the compounds examined (Fig 20) shows that a positive response to Wiesner Reagent A was coincident with the possession of at least 3 conjugated double bonds of which at least one was provided by a carbonyl group. No explanations can be offered for the negative response of the aromatic ketones which had this structure, or for the positive response of furan, 2-methyl furan, and 5-hydroxymethyl furan which did not. It

would not be unreasonable to expect a positive response from other groups of compounds which have this structure, but which were not available to test, eg terpenes such as Vitamin A aldehyde.

Examination by TLC of the components of green and roasted coffee beans that are sensitive to the Wiesner Reagents.

Materials.

The following green and roasted beans ground to pass 20 mesh.

Arabica Santos,	green, and roasts 1 to 4.
Arabica Sao Paulo,	green, and roasts 1 to 4.
Robusta Ghana,	green, and roasts 1 to 4.
Robusta Uganda,	green, and roasts 1 to 4.

Silica gel G TLC plates.

PVP TLC plates.

Solvent Systems.

1. Toluene, ethyl formate, formic acid 5:4:1 $^v/_v$.
2. Diethyl ether, petroleum ether (40°-60°) 5:2 $^v/_v$.
3. Butan-2-one, methyl phenyl ketone, 50% acetic acid 5:5:4 $^v/_v$ used for the PVP plates.

Locating Reagents.

Wiesner Reagents A and B.

DNPH Reagent.

DSA.

Periodate Reagent 0.5%, and 5.0%.

Molybdate Reagent.

Standard Solutions.

Aromatic aldehydes in 70% propan-2-ol 5mg/ml.

Furans in 70% propan-2-ol 5mg/ml.

Procedure.

A 10.0g sample of each ground bean was extracted four times with 100ml aliquots of diethyl ether. Each extraction was carried out in an operating fume cupboard. The extracts were bulked and the solvent removed at reduced pressure. The residue was dissolved in 2.0ml 70% propan-2-ol and transferred to a small glass vial.

These extracts were examined by TLC on PVP, and two dimensionally on silica gel using the ethereal solvent first. Location of the components was initially by UV fluorescence followed as required by a chromogenic reagent. The standard compounds were examined by one dimensional chromatography on silica gel.

Results and discussion.

Table 28 shows the Rf values of the standard compounds after silica gel chromatography, with reported Rf values from the literature where available. Table 29 shows the Rf values, and responses to the various locating reagents, of the components separated from the green beans by two dimensional chromatography. Figures 20-23 are drawings of these chromatograms.

Roasting produced a background haze of DNPH-sensitive material (probably aliphatic carbonyls) and a tendency for the Wiesner-sensitive spots to diffuse. However, the green bean patterns survived roasting without any obvious quantitative changes.

The Arabicas gave distinctly different patterns from the Robustas, but the members of each group were similar. In addition to the Wiesner-sensitive carbonyls (components 1-10) a total of 7 fluorescent compounds were noted. These were insensitive to the location reagents available and their investigation was considered outside the scope of this work.

Components 1, 3 and 6 appear to be phenolic aldehydes since they responded to DSA, but these substances did not correspond to any of the available standards. Consideration of the information obtained from the locating reagents, and from the literature suggests that components 3 and 6 are coniferaldehyde (3M,4HCAl) and sinapaldehyde (3,5DM,4HCAl) respectively.

Chromatography on PVP separated two large DNPH and Wiesner Reagent-sensitive bands from all samples, with Rf values of 0.52 and 0.65. These bands were almost certainly mixtures, but since the

Table 28 One dimensional TLC of standard aromatic aldehydes and furans on Silica gel in the solvents diethyl ether, petroleum ether 5:2 $^{v}/_{v}$ (2) and toluene, ethyl formate, formic acid 5:4:1 $^{v}/_{v}$ (1). Located by Wiesner Reagent A or B*.

Standard	Rf x100	
	Solvent 1	Solvent 2
2HBzAl	43-47 (69)	44-46
3HBzAl	56-59	38-40
4HBzAl	52-55	21-24
2,4DHBzAl	57-60 (55)	22-30
3,4DHBzAl	38-43 (39)	07
3MBzAl	70-75	67-70
4MBzAl	72-76	64-70
3,4DMBzAl	59 (50)	16-20
3M,4HBzAl	53-57	20-28
4M,3HBzAl	53-58	14-16
3,5DM,4HBzAl	56-57	10-14
Phenyl ethanal	73-80	53
4 isopropyl BzAl	86-89	86
CAl	75-80 (67)	76
2MCAl	76-80	70-75
3,4MDCAl	80-82	72-77
* Furan	72	10
Furan-2-aldehyde	76	50
5-hydroxy-methyl-furan-2-aldehyde	60-64	65
* 2-methyl furan	75	38
* 5-hydroxymethyl furan	75	48
Maleic anhydride (Furan-2,5-dione)	80	00

The values given in brackets are taken from the results of Van Sumere *et al*. (185).

Table 29 The components of green and roasted coffee beans separated by two dimensional TLC on Silica gel G using toluene, ethyl formate, formic acid 5:4:1 $^{v}/_{v}$ and diethyl ether, petroleum ether (40°–60°) 5:2 $^{v}/_{v}$ as solvents.

Spot number and sample *				Periodate		DSA	Wiesner Reagent A or B	DNPH	Fluorescence	Rf x100 solvent		Probable Identity
A	B	C	D	0.5%	5.0%					1	2	
1	1			–	–	Brown	Yellow	Orange	–	00	46–48	Aromatic aldehyde
2	2			–	–	–	Yellow	Orange	Yellow	03–06	50–52	Carbonyl
3	3	3	3	Red	Red	Brown	Purple with red halo	Orange	Purple	00	54–56	Coniferaldehyde
4	4			–	–	–	Yellow	Orange	Yellow	00	65–67	Carbonyl
5	5			–	–	–	Yellow	Orange	–	40–42	62–64	Carbonyl
6	6			Yellow	Yellow	Pink	Blue	Orange	Yellow	40–44	80–86	Sinapaldehyde
	7			–	–	–	Orange	Orange	–	84–85	86–90	Carbonyl
		8		–	–	–	Yellow	Orange	–	14–17	62–65	Carbonyl
		9		–	–	–	Yellow	Orange	Yellow	60–62	74–75	Carbonyl
			10	–	–	–	Orange	Orange	Yellow	30–32	65–68	Carbonyl
11				–	–	–	–	–	Yellow	12–14	54–56	Non-carbonyl
12	12			–	–	–	–	–	Yellow	30–36	67–70	Non-carbonyl
13	13			–	–	–	–	–	Yellow	67–70	83–85	Non-carbonyl
14				–	–	–	–	–	Pink	14–17	62–65	Non-carbonyl
	15			–	–	–	–	–	Yellow	30–32	65–67	Non-carbonyl
		16		–	–	–	–	–	Blue	66–69	65–67	Non-carbonyl
		17		–	–	–	–	–	Yellow	55–59	74–78	Non-carbonyl
From the literature:-												References
Coniferaldehyde							Yellow	Red-lilac	Blue-green	51		(168)
Sinapaldehyde							Rose	Lilac	Weak yellow-green	–		(185)

* Key:- A = Arabica Santos. B = Arabica Sao Paulo. C = Robusta Ghana. D = Robusta Uganda.

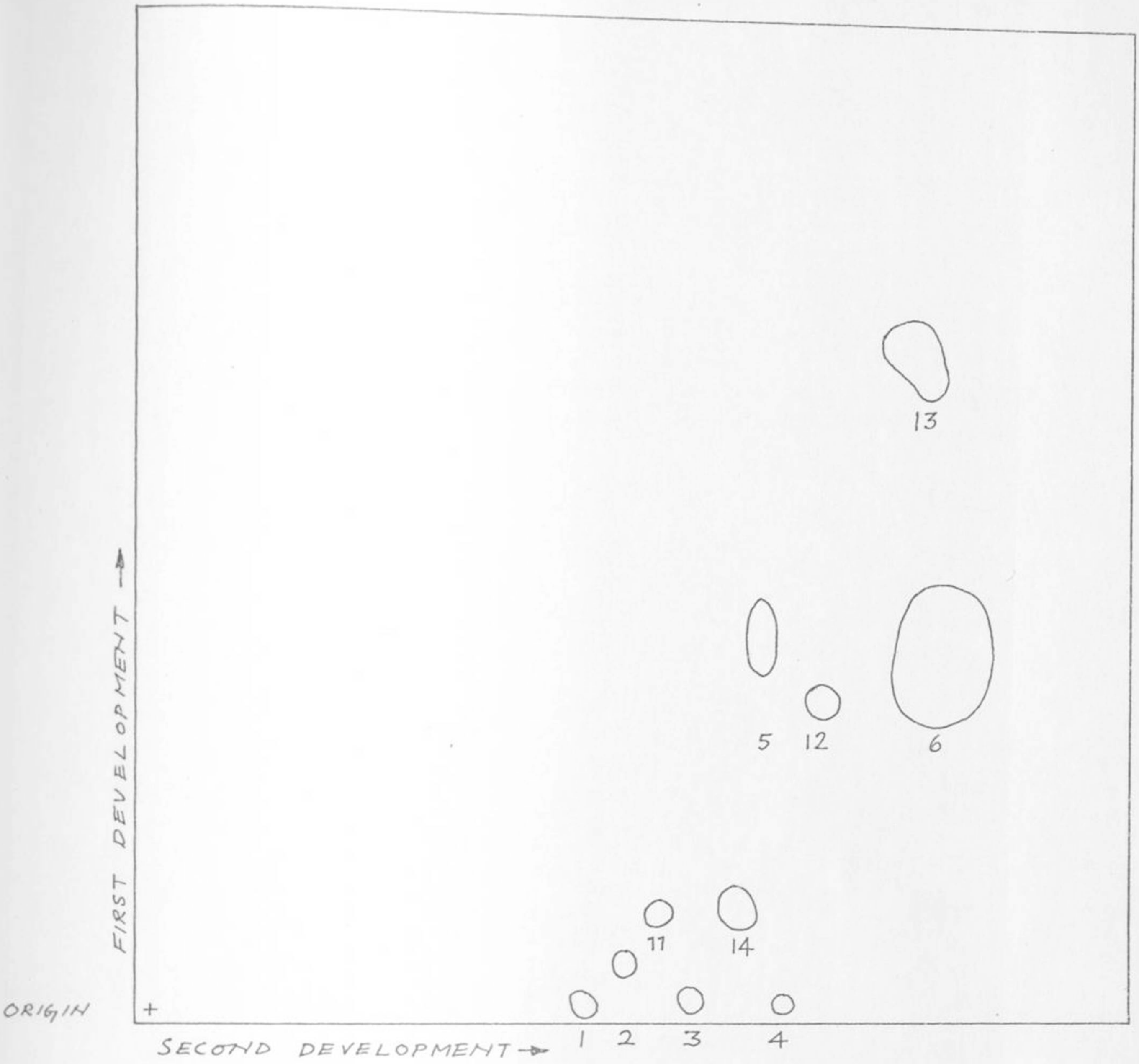

FIG 20 TWO DIMENSIONAL THIN LAYER CHROMATOGRAM OF THE ETHER EXTRACT FROM GREEN ARABICA SANTOS COFFEE BEANS.

LAYER – SILICA GEL G

SOLVENT. 1 – TOLUENE, ETHYL FORMATE, FORMIC ACID 5:4:1 V/V

2. – DIETHYL ETHER, PETROLEUM ETHER (40°-60°) 5:2 V/V

FIG 27 TWO DIMENSIONAL THIN LAYER CHROMATOGRAM OF THE ETHER EXTRACT FROM GREEN ARABICA SAO PAULO COFFEE BEANS.

LAYER - SILICA GEL G

SOLVENT. 1 - TOLUENE, ETHYL FORMATE, FORMIC ACID 5:4:1 V/V

2 - DIETHYL ETHER, PETROLEUM ETHER (40°-60°) 5:2 V/V

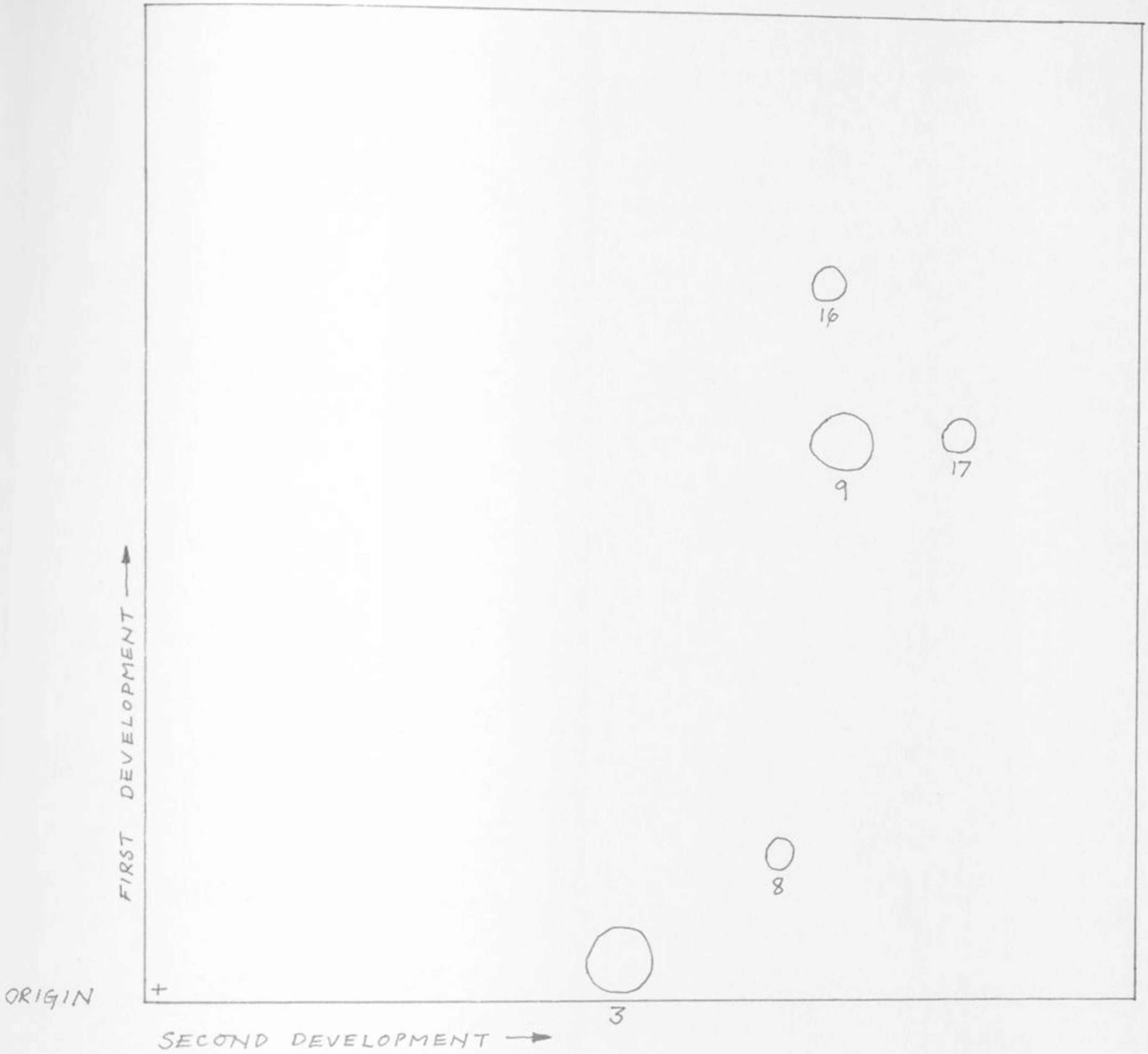

FIG 22 TWO DIMENSIONAL THIN LAYER CHROMATOGRAM OF THE ETHER EXTRACT FROM GREEN ROBUSTA GHANA COFFEE BEANS.

LAYER - SILICA GEL G.

SOLVENT - 1 - TOLUENE, ETHYL FORMATE, FORMIC ACID 5:4:1 v/v

2 - DIETHYL ETHER, PETROLEUM ETHER (40°-60°) 5:2 v/v.

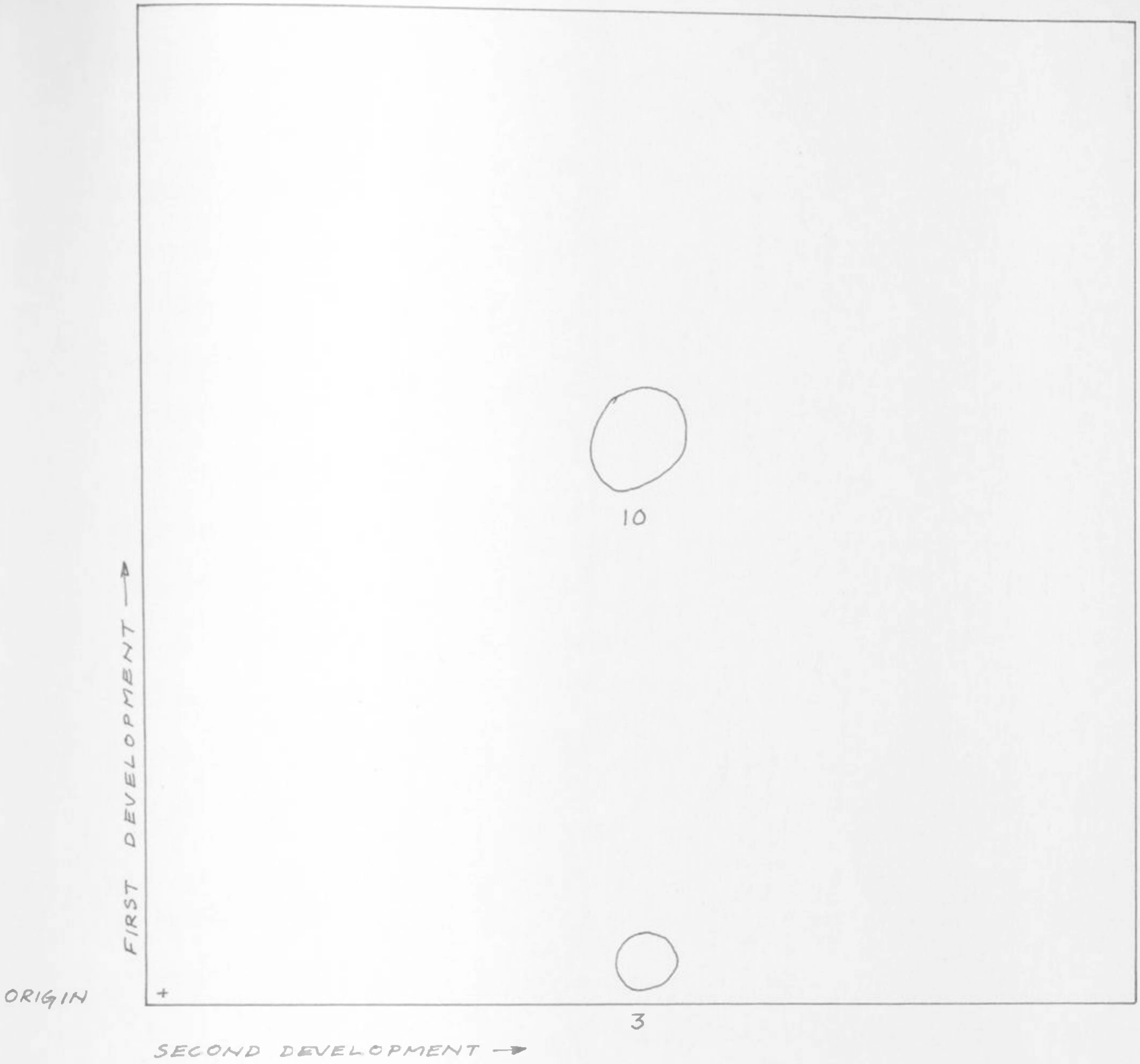

FIG 23 TWO DIMENSIONAL THIN LAYER CHROMATOGRAM OF THE ETHER EXTRACT FROM GREEN ROBUSTA UGANDA COFFEE BEANS.

LAYER - SILICA GEL G.

SOLVENT. 1 - TOLUENE, ETHYL FORMATE, FORMIC ACID 5:4:1 V/V

2 - DIETHYL ETHER, PETROLEUM ETHER (40°-60°) 5:2 V/V

upper band was sensitive to the Periodate Reagent and DSA, it was assumed to contain the phenolic components. Reference to Table 15 shows that aromatic aldehydes containing one hydroxyl group can be expected to have an Rf value in the range 0.65-0.80. This information is in agreement with the suspected presence of coniferaldehyde and/or sinapaldehyde. Since by PVP chromatography only one DSA-sensitive band was located it would appear that Spot 1 from the two dimensional plates also contained one hydroxyl. This compound was insensitive to the Periodate Reagent and therefore it can be deduced that no methoxyl groups were present in the positions ortho- and para- to the hydroxyl. From the information available it was not possible to reach any more positive conclusions about the structure of this compound.

Components 2, 4, 5 and 7-10 could not be identified. It is possible that they are terpenes since many such compounds are found in essential oils.

Conclusions.

1. Green coffee beans contain a range of compounds that are sensitive to the Wiesner Reagents. These substances survive roasting. Arabicas contain a wider range and greater quantity than Robustas.

2. Two of the components appear to be coniferaldehyde (present in all four beans) and sinapaldehyde (present only in the Arabicas). It is possible that the Arabicas also contain another monohydroxy aromatic aldehyde.

CHAPTER THREE

GENERAL DISCUSSION

CONCLUSIONS

SUGGESTIONS FOR FUTURE WORK

SUMMARY

REFERENCES

APPENDICES

GENERAL DISCUSSION.

Examination of the phenolic components of two green Arabicas and two green Robustas by the Differential Technique showed that the total CQ and FQ acids content was in the range 5.8-9.3% (dmb). Robustas contained more CQ acids (7.8-8.0% dmb) than Arabicas (5.6-6.2% dmb), and similarly more FQ acids (0.8-1.3% dmb compared to 0.23-0.25% dmb).

Examination of these same beans, quantitatively and chromatographically, after subjecting each to four degrees of roasting showed that both types of bean suffered a very similar progressive loss of CQ acids. In the case of the Robusta Uganda this loss was 65%, and in the other three cases about 80%. In contrast the FQ acids showed very little loss. This retention of FQ acids leads to roasted Robusta beans having a considerably greater content of depsides than an Arabica bean subjected to the same degree of roast.

The quantitative results obtained by previous workers are very similar if allowances are made for the natural variations in raw materials, and the inherent limitations of their techniques. This point has been fully discussed in Chapter 2, Section 6.

Aqueous extracts from roasted Arabica beans have been shown to have a lower pH value than similar extracts from roasted Robusta beans (11, 74, 192, 209, 210) despite the higher content of phenolic acids present in the Robusta beans. At least a portion of the depsides of green coffee beans is present as a potassium and caffeine complex. Smith (29, 30) has reported that the 3CQ caffeine potassium salt is more heat stable than the free acid, and the stability of the phenolic portion of this complex is supported by the results of the model systems (See Table 19). Such salts present in the roasted bean will not contribute to the acidity of aqueous extracts or brews and their preferential retention after roasting could explain the apparently anomalous relationship between extract pH value and content of phenolic 'acids.' Hassan (192) believed that the lower pH value of Arabica extracts was due to a greater content of non-phenolic acids produced by sugar pyrolysis.

The results obtained by examining model systems containing phenolic compounds showed that caffeic acid was more heat sensitive than 3CQ. In the essentially anhydrous model systems 3CQ did not hydrolyse to caffeic acid and quinic acid, but when heated with dilute hydrochloric acid at moderate temperatures hydrolysis readily occurred. Hassan (192) reported that sufficient moisture was present in the coffee bean during the early stages of roasting for sucrose hydrolysis to occur. It is reasonable to assume that the heat sensitive CQ acids would initially degrade by hydrolysis to caffeic acid and quinic acid. Caffeic acid did not accumulate in the hydrochloric acid hydrolyses but degraded further. Similar degradation of caffeic acid in the anhydrous model systems and its absence from the roasted bean suggest that such a degradation occurs during roasting of coffee beans.

Lack of material prevented a comparison in model systems of the heat sensitivities of FQ acids and CQ acids. When ferulic acid was heated in a model system with another organic acid it was less heat sensitive than caffeic acid. These results suggest that FQ acids will be less heat sensitive than CQ acids, and support the results obtained by quantitative and chromatographic examination of coffee bean extracts.

TLC of the roasted organic acid-containing model systems demonstrated a range of caffeic acid and ferulic acid degradation products. Amongst the former were thought to be pyrogallol, pyrogallic acid, and perhaps protocatechuic acid and aldehyde. Amongst the latter were thought to be 4-hydroxybenzoic acid and methyl vanilloyl ketone. Methyl vanilloyl ketone and protocatechuic aldehyde have previously been reported as ferulic acid and caffeic acid degradation products respectively (29, 111, 148). Hogl (111), and Smith (29) believed that pyrogallol was a chlorogenic acid degradation product derived from the quinic acid moiety. Detection of pyrogallol in roasted model systems containing caffeic acid or 3CQ, but not quinic acid suggests that the caffeic acid is a more likely origin.

When depsides were heated in 'dry' model systems and hydrolysis did not occur, traces of three degradation products were found after triple development of the chromatograms. The evidence of the locating reagents suggested they might contain a pyrogallol unit (3,4,5THBz), and it is possible that a pyrogalloyl quinic acid depside was produced by a reaction paralleling the production of pyrogallic acid from caffeic acid. Lorant (182) reported that heating 3CQ at 260° produced a depside of caffeic acid and 3-hydroxybenzoic acid. Corse et al (177) reported that 5CQ when heated in acidic conditions gave rise to its lactone (Hauschild's Substance.) It is possible that Lorant's Depside and Hauschild's Substance were produced by heating the model systems but it was not possible to establish the presence or absence of such products in the roasted bean.

The only phenolic degradation products that could be demonstrated in the roasted bean were traces of pyrogallol and pyrogallic acid and not the range to be expected if depside hydrolysis and cinnamic acid degradation had occurred as observed in the model systems. Dialysis of an aqueous extract from the roasted coffee beans yielded tanned protein(s) (molecular weight in excess of 20,000) which contained hydrolysable phenolic compounds. The increase from a trace to about 60% closely paralleled the loss of depsides during roasting, and it is suggested that depsides and/or depside degradation products are consumed by interaction with proteins. The amount of tanned protein isolated could not account for all the depsidic material that had been lost, but it is suggested that some water-insoluble tanned protein remained unextracted (See Fig 24). The maximum yield of tanned protein(nearly 4% green bean dmb) compared favourably with the 5% yields of water-soluble 'humic acids' reported by Klocking _et al_ (55) and Obenaus (197).

Klocking _et al_ reported the presence of six hydrolysable phenolic compounds and identified caffeic acid, 4-hydroxybenzoic acid and catechol. The current investigation confirmed the presence of caffeic acid and 4-hydroxybenzoic acid, but not catechol amongst the eleven products detected. The range of phenolic compounds released from the HMW material by hydrolysis was very similar to the degradation products found in various model systems. Two products of the HMW hydrolyses were not found in the hydrolysis controls, and therefore were not artefacts. It is possible therefore that the other nine products were not artefacts, but had arisen via depside degradation, and then been incorporated into the tanned protein.

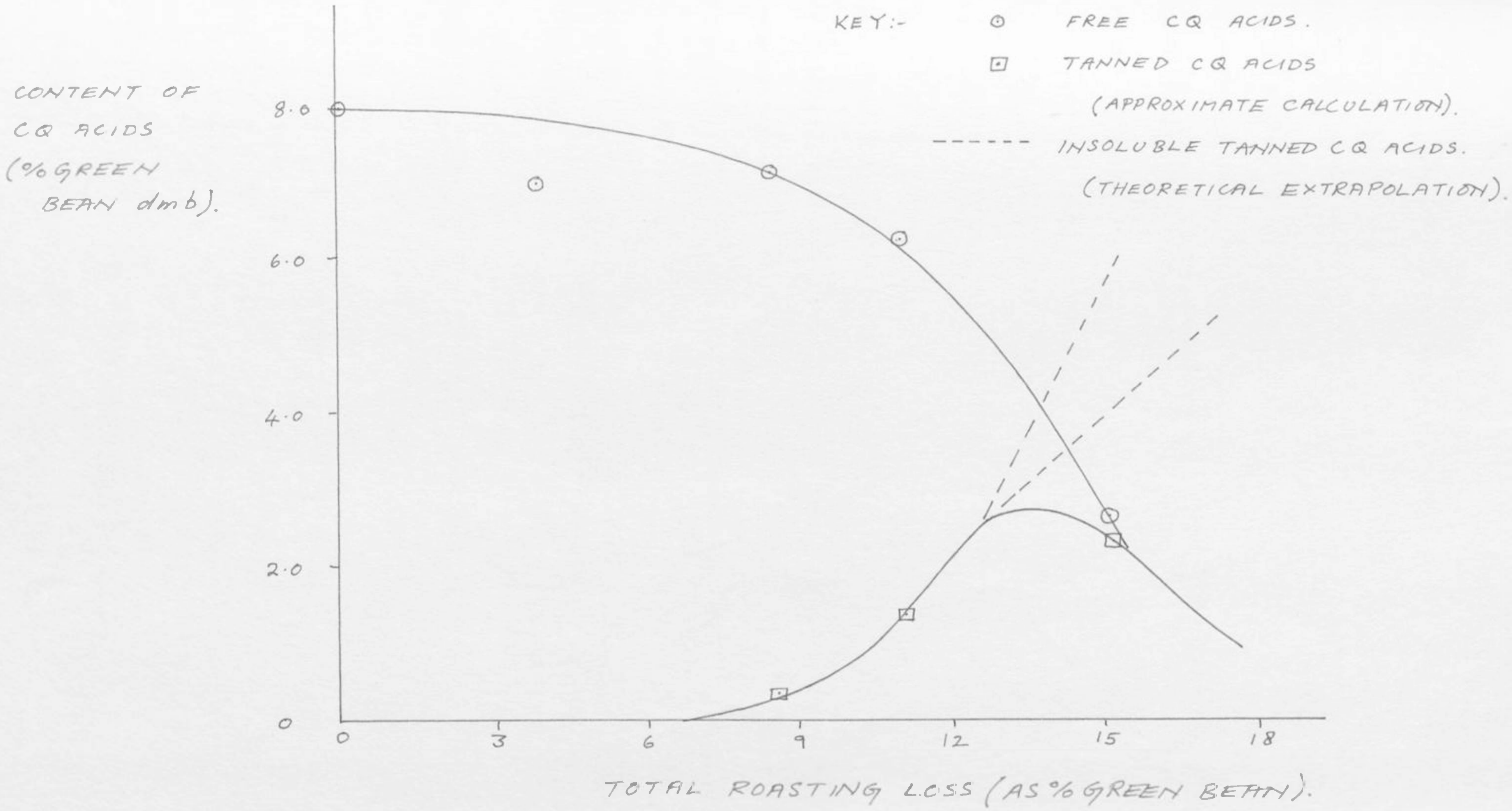

FIG 24 CHANGES IN THE PROPORTIONS OF FREE AND TANNED CQ ACIDS DURING THE ROASTING OF ROBUSTA UGANDA COFFEE BEANS.

Amongst the tanned protein hydrolysis products were traces of two ferulic acid (or FQ) degradation products. Yield of these was far less than yield of CQ degradation products especially in the case of Arabicas. This observation is consistent with the recorded stability of ferulic acid (and FQ acids) compared with caffeic acid and CQ acids, and with the lower content of monomethyl ethers in Arabica beans.

The weight loss noted for caffeic acid, ferulic acid and 3CQ heated alone in model systems indicates the production of some volatiles, although these would not necessarily have been phenolic. Walter and Weidemann (67) reported that 26 phenolic compounds have been detected in coffee volatiles by various workers, and these volatiles included nine aromatic carbonyls (ie carbonyls with a benzene nucleus). Pypker and Brouwer (211) reported that headspace volatiles of a Robusta coffee contained a greater quantity of phenols than an Arabica coffee *(See Table 30). This observation is probably related to the greater destruction of depsides occurring when Robustas are roasted.

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*Table 30 Some phenols present in coffee headspace volatiles.
(After Pypker and Brouwer)(211)

Compound	Relative Content (Approximate)*	
	Arabica	Robusta
4-vinyl guaiacol.	660	5,500
4-ethyl guaiacol.	12	15
4-vinyl catechol.	300	45
Phenol.	25	45
Guaiacol.	35	45

* Deduced from a GLC peak areas.

Compounds of this type are typical of wood smoke (139,183), and the aroma of smoked foods (212), and 4-vinyl phenol has been reported to impart a smoky aroma to popcorn (213). It is suggested that this type of phenol has a completely different role in aroma quality compared with phenolic carbonyls, and that it may impart a smoky and perhaps unpleasant aroma to roasted coffee beans, especially in the case of Robusta beans.

Radtke (122-124) examined coffee volatiles by separating them into four fractions - acids, nitrogenous compounds, phenols, and carbonyls. Mixing the isolated phenols and carbonyls, or carbonyls and nitrogenous compounds gave aromas reminiscent of coffee and indicated the importance of such classes of compound in coffee aroma. Hassan (192) believed that the proportions of these classes, and perhaps the proportions of individual components within a class, was of greater importance in determining the aroma quality than was the total quantity of such compounds. Hassan reported that roasted Arabica volatiles had a greater content of carbonyls than roasted Robusta volatiles and he felt that this difference was partially responsible for the more pleasant aroma of Arabica coffees.

When this information is considered along with the quantitative results of Pypker and Brouwer (211) it is reasonable to suggest that the different aromas of roasted Arabicas and Robustas are partially due to the different proportions of phenols and carbonyls present in the volatiles.

Four aromatic aldehydes have been reported in roasted barley volatiles. Two of these, vanillin and protocatechuic aldehyde, were thought to make a very important and desirable contribution to the character of the aroma (149, 231).

During the course of this current research, methyl vanilloyl ketone has been detected as a degradation product of ferulic acid, and a trace has been detected in roasted coffee beans. Methyl vanilloyl ketone is the ketone corresponding to the aldehyde vanillin, and has a very similar aroma. Protocatechuic aldehyde might also be present in the roasted coffee bean, but this could not be demonstrated with certainty. It is possible that these phenolic aldehydes make an important and desirable contribution to the aroma of coffee, as well as barley.

Green Arabica beans were found to contain three cinnamaldehydes, tentatively identified as coniferaldehyde, sinapaldehyde and a monohydroxy cinnamaldehyde. Only coniferaldehyde, at a much lower level, was found in Robustas. These aldehydes were shown to survive roasting with little quantitative change, an ability probably related to their high boiling point. (See Table 31).

Cinnamaldehydes and benzaldehydes have been reported to be components of the essential oils of many spices and beverages (214-229, See Table 31). Vanillin, found in the vanilla bean is a particularly good example. It is suggested that similar cinnamaldehydes and benzaldehydes present in coffee beans would make a pleasant contribution to the aroma of the coffee.

It has been reported that a high liquoring quality of roasted coffee beans is related to a high content of aldehydes in the green coffee beans (104-106). Although these workers did not specify what type of aldehyde was involved, it is possible that benzaldehydes and cinnamaldehydes were responsible.

Table 31 The Occurrence of benzaldehydes and cinnamaldehydes in essential oils.

Substance			Found in			
	M.Pt	B.Pt	Botanical Name	Trivial Name	% dmb	Reference
Benzaldehyde	-56°	179°	*Perilla spp.*		0.4-1.3	214
Salicylaldehyde) 2HBzAl)	-11°	197°	*Tagetes minuta L.*			215
4HBzAl	119°	-	*Thea sinensis*	Tea		216
4-anisaldehyde) 4MBzAl)	1°	248°	*Ocimum basilicum*	Sweet Basil	2.0	217
Vanillin, 3M4HBzAl	84°	285°	*Aracus aromaticus*	Vanilla bean		218
Cuminal) 4-isopropyl BzAl)	-	235°	*Aegla marmelos* *Corr (Rutaceae)*			219
Dehydrocuminal) 4-isopropenyl BzAl)	-	-	*Cuminum cyminum*	Cumin Seed	2.0-4.0	220
			Homalonema aromatica *Schott.*		4.17	228
Phenyl ethanal	-	-	*Morina longifolia* *Wall. Dipsaceae*		12.6	221
			Rose teplitz			222, 223
			Tagetes minuta L.			215
Cinnamaldehyde	-7.5°	252°	*Thea sinensis*	Tea		216
			Cinnamonum spp.	Cinnamon, Cassia		224, 225
2MCAl	-	295°	*C.cassia*	Cassia		225
4MCAl	58°	-	*Sphaeranthus Indicus L.*			226, 227
			Ocimum basilicum	Sweet Basil	4.0	217
			Artemisia dracunculus	Tarragon		219
Coniferaldehyde) 3M4HCAl)	83°	-	*Cinnamonum Spp.*	Cinnamon		229

Meilgaard _et al_ (230) has reported that the taste threshold, in beer, for cinnamaldehyde and several other aromatic aldehydes is about 1 ppm. It would appear therefore that only traces of benzaldehydes and cinnamaldehydes need be present to have an effect on the taste and aroma of coffee brew.

It is suggested that the substituted cinnamaldehydes, detected in green Arabica coffee beans at a higher level than in green Robusta coffee beans, is one factor which contributes to the higher quality of roasted Arabica coffees. Integrating this hypothesis with the findings of Hassan, and Pypker and Brouwer, suggests the following requirements for a high quality green coffee bean:-

1. A high sugar, low amino acid content to give a high yield of volatile carbonyls.
2. Low depside content to give a low yield of 'smoky' phenols.
3. A high cinnamaldehyde content.

Further support for the importance of the cinnamaldehydes can be obtained from reports by Ray (1660) and Ellison(1968), although neither can be considered of a truly scientific nature.

Ray reported (232) "Those who go about the country looking for plants, if they happen upon acid or nasty tasting beer, can improve it best for the palate and digestion by an infusion of the Common Wormwood." The Common Wormwood _Artemisia absinthum_ is closely related to _A. dracunculus_ or Tarragon which has been reported to contain 4-methoxy cinnamaldehyde (219).

Ellison (234) states "Black coffee or cocoa stirred with sticks of cinnamon bark is imparted with a very pleasant flavour and aroma." _Cinnamonum spp._ have been reported to contain cinnamaldehyde, 2-methoxy cinnamaldehyde, 4-methoxy cinnamaldehyde and coniferaldehyde (224-227, 229).

It is reasonable to assume that the improvements in quality observed by Ray and Ellison were due to extraction of essential oils, and possibly due to the cinnamaldehydes contained therein.

CONCLUSIONS

1. Green Arabica coffee beans have a lower content of caffeoylquinic acids and feruloylquinic acids, than green Robusta coffee beans.
2. The feruloylquinic acids are quite stable during roasting compared to the caffeoylquinic acids.
3. Degradation of these depsides heated in model systems is catalysed by other green bean components.
4. Degradation of these depsides heated in model systems gives rise to a range of non-volatile phenolic compounds, and volatile compounds some of which may be phenolic.
5. Similar degradation occurs during the roasting of the coffee bean, and the bulk of the phenolic degradation products are consumed by interaction with proteins.
6. Green Arabica coffee beans contain three substituted cinnamaldehydes, probably coniferaldehyde, sinapaldehyde, and a hydroxycinnamaldehyde, which are sufficiently non-volatile to survive roasting. Green Robusta coffee beans contain only a smaller quantity of coniferaldehyde.
7. A high quality green bean has certain basic requirements:-
    (1) High sugar:amino acid content giving a high yield of volatile carbonyls.
    (2) Low depside content giving a low yield of 'smoky' volatile phenols.
    (3) A high content of non-volatile cinnamaldehydes.

SUGGESTIONS FOR FUTURE WORK.

1. It would be very informative to examine Arabica and Robusta beans (and Liberica if available) to determine the proportions of amino acids, sugars, CQ and FQ acids, and cinnamaldehydes.

2. Subsequently to roast these beans and to determine the proportions of carbonyls and phenols present in the volatiles. The beans could be roasted in a closed system and the volatiles drawn through a series of traps containing for example DNPH and the Periodate Reagent. Reversing the order of these reagents might serve to give some indication of the proportion of phenolic carbonyls to'smoky' phenols.

Alternatively, use could be made of an aqueous carbonyl-free solution of poly-N-vinyl pyrrolidone (of a lower degree of polymerisation than the insoluble form used for TLC) to trap the phenolic volatiles.

It might be possible to examine this solution spectrophotometrically or colorimetrically after treatment with periodate.

3. The green coffee bean "essential oils" should be isolated and the components characterised by GLC and MS. There may be components other than cinnamaldehydes which contribute to the better quality of Arabica beans, eg terpenes.

4. Model systems of the type described in this thesis should be examined in much greater detail. It is possible that adjusting the roasting conditions might increase the yield of phenolic aldehydes at the expense of 'smoky' phenols. This may suggest some modification that could be made to green coffee beans.

SUMMARY

The phenolic components of green and roasted, Arabica and Robusta coffee beans were extracted, quantitated colorimetrically, and examined chromatographically. Green Robusta beans had a greater content of caffeoylquinic acids and feruloylquinic acids than green Arabic beans. Roasting caused a progressive loss of 65-80% of the caffeoylquinic acids, but the feruloylquinic acids were not detectably destroyed. After a given degree of roast, Robusta beans had a higher content of phenolic compounds than Arabica beans.

To further study the effect of heat on this type of phenolic compound a series of model systems were prepared and heated at 180° for five to twenty minutes. The effect of including certain other green bean components was also examined. The heated model systems were examined gravimetrically, colorimetrically and chromatographically. Of the fifteen phenolic degradation products separated by chromatography, eight were identified, but only two of these were found in the roasted beans.

The water-soluble high molecular weight components of green and roasted beans were extracted and purified by dialysis. The freeze-dried material was fractionated and the fractions examined by hydrolysis and thin layer chromatography for the presence of sugars, amino compounds and phenolic compounds. The alcohol-insoluble fraction contained sugars and amino compounds, and the alcohol-soluble fraction contained amino compounds and phenolic compounds. The content of phenolic compounds in this fraction increased during roasting.

The minor phenolic components of the green and roasted bean were examined chromatographically. Structure-specific locating reagents were employed in an attempt to characterise them, and the specificity of the Wiesner Reagent was investigated. This reagent was found to react with certain furans as well as aromatic aldehydes. Three cinnamaldehydes were tentatively identified in green Arabica beans, only one of which was found in Robusta beans. Their behaviour during roasting was investigated.

During the course of this work two new techniques were developed. These were;-

1. A colorimetric reagent for quantitation of certain phenolic compounds, and for spot location in chromatography.
2. An adsorbant(poly-N-vinyl pyrrolidone)for thin layer chromatographic separation of phenolic compounds.

To facilitate the investigation of the high molecular weight components a continuous, semi-automatic, preparative, thin-film, countercurrent dialyser was designed and commissioned.

REFERENCES.

1. MERORY, J., "Food Flavourings, Composition, Manufacture and Use", Avi, Westport, Conn., 1960, p.125.
2. PRIME, C.T. and DEACOCK, R.J., "The Shorter British Flora," Methuen, London, 1957, p.122.
3. SIVETZ, M. and FOOTE, H.E., "Coffee Processing Technology", Vol. 1, Avi, Westport, Conn., 1963, p.1.
4. Pan-American Coffee Bureau, "Annual Coffee Statistics", Publication No. 26, Pan-American Coffee Bureau, New York, 1963.
5. Indian Coffee, 1968, 32, 273.
6. CLARKE, R., Process Biochem., 1967, 15.
7. D'ORNANO, M., CHASSEVENT, F. and PONGNEAUD, S., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.101.
8. LOCKHART, E.E., Coffee Brewing Institute publication 25, 1957.
9. MONCRIEFF, R.W., Fd Mf., 1943, 18, 342.
10. LITTLE, A.C. and MACKINNEY, G., Fd Technol., Champaign, 1956, 10, 503.
11. KAUFMANN, C.W., Fd Technol., Champaign, 1951, 5, 159.
12. SIVETZ, M. and FOOTE, H.E., "Coffee Processing Technology", Vol. 1, Avi,Westport, Conn., 1963, p.226.
13. THALER, H. and GAIGL, R., Z. Lebensmittelunters U. Forsch., 1963, 119, 10.
14. HARTMAN, L., LAGO, R.A.C., TANGO, J.S. and TEIXEIRA, C.G., J. Amer. Oil Chem. Soc., 1968, 45, 571.
15. KAUFMANN, H.P. and SEN GUPTA, A.K., Fette; Seifen; Anstrichmittel, 1964, 66, 461.
16. CARISANO, A. and GARIBALDI, L., J. Sci. Fd Agric.,1964, 15, 619.
17. GALZOLARI, C. and CERNA, E., Riv. Ital. Sostenze grasse, 1963, 40, 176.

18. DERBESEY, M., VENST, C., CORTE DOS SANTOS, A., BENFREDJ, A. and BUSSON, F., Qualitas Plantarum et Materiae Vegetablis, 1969, 18, 367.

19. NAVELLIER, P., Acta. Chim. Hung., 1960, 23, 303.

20. BRESSANI, R., FIESTER, D., NAVARETTE, D.A. and SCHRIMSHAW, N.S., Fd Technol., Champaign, 1961, 15. 306.

21. KAUFMANN, H.P. and HAMSAGER, R.S., Fette; Seifen; Anstrichmittel, 1962, 64, 734.

22. KAUFMANN, H.P. and SCHICKEL, R., Nutr. Abstr. Rev., 1965, 35, 5549.

23. WINTON, A.L. and WINTON, K.B., "Structure and Composition of Foods," Vol. 4, Wiley, New York, 1939, p.139.

24. BERTRAND, G. and BERTRAND, C.R., C.R.Acad. Sci. Paris, 1954, 238, 1684.

25. BERTRAND, G. and SILBERSTEIN, L., C.R. Acad. Sci. Paris, 1954, 258, 1853.

26. TEPLEY, L.J. and PRIER, R.F., J. agric. Fd Chem., 1957, 5, 375.

27. SIVETZ M., "Coffee Processing Technology," Vol. 2, Avi, Westport, Conn., 1963, p.168.

28. NATARAJAN, C.P., BHATIA, D.S., RAO, N.G., VIRAKTAMATH, C.S., NAIR, R.B. and NARAYANAN, K.M., Fd Sci., Mysore, 1958, 7, 53.

29. SMITH, R.F., Cafe, Cacao, The, 1963, 7, 245.

30. NORTHMORE, J.M., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.405.

31. PLUNKETT, R.A., Dissert. Abstr., 1956, 16, 455.

32. WOLFROM, M.L., PLUNKETT, R.A. and LAVER, M.L., J. agric. Fd Chem., 1960, 8, 58.

33. SIVETZ, M., "Coffee Processing Technology," Vol. 2, Avi, Westport, Conn., 1963, p.165.

34. BARBIROLI, G., Rass. Chim., 1965, 17. 261.

35. FABE, L.A., NERY, J.P. and TANGO, J.S., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.389.

36. COURTOIS, J.E., PECHERON, F. and GLOMAND, J-Cl., Cafe, Cacao, The, 1963, 7, 231.

37. SHADAKSHARASWAMY, M. and RAMACHANDRA, G., Phytochem., 1968, 7, 715.

38. JOLY, R.L., Chim. et Ind., 1951, 65, 335.

39. THALER, H., Deutsch Lebensm. Rundschau, 1957, 53. 126.

40. LAVER, M.L., Dissert. Abstr., 1960, 30, 3955.

41. WOLFROM, M.L. and PATIN, D.L., J. agric. Fd Chem., 1964, 12. 376.

42. PATIN, L.D., Dissert. Abstr., 1965, 25. 6965.

43. NEUKOM, H., Bakers Digest, 1966, 41, 52.

44. THALER, H. and ARNETH, W., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.127.

45. - Ibid - Z. Lebensmittelunters U. Forsch., 1968, 138. 26.

46. WOLFROM, M.L. and ANDERSEN, L.G., J. agric. Fd Chem., 1967, 15, 685.

47. MAIER, H.G., DIEMAIR, W. and GANSMANN, T., Z. Lebensmittelunters U. Forsch., 1968, 137. 282.

48. SHADAKSHARASWAMY, M. and RAMACHANDRA, G., Curr. Sci., 1968, 37, 583.

49. UNDERWOOD, G.E. and DEATHERAGE, F.E., Fd Res., 1952, 17, 415.

50. URS, M.K., NATARAJAN, C.P. and BHATIA, D.S., Bull. Cent. Fd Technol. Res. Inst. Mysore, 1955, 4, 260.

51. UNDERWOOD, G.E. and DEATHERAGE, F.E., Fd Res., 1952, 17, 425.

52. CENTIGROSSI, M., TASSI-MICCO, C. and SILANO, V., Phytochem., 1969, 8, 1749.

53. THALER, H. and GAIGL, R., Z. Lebensmittelunters U. Forsch., 1963, 120, 357.

54. URS, M.S., NATARAJAN, C.P. and BHATIA, D.S., J.Sci. Industr. Res. India, 1953, 12B, 124.

55. KLOCKING, R., HOFMANN, R. and MUCKE, D., Z. Lebensmittelunters U. Forsch., 1967, 135. 59.

56. MEICHELBECK, H. and ZAHN, H., Z.Naturf., 1968, 23, 879.

57. SIVETZ, M., "Coffee Processing Technology," Vol. 2, Avi, Westport, Conn., 1963, p.115.

58. LEHMANN, C. and MARTINOD, P., Z. Physiol.Chem., 1965, 341, 155.

59. FRANZKE, C., GRUNET, S., HILDELSRANDT, U. and GRIEHL, H., Z.Physiol.Chem., 1968, 348, 1725.

60. CZOK, G., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.269.

61. - Ibid - Nutr. Abstr. Rev., 1963, 33. 42.

62. HUGHES, E.B. and SMITH, R.F., J. Soc. Chem. Ind. Lond.,1946, 65, 284.

63. SIVETZ, M., "Coffee Processing Technology," Vol. 2, Avi, Westport, Conn., 1963, p.163.

64. GOLDSMITH, G.A., MILLER, O.N.,UNGLAUB, W.G. and KERCHERAL, K., Nutr.Abstr.Rev., 1960, 30, 1039.

65. LEHMANN, G. and HAHN, H.G., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.115.

66. ORDYNSKY, G., Z. Ernahrungs., 1965, 5, 203.

67. WALTER, W. and WEIDEMANN, H:L., Z. Ernahrungs., 1969, 9, 123.

68. MERRIT, M.C. and PROCTOR, B.E., Fd Res., 1959, 24, 672.

69. SIVETZ, M. and FOOTE, H.E., "Coffee Processing Technology," Vol.1, Avi, Westport, Conn., 1963, p.233.

70. PEKKURINEN, L., and PORKA, E., Z. Lebensmittelunters U. Forsch., 1963, 120, 20.

71. NATARAJAN, C.P., BALAKRISHNAN, R., NAIR, R., RAO, G., VIRAKTAMATH, C.S., BHATIA, D.S. and SANKARAN, A.N., J.Sci. Indust. Res., 1960. 198, 32.

72. SONDHEIMER, E., SZYMANSKI, C.D. and CORSE, J.W., J. agric. Fd Chem.,1961, 9, 146.

73. SCARPATTI, M.L. and ESPOSITO, P., Tetrahedron L., 1963, 1147.

74. LENTNER, C. and DEATHERAGE, F.E., Fd Res., 1959, 24. 483.

75. SIVETZ, M., "Coffee Processing Technology," Vol. 2, Avi, Westport, Conn., 1963, p.118.

76. MONCRIEFF, R.W., Food, 1950, 19, 124.

77. MABROUK, R.F. and DEATHERAGE, F.E.,Fd Technol., Champaign, 1956, 10, 194.

78. CLEMENTS, R.G. and DEATHERAGE, F.E.,Fd Res., 1957, 22, 222.

79. SCHORMULLER, J., BRANDENBURG, W. and LAGNER, H., Z. Lebensmittelunters U. Forsch.,1961, 115, 226.

80. WOODMAN, J.S., GIDDEY, A. and EGLI, R.H., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.137.

81. TURNER, E.E. and HARRIS, M.M., "Organic Chemistry," Longmans, London, 1960, p.237.

82. ROBIQUET, P.J. and BOUTRON, C., 1837, cited by Reichstein and Staudinger,(83).

83. REICHSTEIN, T. and STAUDINGER, H., Perfum. essent. Oil Rec., 1955, 46, 91.

84. ROCHLEDER, F., 1866, cited by Reichstein and Staundinger,(83).

85. PAYEN, A., 1846, and 1849, cited by Reichstein and Staundinger, (83).

86. HLASOWITZ, cited by Reichstein and Staundinger,(83).

87. FREUDENBERG, K., Ber., 1920, 53, 232.

88. FISCHER, H.O.L. and DANGSCHAT, G., Ber., 1932, 65, 1009 and 1037.

89. BARNES, H.M., FELDMAN, J.R. and WHITE, W.V., J. Amer. Chem. Soc., 1950, 72, 4178.

90. SMITH, R.F., Chem. Ind., 1958, 215.

91. SONDHEIMER, E., Arch. Biochem. Biophys., 1958, 74, 131.

92. CORSE, J., Nature, Lond., 1963, 172. 771.

93. KRASEMANN, R., Arch. Pharm. Berl., 1960, 293, 721.

94. PICTET, G. and BRANDENBURGER, A., J. Chromatog., 1960, 4, 396.

95. CORSE, J., SONDHEIMER, E. and LUNDIN, R., Tetrahedron, 1962, 18, 1207.

96. SCARPATTI, M.L. and GUISO, M., Ann. Chim. Roma, 1963, 53, 1315.

97. SCARPATTI, M.L. and GUISO, M., Tetrahedron L., 1964, 2851.

98. NAKANISHI, K., "Symposium on the Advances in Plant Phenolics Research," New Delhi, India, 1964.

99. CORSE, J., Lundin, R.E. and WAISS, A.C., Jr., Phytochem., 1965, 4, 527.

100. KUNG, J.T., RYDER, W.S. and FELDMAN, J.R., 3rd. Int. Coll. Coffee Chem. Trieste, 1967, A.S.I.C. Paris, 1967, p.223.

101. CORSE, J. and PATTERSON, D.C., Phytochem., 1969, 8, 203.

102. LINDNER, M.W., "Warenkunde und Untersuchung Kaffee," Verlag A.W. Heyns Erben, Berlin, 1955.

103. THIER, H.P., BRICONT, J., VIANNI, R., REYMOND, D. and EGLI, R.H., Z. Lebensmittelunters U. Forsch., 1968, 137, 1.

104. DE AMORIM, H.V. and SILVA, D.M., Nature, Lond., 1968, 219, 381.

105. FORSYTH, W.G.C., Ann. Rev. Plant Physiol., 1964, 15, 443.

106. CALLE, H.V., Cenicafe, 1963, 14, 187.

107. PIERPOINT, B.W., Biochem. J., 1966, 98, 567.

108. JURICS, E.W., Z. Ernahrungs., 1967, 12, 427.

109. WAISS, R.C., J. Amer. Oil Chem. Soc., 1953, 36. 663.

110. KRASEMANN, R., Arch. Pharm. Berlin., 1961, 294, 266.

111. HOGL, O., Mitt. Lebensm. Hyg., Bern., 1958, 49, 433.

112. NATARAJAN, C.B., BALAKRISHNAN, R., NAIR, N., RAO, G. and BHATIA, D.S., Food Sci. Mysore, 1958, 7, 27.

113. SEGALL, S. and PROCTOR, B.E., Fd Technol., Champaign, 1959, 13, 266.

114. BERNHEIMER, O., Mh. Chem., 1880, 1, 456, cited by Walter and Weidemann, (67).

115. MONARI, A. and SCOCCIANTI, L., Gazz chim. ital. 1895, 25, 115, cited by Walter and Weidemann, (67).

116. JAECKLE, H., Z. Unters, Nahr - Genussmitt., 1898, 1, 457, cited by Walter and Weidemann, (67).

117. ERDMANN, E., Ber. dtsch. chem. Ges., 1902, 35, 4846, cited by Walter and Weidemann, (67).

118. STRAUDINGER, H. and REICHSTEIN, T., U.S. Patent, 1,696,419. 1928.

119. HUGHES, E.B. and SMITH, R.F., J. Soc. chem. Ind. London, 1949, 68, 322.

120. RHOADES, J.W., Fd Res., 1958, 23, 254.

121. MERRIT, C., BAZINET, M.C., SULLIVAN, J.H. and ROBERTSON, D.H., J. agric. Fd Chem., 1963, 11, 152.

122. RADTKE, R., SPRINGER, R., MOHR, W. and HEISS, R., Z. Lebensmittelunters U. Forsch., 1963, 119, 293.

123. RADTKE, R., SPRINGER, R. and MOHR, W., Z. Lebensmittelunters U. Forsch., 1966, 128, 321.

124. RADTKE, R., MOHR, W. and SPRINGER, R., Z. Lebensmittleunters U. Forsch., 1966, 129, 349.

125. BONDAROVITCH, H.A., FRIEDEL, P., KRAMPL, V., RENNER, J.A., SHEPHARD, F.W. and GIANTURIO, M.A., J. agric. Fd Chem., 1967, 15, 1093.

126. MARION, J.P., MUGGLER-CHAVAN, F., VIANNI, R., BRICONT, J., REYMOND, D. and EGLI, R.H., Helv. Chim. Acta, 1967, 50, 1509.

127. FLAMENT, I., WILLHALM, B. and STOLL, M., Helv. Chim. Acta, 1967, 50, 2233.

128. MASON, M.E., JOHNSON, B. and HAMMING, M., J. agric. Fd Chem., 1966, 14, 454.

129. GRIMMETT, M.R., Rev. Pure and Appl. Chem., 1965, 15, 101.

130. FUJII, S., TSUCHIDA, H. and KOMOTO, M., Agr. Biol. Chem., 1966, 30, 73.

131. TSUCHIDA, H. and KOMOTO, M., Agr. Biol. Chem., 1967, 31, 185.

132. KOMOTO, M. and TSUCHIDA, H., Agr. Biol. Chem., 1968, 32, 983.

133. NEWALL, J., MASON, M.E. and MATLOCK, R.S., J. agric. Fd Chem., 1967, 15, 767.

134. GAUTSCHI, F., WINTER, M., FLAMENT, I., WILHALM, B. and STOLL, M., 3rd. Int. Coll. Coffee Chem., Trieste, 1967, A.S.I.C. Paris, 1967, p.269.

135. SUGISAWA, H. and EDO, H., Chem. Ind., 1964, 892.

136. SUGISAWA, H., J. Food Sci., 1966, 31, 381.

137. KATO, K., Agr. Biol. Chem., 1967, 31, 657.

138. KATO, K. and KOMORITA, H., Agr. Biol. Chem., 1968, 32, 21.

139. KATO, K., DOIHARA, T. and SAKAI, F., J. Agri. Chem. Soc. Japan, 1966, 40, 443.

140. KATO, K. and TAKOHASHI, N., Agr. Biol. Chem., 1967, 31, 519.

141. KATO, K. and KOMORITA, H., Agr. Biol. Chem., 1968, 32, 715.

142. KOBAYASHI, N. and FUJIMAKI, M., Agr. Biol. Chem., 1965, 29, 698.

143. KATO, H., Agr. Biol. Chem., 1967, 31, 1086.

144. FUJIMAKI, M., KATO, S. and KURATA, T., Agr. Biol. Chem., 1969, 33, 1144.

145. KATO, K., SAKAI, F. and NAKAHATA, T., Chem. Abstr., 1966, 64, 11567.

146. WALTER, R.H. and FAGERSON, I.S., J. Food Sci., 1968, 33, 294.

147. JONES, T.C. and SCHMELTZ, I., Chem. Ind., 1968, 1480.

148. FIDDLER, W., PARKER, W.F., WASSERMAN, P.E. and DOER, R.I., J. agric. Fd Chem., 1967, 15, 757.

149. SHIMIZU, Y., MATSUTO, S., ITO, Y. and OKADA, I., Agr. Biol. Chem., 1968, 32, A2.

150. BATZER, O.F., SANTORO, A.T. and LANDMANN, W.A., J. agric. Fd Chem., 1962, 10, 94.

151. BATZER, O.F., SANTORO, A.T., TAN, M.C., LANDMANN, W.A. and SCHEIGERT, B.S., J. agric. Fd Chem., 1960, 8, 498.

152. KOEHLER, H.H. and JACOBSON, M., J. agric. Fd Chem., 1967, 15, 707.

153. HORNSTEIN, I. and CROWE, P.F., J. Agric. Fd Chem., 1963, 11, 147.

154. ROHAN, T.A., J. Sci. Fd Agric., 1963, 14, 799.

155. WANG, P. and SAKURAI, Y., J. Fd Sci. Technol. Tokyo, 1968, 15, 514.

156. MASON, M.E., Dissert. Abstr., 1964, 25, 740.

157. MASON, M.E. and WALLER, G.R., J. agric. Fd Chem., 1964, 12, 274.

158. HASSAN, M., Pakistan Council of Scientific and Industrial Research, Karachi, Pakistan, Personal Communication.

159. ROHAN, T.A. and CONNEL, M., J. Food Sci., 1964, 29, 460.

160. GNAGY, M.J., J. Amer. Oil Chem. Soc., 1961, 44, 272.

161. KHANA, S.K., VISWANATHAN, P.N., KRISHNAN, P.S. and SANWAL, G.C., Phytochem., 1968, 7, 1513.

162. SWAIN, T. and GOLDSTEIN, J.L., in "Methods in Polyphenol Chemistry," Pridham, J.B., Pergamon Press, London, 1963.

163. KRUPNIKOVA, T.A., DRANICK, L.I. and SHKOL'NIK, M. Ya., Dokl. Akad. Nalk., S.S.S.R., 1968, 180, 1497.

164. HUGHES, J.C. and SWAIN, T., J. Sci. Fd Agric., 1962, 13, 358.

165. WHITING, G.R., Chem. Ind., 1958, 214.

166. ARAI, S., Agr. Biol. Chem., 1965, 29, A8.

167. BROWNING, B.L., "Methods in Wood Chemistry," Vol. 1, Interscience 1967, p.223.

168. BARDINSKAYA, M.S. and SHUBERT, T.A., J. Chromatog., 1964, 15, D20.

169. SCHELINE, R.R., J. Pharm. Pharmacol., 1966, 18, 665.

170. SWAIN, T. and HILLIS, W.E., J. Sci. Fd Agric., 1959, 10, 63.

171. HODGE, J.E., J. agric. Fd Chem., 1953, 1, 928.

172. CARTWRIGHT, R.A. and ROBERTS, E.A.H., Chem. Ind., 1955, 230.

173. ADLER, E., Angew. Chemie, 1959, 71, 580.

174. ADLER, E., MAGNUSSON, R., BERGMANN, E. and THOMELIUS, H., Acta. Chem. Scand., 1960, 14, 515.

175. ADLER, E. and MAGNUSSON, R., Acta. Chem. Scand., 1959, 13, 505.

176. ADLER, E. and BERGMANN, E., Acta. Chem. Scand., 1960, 14, 529.

177. CORSE, J., LAYTON, L.L. and PATTERSON, D.C., J. Sci. Fd Agric., 1970, 21, 164.

178. CHASSEVENT, F., VINCENT, J-C., HAHN, D., PONGUEARD, S. and WILBRAUX, R., 4th. Int. Coll. Coffee Chem., Amsterdam, 1969, A.S.I.C. Paris, 1969, p.179.

179. PAUSEN, P.L., Freeland Professor of Organic Chemistry, University of Strathclyde, Glasgow, Personal Communication.

180. MERRIT, C., ROBERTSON, D.H. and MACADOO, D., 4th. Int. Coll. Coffee Chem. Amsterdam, 1969, A.S.I.C. Paris, 1969, p.144.

181. SEVERIN, T. and BRAUTIGEN, K.H., Z. Lebersmittelunters U. Forsch., 1970, 144, 321.

182. LORANT, B., Nahrung, 1968, 12, 351.

183. FARMER, R.H., "Chemistry in the Utilization of Wood," Pergamon Press, 1967, p.178.

184. GRODZINSKA-ZACHWEIJA, Z., KARL, W. and WARCHOL, A., J. Chromatog., 1967, 29, 362.

185. VAN SUMERE, C.F., WOLF, G., TEUCHY, H. and KINT, J., J.Chromatog., 1965, 20. 48.

186. SANDERSON, G.W. and PERERA, B.P.M., Analyst Lond., 1966, 91, 335.

187. ANDERSON, R.A. and SOMERS, J.A., Phytochem., 1968, 7, 293.

188. QUARMBY, C., J. Chromatog., 1968, 34, 52.

189. DOUGHERTY, T.M. and SCHEPARTZ, A.I., J. Chromatog., 1969, 42, 415.

190. LERNER, S., DOUGHERTY, T.M. and SCHEPARTZ, A.I., J. Chromatog., 1968, 37, 453.

191. WROLSTADT, R.E. and PUTNAM, T.B., J. Food Sci., 1969, 34, 154.

192. HASSAN, M., "The Precursors of Coffee Aroma," Ph.D. Thesis, University of Strathclyde, Glasgow, Scotland, 1970.

193. RINDERKNECHT, H. and JURD, L., Nature, Lond., 1958, 181, 1268.

194. BURTON, H.S., MCWEENY, D.J. and PANDHI, P.N., Nature, Lond., 1963, 199, 659.

195. EL ODE, K.E., DORNSEIFER, T.P., KEITH, E.S. and POWERS, J.J., J. Food Sci., 1966, 31, 351.

196. CAREY, J.C., SELF, R. and SWAIN, T., J. Food Sci., 1965, 30, 33.

197. OBENAUS, R., NEUMANN, H.-J. and MUCKE, D., Naturwissenschaften, 1966, 13, 19.

198. STREULI, H., Chimia, 1962, 16, 371.

199. THALER, H., Z. Lebensmittelunters U. Forsch., 1970, 143, 342.

200. COULSON, C.B., DAVIES, R.I. and KHAN, E.J.A., Soil Sci., 1959, 88, 191.

201. THALER, H., Z. Lebensmittelunters U. Forsch., 1957, 106, 128.

202. THALER, H., Z. Lebensmittelunters U. Forsch., 1959, 110, 442.

203. THALER, H. and ARNETH, W., Z. Lebensmittelunters U. Forsch., 1969, 140, 101.

204. SIVETZ, M., "Coffee Roasting Technology," Vol. 2, Avi, Westport, Conn., 1967, p.167.

205. FORSYTH, W.G.C., QUESNEL, V.C. and ROBERTS, J.B., J. Sci. Fd Agric., 1958, 9, 181.

206. MORRISON, W.L., Food Science Department, University of Strathclyde, Glasgow, Personal Communication.

207. WOLFROM, M.L., SCHUETZ, R.D. and CAVALIERI, L.F., J. Amer. Chem. Soc. 1948, 70, 514.

208. TURNER, E.E. and HARRIS, M.M., "Organic Chemistry," Longmans, London, 1960, p.237.

209. SIVETZ, M. and FOOTE, H.E., "Coffee Processing Technology," Vol. 1, Avi, Westport, Conn., 1963, p.232.

210. NATARAJAN, C.P., IYENGAR, J.R. and BHATIA, D.S., J. Sci. Indust. Res., 1957, 42, 16.

211. PYPKER, J. and BROUWER, H., 4th. Int. Coll. Coffee Chem., Amsterdam, 1969, A.S.I.C. Paris, 1969, p.122.

212. ISSENBERG, P., KORNREICH, M.R. and LASTRA, A.O., J. Food Sci., 1971, 36, 107.

213. WALRADT, J.P., LINDSAY, R.L. and LIBBEY, L.M., J. agric. Fd Chem., 1970, 18, 926.

214. FUJITA, Y., FUYITA, S. and HAYAMA, Y., J. Agric. Chem. Soc., Japan, 1970, 44. 428.

215. HONDA, K.L., CHOPRA, M.M. and NIGAM, M.C., Perfum. essent. Oil Rec.,1963, 54, 372.

216. MOKHNACHEV, I.G., KAMENSHCHIKOVA, S.V. and KOVTUN, V.S., Chem. Abstr.,1966, 67. 18144.

217. POGANY, D., BELL, C.L. and KIRCH, E., Perfum. essent. Oil Rec., 1968, 59, 858.

218. BENDER, A.E., "Dictionary of Nutrition and Food Technology," Butterworths, London, 1965, p.204.

219. BASLAS, K.K., Perfum. essent. Oil Rec., 1967, 58, 437.

220. BENDER, A.E., "Dictionary of Nutrition and Food Technology," Butterworths, London, 1965, p.59.

221. BASLAS, K.K., Perfum. essent. Oil Rec., 1968, 59, 12.

222. BASLAS, K.K., Perfum. essent. Oil Rec., 1968, 59, 259.

223. AGARARAL, V.B. and GUPTON, G.N., Indian Soap J., 1967, 27, 78.

224. BENDER, A.E., "Dictionary of Nutrition and Food Technology," Butterworths, London, 1965, p.48.

225. LAWRENCE, B.M., Can. Inst. Fd Technol. J., 1969, 2, 178.

226. BASLAS, K.K., Perfum. essent. Oil Rec., 1959, 50, 765.

227. NIGAM, S.S. and RAO, A.K., Chem. Abstr.,1968, 69, 12881.

228. BAHADUR, R. and GUPTA, G.N., Perfum. essent. Oil Rec., 1966, 57, 421.

229. BRAMARAMBA, A. and SIDHU, G.S., Perfum. essent. Oil Rec., 1963, 54, 732.

230. MEILGAARD, M., ELIZONDO, A. and MOYA, E., Master Brewers Association of America Technical Quarterly, 1970, 7, 143.

231. SHIMIZU, Y., MATSUTO, S., MIZUANA, Y. and OKADA, I., Agr. Biol. Chem., 1970, 34, 437.

232. RAY, 1660, cited by Prime and Deacock, (233).

233. PRIME, C.T. and DEACOCK, R.J., "The Shorter British Flora," Methuen, London, 1957, p.147.

234. ELLISON, A., "A Bouquet of Herbs," Pannet and Needham, London, 1968.

235. PEARL, I.A., "The Chemistry of Lignin," Edward Arnold, London, 1970, p.41.

APPENDICES.

Appendix A.

1. Preparation of Locating Reagents.

A. Periodate Reagent 0.5% and 5.0%.

Dissolve the appropriate quantity of analytical grade potassium metaperiodate in deionised water.

B. Molybdate Reagent (170).

To prepare 1 litre of reagent dissolve the following in deionised water:-

1. 16.5g sodium molybdate $Na_2MoO_4\ 2H_2O$.
2. 8.02g disodium hydrogen phosphate $Na_2HPO_4\ 2H_2O$.
3. 7.43g sodium dihydrogen phosphate $NaH_2PO_4\ 2H_2O$.

C. Diazotised Sulphanilic Acid Reagent (167).

Add freshly prepared sodium nitrite (5%, 25ml), slowly at 0° to 5.0ml sulphanilic acid solution (0.9g sulphanilic acid dissolved in 9ml concentrated hydrochloric acid diluted to 100ml with deionised water).

Air dry the plates after spraying.

D. Dinitrophenylhydrazine Reagent (DNPH) (192).

Dissolve 2,4 dinitrophenylhydrazine (2g) in 180ml concentrated hydrochloric acid and dilute to one litre. The DNPH is slow to dissolve - the reagent should be filtered before use to remove particles which may clog the spray nozzle.

E. Wiesner Reagent A and B (168, 206).

Dissolve 1g phloroglucinol (2,4,6THBz) in 100ml 12% hydrochloric acid to prepare Reagent A. For Reagent B, use concentrated hydrochloric acid. This reagent turns yellow, and then red during storage, and should be discarded if discoloured.

F. Vanillin Reagents.

1. Reagent of Swain and Hillis (170).

Vanillin(3M,4HBzAl) (1%) dissolved in 70% sulphuric acid.

2. Reagent developed during this project.

Vanillin (1%) dissolved in 12% hydrochloric acid. If the reagent becomes discoloured during storage, it should be discarded.

Table 32 Response of standard compounds to Vanillin Reagent 2.

Compound		Colour Response (1μg)
2,4 DHBA	resorcylic acid	faint red
3,5 DHBA	resorcylic acid	faint red
2,6 DHBA	resorcylic acid	faint red
2,4 DHBzAl	resorcylaldehyde	faint red
2,4 DHBz	resorcinol	red
2,4,6 THBz	phloroglucinol	red
3,4,5 THBz	pyrogallol	faint purple
3,4,5 THBA	pyrogallic acid	faint purple

G. Alpha-naphthol Reagent. (192).

Dissolve 1g alpha-naphthol in 10ml methanol. Add 90ml sulphuric acid (66%) and mix carefully.

Appendix B.

Preparation of TLC Plates.

1. Plain Silica gel G plates.

Silica gel G (BDH or Merck) (30g) was mixed with 65ml deionised water and mixed at high speed in a homogeniser for about thirty seconds. This slurry was rapidly transferred to a proprietry TLC spreader and a layer of 250 nm applied to five 20cm x 20cm plates, (or equivalent number of smaller plates). If a deeper layer was required the quantities were adjusted accordingly.

The plates were allowed to dry at room temperature for 20-30 minutes and then heated at 100° for 15-20 minutes. These plates were stored in a desiccator until required. After prolonged storage the plates were reactivated before use.

2. Bisulphite impregnated plates.

These were prepared using 0.1M sodium metabisulphite instead of deionised water.

3. Poly-N-vinyl-pyrrolidone plates.

Insoluble PVP (12.0g) and $CaSO_4\frac{1}{2}H_2O$ (2.4g) were mixed thoroughly in the dry and then homogenised with 96ml deionised water for 1-1½ minutes. The slurry was spread over five 20cm x 20cm plates to a depth of 500 nm. The plates were allowed to dry for 1 hour at room temperature, then heated at 110° for 15-20 minutes. They were stored in a desiccator until required.

Appendix C.

Preparation of the dialyser.

Assembly.

1. Soak eighteen inches of 8/32" diameter 'Visking' dialysis tubing in water until pliable.
2. Pass one end carefully through the PTFE washer, silicone rubber ring and the plastic screw cap.
3. Moisten a tapered glass tube and insert carefully into the dialysis tubing and through the plastic cap, rubber ring and PTFE washer. Insert this assembly into the condenser jacket. This is the assembly stage shown in the diagram.
4. Screw the plastic cap loosely onto the condenser body.
5. Remoisten the free end of the dialysis tubing and repeat step 2.
6. Repeat step 3.
7. Repeat step 4.
8. Adjust the dialysis tubing so that it is not twisted, and not in contact with the sides of the condenser jacket. The dialysis tubing must be kept damp or it will tear.
9. Connect one of the tapered glass tubes to the peristaltic pump and the reservoir containing the material to be dialysed. Connect tubing from the other tapered glass tube back to the same reservoir.
10. Similarly connect the inlet and outlet of the condenser jacket to the peristaltic pump and solvent reservoir. Arrange the supplies to allow countercurrent flow of solvent and material to be dialysed.

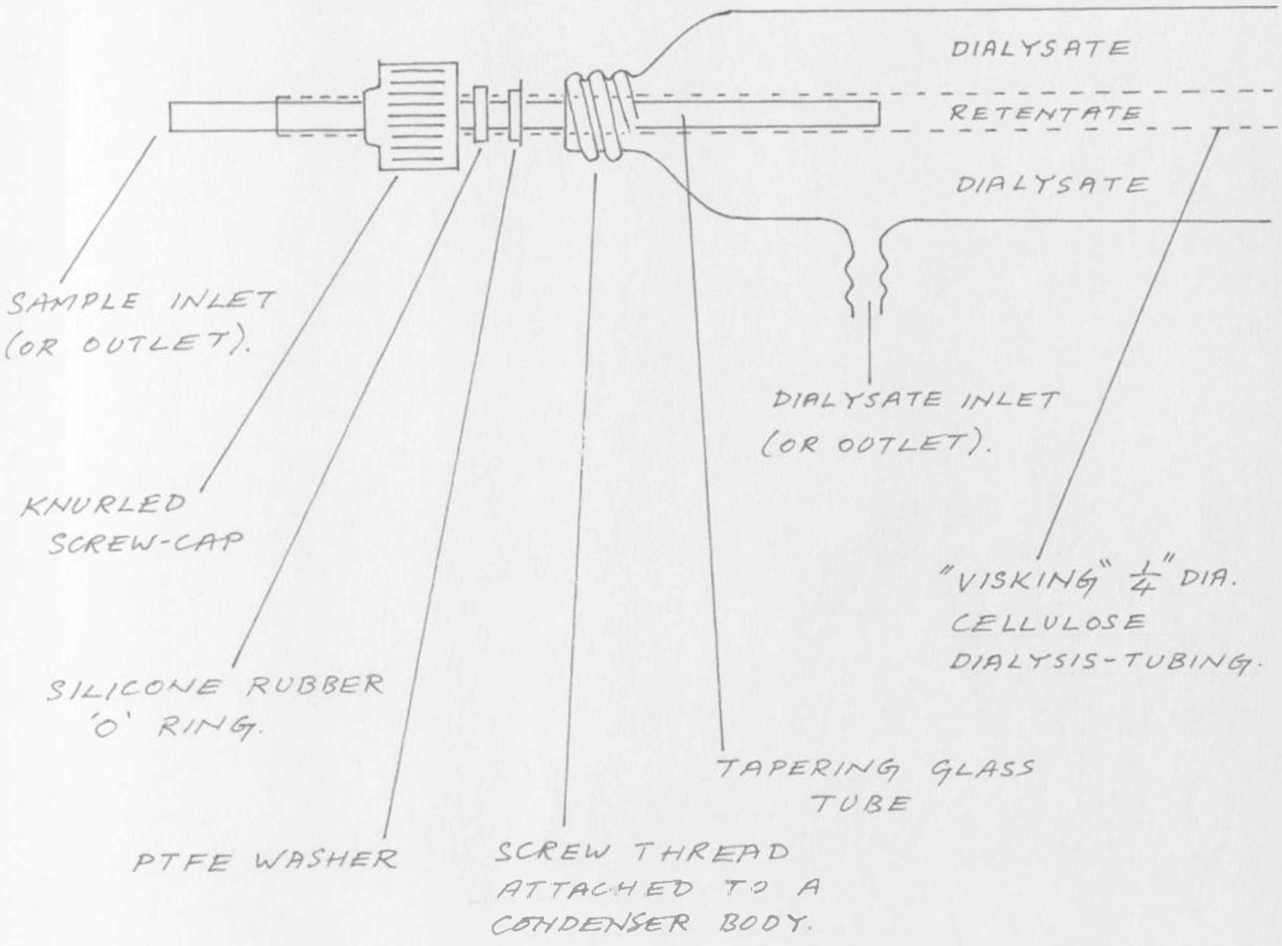

FIG25 EXPLODED DRAWING SHOWING CONSTRUCTION OF DIALYSER (APPROXIMATELY TO SCALE - BOTH ENDS ALIKE).

GENERAL DESCRIPTION :-

THE APPARATUS IS BASED UPON THE OUTER JACKET OF A CONVENTIONAL CONDENSER TO WHICH SCREW THREADS HAVE BEEN FITTED.

11. Make final adjustments to dialysis tubing and tighten the screw caps.
12. Switch on the peristaltic pump.
13. Top up the level of solvent as necessary. Dilute the retentate as required for easy pumping. Diffusion into the 70% propan-2-ol concentrates the retentate.

Appendix D.

The quantitation of lignin in green and roasted coffee beans.

Introduction.

According to Pearl (235), lignin is a system of tridimensional polymers which permeate the membranous polysaccharide and the spaces between plant cells. Lignin cannot be fully defined chemically, but is known to consist of a group of amorphous chemically-related aromatic substances. Since this substance cannot be chemically defined, only approximate methods are available for its quantitation. Pearl (235) reported that the best methods available are based on the insolubility of lignin in concentrated sulphuric acid, and the simplest of this type is the Russian Standard Method.

The Russian Standard Method for the determination of lignin in woody materials. (235).

Treat the sample (1g) with hot distilled water (10ml) and allow to stand for fifteen minutes. Hydrolyse with 86% sulphuric acid (25ml) at room temperature for three hours. After hydrolysis dilute the suspension with 250ml water and boil for five minutes. Filter the suspension through a sintered glass filter, wash the precipitate and dry to constant weight. The residue is assumed to be lignin.

Materials.

Ground green Robusta Uganda	4 x 1.000g
Ground roasted Robusta Uganda	4 x 1.000g
Skim milk powder	2 x 1.000g
Analytical grade Sulphuric acid 86% $^{v}/_{v}$	

Results and discussion.

Lignin is found in woody tissues, which are dead, and in vascular tissues which are present in all plant material. In woody tissues the protoplast has been destroyed and the protein content is very low, perhaps zero. Vascular tissue is associated with living tissue which will contain a certain amount of protein. This is the form in which lignin may be expected in the coffee bean, along with protein 10-14% (dmb) (35, 38, 44). It was felt that a method designed for tissues of low protein content might not be suitable for examining tissues of high protein content, so skim milk powder (approximately 33% protein) was used as a control.

The results are set out in Table 33. The milk powder appeared to contain slightly more lignin than the green or roasted coffee beans.

Since milk powder does not contain lignin one must assume that some component of skim milk powder interferes in this technique, and that a similar interference could have occurred with the coffee bean samples. It was decided that a detailed investigation of the methods of lignin determination was outside the scope of this project, and no further action was taken in this respect.

Table 33 The lignin concent of green and roasted coffee beans determined by the Russian Standard Method.

Sample and replicate number.		Lignin Content (% dmb) Range	Mean
Green Robusta Uganda	1	22.0	20.45
	2	17.2	
	3	19.5	
	4	23.1	
Roasted Robusta Uganda	1	24.5	23.02
	2	22.0	
	3	23.7	
	4	21.9	
Skim Milk Powder	1	23.5	22.80
(Control)	2	23.5	

Conclusions.

This technique is not suitable for determining lignin in coffee beans or in other protein-rich materials.

Appendix E.

Laboratory roasting of coffee beans.

A hand-operated, gas-heated, laboratory coffee roaster was employed to prepare all the roasted samples used during the course of this work. The roaster (Type 260, capacity 3 lb.) was supplied by Uno Limited of London. The gas jet adjustment was carried out by the manufacturer before despatch, and no further adjustments were made.

The roaster, with the gas jets burning, was operated for about five minutes in order to heat to normal roasting temperature before the green coffee beans were placed in the roasting chamber. The degree of roast was assessed visually - a task which required a little practice - and when roasting was considered to be complete the gas jets were shut off, and the beans discharged into the cooling tray. Cooling was speeded by exposure to a blast of cool air from a fan.

PCT (SPAI)

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