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THE FATTY ACIDS AND STEROLS OF COFFEE AND MINT SUSPENSION CULTURES

by

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ABSTRACT

The cells of two plants, Coffea arabica and an unknown Mentha species, were grown as suspension cultures in liquid media, in order to analyse and compare the fatty acids and sterols of the cell cultures to those found in the parent plants. The cell growth, the parameters of pH and conductivity of the media, and the composition of the neutral lipid fraction were examined. In the case of the coffee cell cultures, the cell growth and the media pH and conductivity were studied in three different media, two defined and one undefined, while the mint cell cultures were studied in one other defined medium. Both coffee and mint cell cultures were grown in the absence of light (normal cultural conditions) and in the presence of light.

The coffee cells showed no differences in growth rate due to variations in media composition. Exposure to light affected neither the growth rate nor initiated chlorophyll formation in the coffee cell cultures, although a distinct green pigmentation formed in the mint cells. A plot of the ionic conductivity of coffee and mint cell suspension cultures was essentially a mirror image of the growth curve of the respective culture.

The fatty acids present in the neutral lipid fraction of the cells were studied via gas chromatography, and were

compared to the fatty acids found in the seeds and tissues of the parent plants. Palmitate, stearate, oleate, linoleate and linolenate were found in all coffee and mint cell cultures, independent of the composition of the media and of the presence or absence of light. The appearance of short chain fatty acids (less than C-16) occurred during the dying phases of culture. The fatty acid composition of the coffee cell cultures resembled the analyses of the leaf and stem tissues of the coffee plant rather than the coffee bean. The cell cultures all contained linolenate, not found in the coffee bean, and lacked arachidate, which was present in the bean. In contrast to the coffee cell, the mint cell fatty acids resembled the fatty acid composition of the mint seed rather than the parent plant tissues which contained substantial quantities of the short chain fatty acids (less than C-16). The total fatty acid content of the coffee and mint cell cultures was lower than the seeds of the parent plant, but was comparable to parent plant tissues. A decrease in the total fatty acid content of the neutral lipid fraction of both cultures was noted during the death phase of culture. The total fatty acid content of the coffee cell cultures was not altered by changes in media composition, nor by growth of the cultures in the presence of light. However, the growth of mint cell cultures in the presence of light had a marked effect on the fatty acid content, which increased approximately four fold in comparison to cultures grown in the dark.

The sterol composition of the unsaponifiable lipid found in the extracts of coffee and mint cell cultures was

investigated via gas chromatography and thin layer chromatography. The sterols present were compared to those found in the seeds of the parent plants. The sterols found in large concentration in the coffee cells were β-sitosterol, stigmasterol and campesterol, while in the mint cells only β-sitosterol was conspicuous. The predominant sterols found in the seeds of the parent plants and in the plant cell cultures were identical. However, the cell cultures of both coffee and mint contained larger amounts of sterols in their saponified lipid extracts than found in the seeds. Furthermore, in comparison to the seeds, the lipid extracts of the cell cultures contained greater quantities of unesterified sterols. A wide variety of sterols other than desmethyl sterols were located in the seeds and cell cultures, but were not identified as they constituted only a minor portion of the total plant sterols present.

A large portion of the unsaponifiables of the coffee bean was characterized as non-steroidal. This non-steroidal material was identified as a mixture of two diterpenoid alcohols, cafestol and kawheol, both known to be major constituents of the unsaponifiables of coffee bean oil. The coffee cell oil unsaponifiables were also found to contain these two diterpenoid alcohols.

ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. P.M. Townsley for his interest and guidance during the course of this study. Thanks are also expressed to Dr. W.D. Powrie, Dr. J.F. Richards and Dr. C.A. Hornby for their suggestions.

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INTRODUCTION

Relatively few investigations regarding the tissue culture of either coffee or mint have been reported in the literature. In 1970, Staritsky[43] described the first callus culture of coffee. Keller et al.[24], in 1972, reported the production and release of caffeine in the culture medium by the callus of Coffea arabica. In the same year, Buckland[6] studied the caffeine, chlorogenic acid and amino acid content of the same coffee species. The culture of peppermint and spearmint as calluses and suspension cultures was first described by Lin and Staba[31] in 1961. Further investigations were also undertaken by Staba[12,28,52] concerning the growth characteristics, chlorophyll production and the effect of antibiotics on mint tissue cultures. However, no analyses of cellular constituents of mint cell cultures have been made.

It has been stated by Tattrie and Veliky[49] that "it is possible that the routine cultivation of plant cells in rapidly growing suspension cultures will provide a useful system for the study of lipid metabolism, membrane structure, membrane transport and permeability". This statement reflects the basic nature of the research required to exploit the potential synthetic capability of the cell. However, in order to determine the suitability of plant cells for the

study of lipid metabolism, comparative data on the lipid constituents of the plant cells and the parent plant tissues will be useful.

The objective of this study was, therefore, to determine the fatty acid and sterol composition of coffee and mint cell cultures, and compare this data to the composition of the parent plant tissues.

LITERATURE REVIEW

Plant cell culture has advanced considerably since Haberlandt[17] first formulated the theory of plant cell culture in an early attempt to study the problems of cell differentiation and cell interrelationships. Although Haberlandt's attempts to culture plant tissues were unsuccessful, his work provided the impetus for other workers interested in the field. Twenty years later, the actual culture of plant tissue in the form of excised root tip tissue was accomplished by Kotte[26,27] and Robbins[37,38], but further work was limited by the inability to continuously culture the plant material. In 1934, White[54] successfully cultured excised tomato roots by changing the carbon source from dextrose to sucrose. Two years later, he reported the continuous culture of Nicotina on agar solidified medium in the form of an undifferentiated mass of tissue termed a callus. In the decade following 1950, a number of workers reported the growth or presence of essentially single cells in submerged culture of a number of plant species. Since that time a large number of plant species have been successfully grown as cell suspensions. From 1960 onwards, the development of a variety of defined media made it possible to study the metabolism and production of plant cell metabolites under controlled conditions, thus eliminating the effects of unknown constituents.

Today, a major emphasis in the plant cell field is the study of the production or presence of secondary metabolites which are associated with the parent plant. Nickell[36] defined secondary metabolites (products) as those compounds which are not produced by all plants, whose functions are not known, and which, while not essential metabolites, have considerable biological activity. Examples of secondary metabolites which have been found in plant cell tissue cultures are glycosides, alkaloids, antibiotics, steroids, pigments and flavenoids[36].

I. Sterols and triterpenoids in plant tissue cultures

Numerous papers have appeared in the last few years concerning the presence of sterols and triterpenoids and their related derivatives in plant cell cultures. Benveniste[4] separated the sterols and triterpenes of Nicotina callus cultures, and identified the major constituents as β-sitosterol, campesterol, stigmasterol and cholesterol, with these components accounting for 150 mg/100 g dry weight cells. Two minor constituents, citrostadienol and 28-nor citrostadienol, were also found. Diosgenin, an important starting compound for the manufacture of pharmaceutically important steroid compounds, was isolated by Kaul and Staba[23] from Dioscorea deltoidea, a culture also found to contain stigmasterol and campesterol. Similarly, Heble et al.[21] reported the presence of diosgenin and β-sitosterol in the cultures of Solanum xanthocarpum, noting that the sterol content in the tissue cultures was higher than in the parent plant. In a later

paper[20], the same authors found lupeol to be the major triterpenoid constituent of these cultures. Tomita et al.[50] looked for sesquiterpenes and sterols in the tissue cultures of Lindera strychnifolia, and found the phytosterols campesterol, stigmasterol and β-sitosterol in a ratio of 6:1:53. They also noted that the ability of sterol and sesquiterpene synthesis was not inhibited by one year of tissue subculture. In their study of nine plant species in suspension culture, Tattrie and Veliky[49] reported that routine thin layer work showed the presence of free sterols and sterol esters in all cultures, with no obvious differences in the thin layer patterns. In a more thorough study of the sterols present in one species (Ipomoea sp), these authors found the cells to contain β-sitosterol, campesterol and stigmasterol, in a ratio of 71:12:17. Laseter et al.[29] studied the sterols of Pinus elliotti callus tissues in comparison to the sterols present in the seeds and seedlings. Cholesterol, desmosterol, campesterol, stigmasterol, β-sitosterol and cycloartenol were present in all the tissues, but lophenol and 24-methylenophenol were found only in the seeds and seedlings. β-sitosterol was the major sterol in all the tissues and comprised eighty percent of the total seed sterols, thirty-eight percent of the callus sterols and forty-seven percent of the seedling sterols.

A new area of study concerning the steroids of plant cells is the biotransformation of extraneous steroids added to the culture medium. Furuya et al.[14] reported

the ability of suspension cultures of Nicotina tabacum and Sophora angustifolia to convert progesterone to 5 α pregnanolone palmitate. These authors also noted that this was the first report of the ability of higher plants to convert an exogenous steroid to its ester form. The conversion of 4-androstene-3,17 dione to 5α-androstan 3β-ol-17 one and 5α-androstan-3β,17β dione, by Dioscorea deltoidea suspension cultures was reported by Stohs and El-Olemy[46], and recently Furuya et al.[15] have shown that progesterone can be converted to a number of pregnane type compounds and their glycosides by Digitalis purpurea. Research of this nature opens up the possibility of utilizing plant cells for the conversion of readily available steroids to more valuable compounds, in the manner microorganisms are used today.

II. The fatty acids of plant tissue cultures

In recent years, a number of studies have been carried out on the fatty acids present in plant cell cultures in relation to the fatty acids of the seeds and parent plant material. Staba et al.[41] studied the fatty acid composition of the triglycerides of rape and turnip rape cultures in comparison to the roots, stems, seedlings and seeds of the parent plant. Erucic acid, a major fatty acid present in the triglycerides of the seed oil of both plants, was missing from the plant cell cultures. The triglyceride content of the stems, roots, seedlings and plant cell cultures of both plants was found to be low, one to five percent of the total

lipids. Weete[53] studied the fatty acids of habituated (normal) and teratoma (tumor) callus cultures of Nicotina and its seedlings. He found the fatty acid distribution for all three tissues to be similar, but noted that the individual fatty acid content of the lipid was different; with the seedlings containing 42.03 mg, teratoma culture 1.31 mg and habituated culture 0.40 mg (all per gram dry weight tissue). Davydova et al.[9,10], while studying the fatty acid composition of flax endosperm and the tissue cultures derived from flax, noted that the tissue cultures had low lipid levels and a low level of fatty acids in the lipid. The lipid level of cultures grown in light and dark stayed the same, but the fatty acid content rose in the cultures grown in light. Tattrie and Veliky[49] studied the fatty acid composition of nine species of plants in suspension culture in order to find a preferable culture to study lipid metabolism and membrane structure. Thin layer studies indicated no obvious differences in total lipid constituents in any culture. In comparing the fatty acids of the cell suspensions of Ipomoea sp and of Glycine max to their corresponding roots, stems, leaves and seeds, the fatty acid pattern of the cell suspensions was found to resemble the fatty acid pattern of the leaves in both cultures. The amount of lipid in Ipomoea and Glycine was 0.33% and 0.50% respectively (fresh weight basis). Laseter et al.[30] compared the fatty acids of callus cultures of Pinus elliotti to the fatty acids of the seeds, needles and seedlings. The fatty acid pattern of the calluses

resembled that of the needles and seedlings, although the general fatty acid composition was similar in all materials. The amount of lipid extracted from the individual tissues was 62.02% for the seeds, 5.03% for the stems, 7.43% for the leaves and 4.01% for the calluses. The authors make note of the fact that the amount of lipid extracted from the Pinus callus was approximately two times higher than that extracted from tobacco and soybean cultures.

III. Fatty acids and sterols in coffee bean oil

The fatty acid composition of coffee bean (seed) oil has been known for many years(5,7,13,19). The major fatty acid constituents of coffee bean oil are palmitate, stearate, oleate, linoleate, linolenate and arachidate. Behenate is present in minor amounts and traces of myristate, palmitoleate, margarate and gadoleate have been detected. The fatty acid compositions of various coffee species are all very similar, with no major differences being observed due to climatic or soil conditions(7). The oil content of coffee beans ranges from seven to sixteen percent, with seventy-five percent of the oil being triglycerides(19). Coffee oil contains an unusually high content of unsaponifiable matter (up to twelve percent), the main constituents of which are two diterpenoid alcohols, cafestol ($C_{20}H_{28}O_3$) and kawheol ($C_{20}H_{24}O_3$)(19). The structures of cafestol and kawheol have been elucidated by Djerassi et al.(11), and are as shown in Figure 1. Cafestol (cafesterol), which has been used as an anti-inflamatory agent in the treatment of

rheumatoid arthritis, and kawheol, also known as tetrahydrocafestol, are present in coffee oil mainly as mono esters of fatty acids, with a small amount present as free alcohols. Two minor constituents of the unsaponifiables are phosphotides and phytosterol esters.

Although the sterol composition of coffee oil has been studied by several workers, the most comprehensive study is a co-operative work done by the two groups of Nagasampagi and Rowe and Simpson and Goad, entitled "Sterols of Coffee"(35). The distribution of sterols in coffee beans, as found by these authors, is illustrated in Table 1(35). The results of the work done by Nagasampagi et al. show that the sterols, the majority of which are in the esterified form, make up 5.4% of the lipids. The major sterols are campesterol, β-sitosterol and stigmasterol, with the rest being present in comparatively minor amounts.

IV. Fatty acids and sterols of mint plants

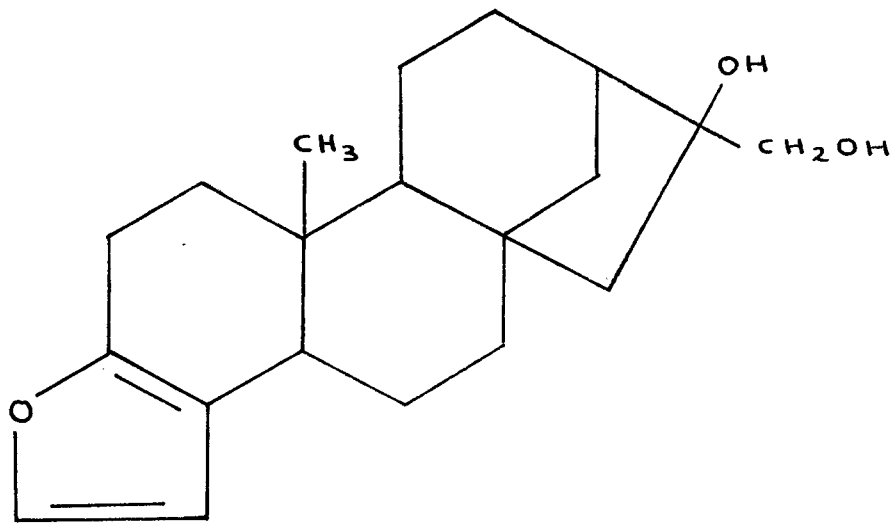
There is apparently no literature available on the fatty acid composition of mint. In a textbook by Bonner(5), the lipid content of mint leaves is stated as being 5%, and in a work by Vidal(51), the fat content of Japanese mint seeds is stated to be 25.28% (w/w). Two papers mention the sterol content of mint plants. Shadakova et al.(40) analysed for sterols in Mentha piperita leaves, rejected plant material and macerated tissue left over after distillation of essential oils. Vitamin D_2 and β-sitosterol were identified using

thin layer chromatography, and the isolated crystal form of β-sitosterol and its benzoate were comparatively identified by spectrophotometry and melting point. The authors mentioned that one sterol was not identified. Battu et al.(3) detected the presence of β-sitosterol in Mentha piperita via thin layer chromatography and visualization by spray reagents, but doubted it to be a pure compound due to a large melting point spread.

Table 1. Distribution of coffee sterols

Sterol	% from beans			% from oil
	Occurring as esters	Occurring free	Total	Total
Cycloartenol	1.2	0.2	1.4	8
24-Methylenecycloartanol	3.0	0.7	3.7	4
Cycloeucalenol	0.4	0.1	0.5	-
Obtusifoliol	0.4	0.1	0.5	-
24-Methylenelophenol	0.3	0.1	0.4	1
Citrostadienol	0.5	0.1	0.6	1
Campesterol	11.3	6.5	17.8	11
Sitosterol	33.5	19.3	52.8	53
Stigmasterol	9.3	12.8	22.1	21
	59.9	39.9	99.8	99

Cafestol



Kawheol

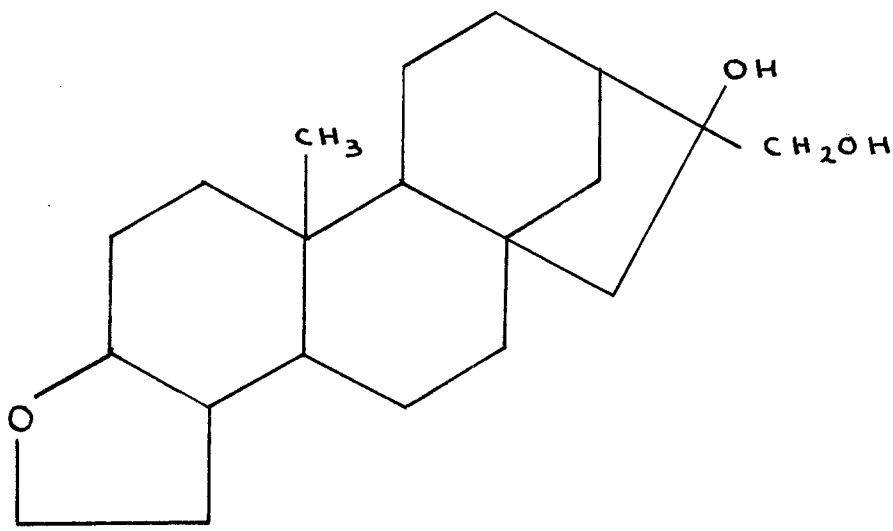


Figure 1. The structures of cafestol and kawheol

MATERIALS AND METHODS

I. Plant origin, media and the preparation of callus and single cell suspensions

A. Origin of the plants

The two plants used throughout this study were Coffea arabica and an unidentified Mentha species. Coffee tissue material was readily available from the University of British Columbia Botanical Garden greenhouse where a number of coffee trees are growing. Mint plants were not available but seeds and dried plant material (recently gathered foliage and stems) of wild mint from the Blue Mountains in Colorado were obtained from the University seed collection.

B. Media used for the production of callus cultures of coffee and mint

The basal agar solidified medium PRL-4, as devised by Gamborg[16], was used to initiate tissue callus cultures of both plants. The composition of this medium is listed in Table 2.

C. Media used for the suspension culture of coffee and mint

Coffee suspension cultures were initiated in liquid PRL-4 media. After suspension formation, the coffee cultures were transferred into two variations of Modified Fox medium[33]. These two altered media are termed Medium #1 and Medium #2, and differ from Modified Fox only in their hormone composition.

Table 2. Composition of PRL-4 Medium[16]

Ingredient	mg/l
NaH_2PO_4 H_2O	90
Na_2HPO_4	30
KCL	300
$(NH_4)_2SO_4$	200
$MgSO_4 \cdot 7H_2O$	250
KNO_3	1000
$CaCl_2 \cdot 2H_2O$	150
KI	0.75
Iron*	28
Micronutrients+	1.0 ml
Vitamins++	10.0 ml
Sucrose	20.0 g
N-Z Amine type A	2.0 mg
2,4-D	2.0 mg
Final pH	6.2

*Sequestrene 330 Fe (Geigy Agric. Chem., Saw Mill River Rd., Ardsley, N.Y.)

+Stock solution. Dissolved in 100 ml water: 1 g $MnSO_4 \cdot H_2O$, 300 mg H_3BO_3, 300 mg $ZnSO_4 \cdot 7H_2O$, 25 mg $Na_2MoO_4 \cdot 2H_2O$, 25 mg $CuSO_4$, 25 mg $CoCl_2 \cdot 6H_2O$.

++Stock solution. Dissolved in 100 ml H_2O: 10 mg nicotinic acid, 100 mg thiamine, 10 mg pyridoxine, 1 g myoinositol.

The composition of Modified Fox medium is presented in Table 3, with the hormone changes listed beneath the table.

Mint suspension cultures were grown only in Murashige and Skoog tobacco medium(34), the composition of which is shown in Table 4.

D. Callus preparation and formation

i. Coffee callus

Unripe coffee cherries and rapidly growing branch tips were taken from the coffee trees at the University of British Columbia Botanical Garden. The tissues were cut into small sections and placed into a 5% hypochlorite solution to sterilize any natural microbial flora. The tissue pieces were aseptically transferred into a sequence of petri plates containing sterile distilled water to remove residual hypochlorite from the tissues. The tissue pieces were transferred into sterile 100 ml dilution bottles containing 20 ml of agar solidified PRL-4 medium and the cultures were then placed in a temperature controlled room, kept at 28°C, with no light present. Within two weeks, callus formation was readily apparent, and within one month the calluses were large enough to transfer into liquid medium. Callus propagation was facilitated by monthly transfers.

ii. Mint callus

Exactly the same sterilization procedure described for coffee was used for the mint seeds, which were also cultured on solid PRL-4 medium. Most mint seeds did not germinate but went directly into callus. Good callus

formation took approximately the same length of time as in the case of the coffee tissues.

E. Preparation of suspension cultures

All suspension cultures were grown in 250 ml erlenmeyer flasks containing 100 ml liquid medium. Callus cultures of coffee were inoculated into PRL-4 liquid medium. The cultures were grown and maintained in the dark at a temperature of 28°C and a relative humidity of 95%. Agitation and aeration of the cultures were provided via a gyratory shaker[1] operating at 110 r.p.m. in a one inch circular orbit. Suspension culture formation evolved slowly as the cells were transferred weekly, and after two months the coffee calluses formed a suspension of an "apple sauce" consistency. The mint suspension culture never attained this state but remained in a rather granular form.

II. Measurement of cellular growth

Cellular growth of both coffee and mint suspension cultures was measured by the following procedure. A sequence of eight duplicate or triplicate flasks was inoculated with a known inoculum, and harvested at convenient intervals (every two to three days) by filtering out the cells through Myracloth[2], using a Buchner funnel. The harvested cells were freeze dried in a Virtis[3] freeze drier to a constant weight.

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[1]New Brunswich Scientific Co., Inc.

[2]Chicopee Mills Inc., 1450 Broadway, New York

[3]Virtis Research Equipment Co., Gardiner, New York

Table 3. Composition of Modified Fox Medium(33)

Ingredient	mg/l
NH_4NO_3	1000
KNO_3	1000
$Ca(NO_3)_2 \cdot 4H_2O$	500
$MgSO_4 \cdot 7H_2O$	300
KH_2PO_4	250
KCL	50
$Na_2EDTA \cdot 2H_2O$	35
$Fe_2(SO_4)_3$	25
$ZnSO_4 \cdot 7H_2O$	7.5
$MnSO_4 \cdot H_2O$	5.0
H_3BO_3	5.0
KI	0.8
Glycine	3.0
Nicotinic acid	0.5
Pyridoxine	0.1
Thiamine	0.1
Inositol	100
Indole-3-acetate	2
Kinetin	0.05
Sucrose	30,000

Medium #1 - 2,4-D was added in a concentration of 2 mg/l

Medium #2 - 2,4-D was added as the only hormone at 2 mg/l (kinetin and indole-3-acetate were eliminated)

Table 4. Composition of Murashige and Skoog Medium(34)

Ingredient	mg/l
NH_4NO_3	1650
KNO_3	1900
H_3BO_3	6.2
KH_2PO_4	170
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CoCl_2 \cdot 6H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$CuSO_4 \cdot 5H_2O$	0.025
Na_2EDTA	37.5
$FeSO_4 \cdot 7H_2O$	27.85
Thiamine hydrochloride	0.1
Nicotinic acid	0.5
Pyridoxine hydrochloride	0.5
Glycine	2.0
Addendum:	
Sucrose	30 g/liter
Myoinositol	100 mg
Indole-3-acetic acid	10 mg
Kinetin	0.04mg

The cell dry weights were plotted against incubation time to obtain the growth curve.

III. Measurement of the pH and conductivity of the medium

After the removal of the cells for growth measurements, the electrolytic conductivity of the medium was measured using a YSI model 31 Conductivity Bridge. The hydrogen ion activity was recorded using a pH meter. This procedure was carried out at each harvest interval.

IV. Materials and methods for fatty acid analyses

A. Neutral lipid extraction

The neutral lipid fraction of the plant materials was isolated by extraction with hexane (b.p. 66-69° C), in order to study the fatty acid composition of the glycerides present in the lipid material. Mint seeds and mint plant material (a mixture of foliage and stem) were extracted with petroleum ether/diethyl ether (50:50 v/v). Prior to lipid extraction, all the plant tissues, with the exception of mint seeds and coffee beans, were freeze dried. To facilitate the extraction, the tissues were ground to pass through a 40 mesh sieve. All extractions were performed using a Goldberg extraction apparatus. After the extraction was completed (one hour), the extracting solvent containing the plant lipids was filtered or centrifuged at room temperature to remove any particulate matter, including any insoluble waxes. The solvent was removed using a stream of dry nitrogen and the residual lipid (oil) weighed in a tarred vial. The

oil weight was expressed as a percentage of the dry weight of the tissue.

B. Saponification of the glycerides in the hexane extracts, and methylation of the component fatty acids

Saponification and methylation of glyceride fatty acids were performed according to the method of Luddy et al[32]. Their procedure, which is recommended for the preparation of samples for gas chromatographic analysis, is a rapid and quantitative method of concurrent saponification and methylation using sodium methoxide. The procedure requires only 1 to 30 mg of oil to be placed in a small vial, to which is added 0.25 ml of 4% anhydrous sodium methoxide solution. The vial is flushed with nitrogen, sealed and heated to 65° C for two minutes, with intermittent shaking. The vial is cooled to room temperature and a 0.6 g mixture of silica gel and calcium chloride (1:1 w/w) is added and stirred into the reactant solution. The silica gel absorbs the free fatty acids and glycerol, while the calcium chloride complexes any excess methanol. To dissolve the methyl esters, 3 ml of CS_2 are added and the solution is centrifuged. The CS_2 supernatant is then transferred to a new vial, the CS_2 volume is reduced under a stream of nitrogen gas to an approximate volume of 0.9 ml, and an internal standard, octadecane, is added.

C. Gas chromatography of fatty acid methyl esters

1. Analysis of fatty acids

The analysis of the fatty acid methyl esters, derived by the saponification and esterification of the oil

extracted from the plant cells, seeds and tissues, was performed on a Becker Research Gas Chromatograph 3810 provided with a flame ionization detector. Fatty acid separation was obtained by using a 15% diethylene glycol succinate liquid phase (HI-EFF 1BP[1]) on 80/100 mesh Gas Chrom P[2] support. The column was made of stainless steel, 6' long by 1/4" i.d. Commercially purified nitrogen was used as the carrier gas at a rate of 54 ml/min, with an isothermal column temperature of 180° C. The component fatty acids were identified by co-chromatography with the appropriate standards and by their corresponding retention times. The areas under the peaks were calculated by using the formula:

height x width at 1/2 peak height

ii. Quantitation of glyceride fatty acids using an internal standard

Method A -- Quantitation of the glyceride fatty acids was performed by using octadecane as an internal standard, since octadecane does not interfere with major fatty acid methyl ester peaks. A standard curve was determined by the following procedure. Two standard solutions, composed of 100 mg of octadecane in 10 ml CS_2 and 10 mg methyl palmitate in 1 ml CS_2, were prepared. Standards were mixed in known concentrations and 5 µl of mixed standard were subjected to gas chromatographic separation. A ratio, which was determined by dividing the area under the palmitate peak by the height

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[1,2]Applied Science Laboratories, Inc.

of the octadecane peak, was plotted against the concentration of methyl palmitate (y axis), producing a linear relationship passing through zero at zero concentration (Figure 2). The equation of the line was found to be: $y=136x$. Using an equimolar mixture of fatty acid methyl esters[1], the areas under the various fatty acid peaks were found to be proportional to their concentrations. The plot obtained from the recorder response was found to be linear, and thus the use of this gas chromatographic quantitation technique allowed reproducible results to be obtained within an acceptable error (± 10%).

Method B -- Quantitation was performed by carrying out the saponification and methylation reaction, described in Method A, on 1 mg of tripalmitate to which had been added 10 µl of octadecane, the internal standard. The methyl esters and the internal standard were taken up in 3 ml of CS_2, and injected into the gas chromatograph. From the resulting gas chromatogram, the ratio of the height of the octadecane peak to the area under the palmitate peak was calculated. The constant ratio obtained was considered to be equal to 1 mg of glyceride fatty acids. Exactly the same procedure described above for tripalmitate was carried out on mint tissue lipids, and the total area under the fatty acid peaks was related to the height of the octadecane standard, in order to calculate the amount of glyceride fatty acids present in the extracted

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[1]Applied Science Laboratories, Inc.

lipid. When calculated, the amount of fatty acid was converted to a percentage of the total weight of the lipid undergoing the reaction.

V. Materials and methods for sterol analyses

A. Lipid extraction

All plant materials used for the analyses were ground to a size sufficient to pass through a 40 mesh sieve. The extraction of the sterols was performed using a 50/50 v/v mixture of petroleum ether and diethyl ether in a Goldberg extraction apparatus. The solvent containing the extracted oil was centrifuged to remove any insoluble matter, then the solvent was removed using a dry stream of nitrogen. The oil residue yield was recorded.

B. Saponification

The crude lipid extract was added to 95% ethanol containing 10% of a 60% KOH solution. The KOH-ethanol solution was refluxed for a minimum of one hour to saponify the lipid, and the preparation was cooled to room temperature.

C. Isolation of unsaponifiables

The saponified mixture was placed in a separatory funnel and diluted with four volumes of water. This solution was extracted with three successive aliquots of ether, and the ether portions were pooled and washed with water three times to remove any residual base. Most of the ether was distilled off, and the remaining solution was put under vacuum in a rotoevaporator to remove the remaining solvent and water. The residual material was taken up in diethyl ether, filtered

and placed in a tarred vial. A stream of dry nitrogen was used to remove the solvent, and the weight of the unsaponifiable residue was recorded.

D. Thin layer chromatography

All thin layer chromatography was done using Polygram[1] sil G precoated plates, with a layer thickness of 0.25 mm. Unsaturated compounds were visualized by using iodine vapour. The major solvent system used was chloroform: ethyl-acetate (9:1 v/v). Qualitative and semi-quantitative thin layers were run on both unsaponifiable material and the petroleum ether/diethyl ether lipid extracts of the various plant materials. Preparative thin layer plates were used to separate the unsaponifiable constituents. The areas corresponding to the iodine positive spots were eluted and derivatized for gas chromatography.

E. Silylation of sterols for gas chromatography

The sterols and diterpene alcohols in the unsaponifiable matter were converted to trimethylsilyl ether derivatives via the method of Sweeley et al.(47). Their method uses anhydrous pyridine as the reaction solvent, hexamethyldisilazane as the reactant and chlorotrimethylsilane as a catalyst. This reaction system converts easily accessible hydroxyl groups to trimethylsilyl ethers in the following manner:

$$3ROH + Me_3SiNHSiMe_3 + Me_3SiCl \longrightarrow 3ROSiMe_3 + NH_4Cl$$

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[1]Macherey-Nagel and Co., Duren

After five minutes of reaction, a heavy precipitate (NH_4Cl) is apparent. The reaction vial is then centrifuged to clear the solvent for direct injection into the gas chromatograph. Satisfactory conversion was attained for the sterols by using a total of 0.5 ml reagents (8 mg sample or less) and 1.0 ml total reagents (greater than 8 mg sample) in a constant ratio of 10:2:1 (v/v) of pyridine, hexamethyldisilazane and chlorotrimethylsilane respectively. In some cases the pyridine solvent was removed with a stream of dry nitrogen after the reaction was completed, and replaced with another solvent such as carbon disulphide or iso-octane to reduce the solvent peak found in gas chromatography.

F. Gas chromatography of sterols

Gas chromatography of sterols was performed using the Becker Research Gas Chromatograph type 3810. The column used for sterol analyses was a ten foot, stainless steel column containing a 5% OV-1 liquid phase adsorbed on Gas Chrom Q[1] solid support. Nitrogen was used as the carrier gas at a flow rate of 31.2 ml/min. The injection port temperature was 300° C, and in order to facilitate the best possible separation of the plant sterols, a temperature program of 240° C for 1.5 hours with an increase in temperature to 280° C for the remainder of the run, was followed. A constant chart speed of 0.1 cm/min was used due to the long duration of the run. Identification of four sterols was possible through the use of available standards and their corresponding retention times.

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[1]Applied Science Laboratories, Inc.

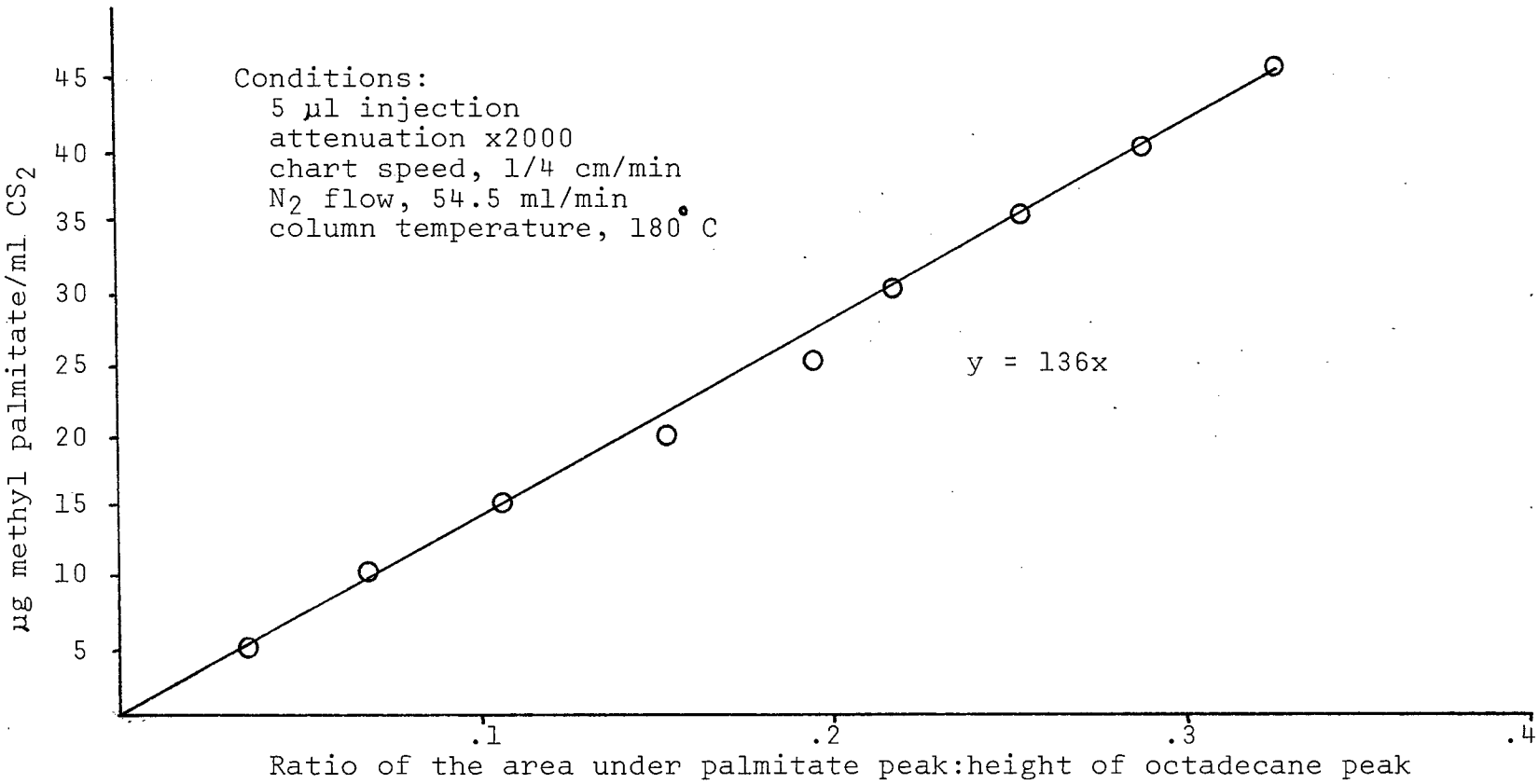

Figure 2. Graph of the standard curve

G. Column chromatography - the isolation of the diterpenoid alcohols, cafestol and kawheol, from coffee bean unsaponifiables

Following a simplification of the procedure used by Nagasampagi et al.[35], a 40 cm x 1 cm column containing 6 gm of Brockman grade III alumina in petroleum ether was made, and a 100 mg sample of coffee oil unsaponifiables was applied to the top of the column. The column was eluted with 100 ml of petroleum ether to remove any hydrocarbons and then eluted with 100 ml of petroleum ether:diethyl ether (60:40 v/v) to remove the sterols. Samples of the final drops of this eluent were subjected to thin layer chromatography to ensure complete removal of the sterols. To release the diterpenoid alcohols[35], the column was eluted with chloroform:methanol (99:1 v/v). This fraction was kept for further examination.

H. Crystallization of cafestol and kawheol

The chloroform:methanol fraction obtained from alumina column chromatography was placed in a rotoevaporator to remove the solvents. The residual material was refluxed in petroleum ether for five minutes, the solvent was transferred to another flask and the petroleum ether was removed under vacuum. The residue was used for ultraviolet spectroscopy.

I. Ultraviolet spectroscopy

The crystallized material obtained from petroleum ether was run in diethyl ether, with a diethyl ether reference, on a Unicam Sp. 800 B ultraviolet spectrophotometer.

EXPERIMENTAL RESULTS

I. Growth Studies

A. Growth study of the coffee cell suspension cultures

Growth studies of coffee cell suspension cultures were initiated in order to characterize the growth pattern of the cultures. The coffee cells were grown in three media, PRL-4 (an undefined medium) and two defined media, Medium #1 and Medium #2, which differ from each other only in hormone composition. A sequence of eight duplicate or triplicate flasks of each media was inoculated with 10 ml (0.2 g dry weight cells) of stock cultures grown up in their corresponding media. Two sets of cultures were inoculated as described above, one grown in the dark (normal cultural conditions) and the other grown in the presence of light (9,840 lumens). Cells were harvested every two to three days, freeze dried and weighed. The pH and conductivity of the culture medium were measured at each harvest.

The changes over a period of fourteen days in cell dry weight, pH and conductivity of the free cell suspension cultures of coffee grown in Medium #1, under conditions of light or dark, are illustrated in Figures 3 and 4. The cell growth curves shown for Medium #1 illustrate the trends observed in all three media, since similar results were obtained for cells grown in Medium #2 and in PRL-4. Maximum cell yield in all cultures occurred around day 8, with the

"exponential" phase of growth occurring between days 4 and 8. The pH of all three media followed similar patterns, with a decrease occurring during the first two days. During the "exponential" phase of growth the pH of the supporting medium increased. This rise in pH was noted in all growth studies. Adjustment of the pH of the medium to pH 5 prior to sterilization, in order to give an initial culture pH of 4, produced a pronounced lag in the growth of the cultures (Figure 5). This lag was eliminated by adjustment of the pH of the unsterilized medium to provide a final pH of 5 to 6 following steam sterilization. The conductivity data obtained for all coffee cultures showed that conductivity was essentially a mirror image of the growth curve.

B. Growth study of the mint cell suspension cultures

The growth of the mint cell cultures in stock media was followed under the conditions of light or dark, and the conductivity and pH were measured at harvest intervals. The growth of the mint cell suspension cultures in the dark is presented in Figure 6. The resulting growth curve illustrated in Figure 6, along with the parameters of pH and conductivity, were exactly the same as for the culture grown in the presence of light (Figure not shown). The only difference observed between light and dark grown mint cultures was the development of a green pigmentation in the cell cultures grown in the presence of light. As in the coffee cultures, the conductivity of the media inversely reflected the growth rate of the culture.

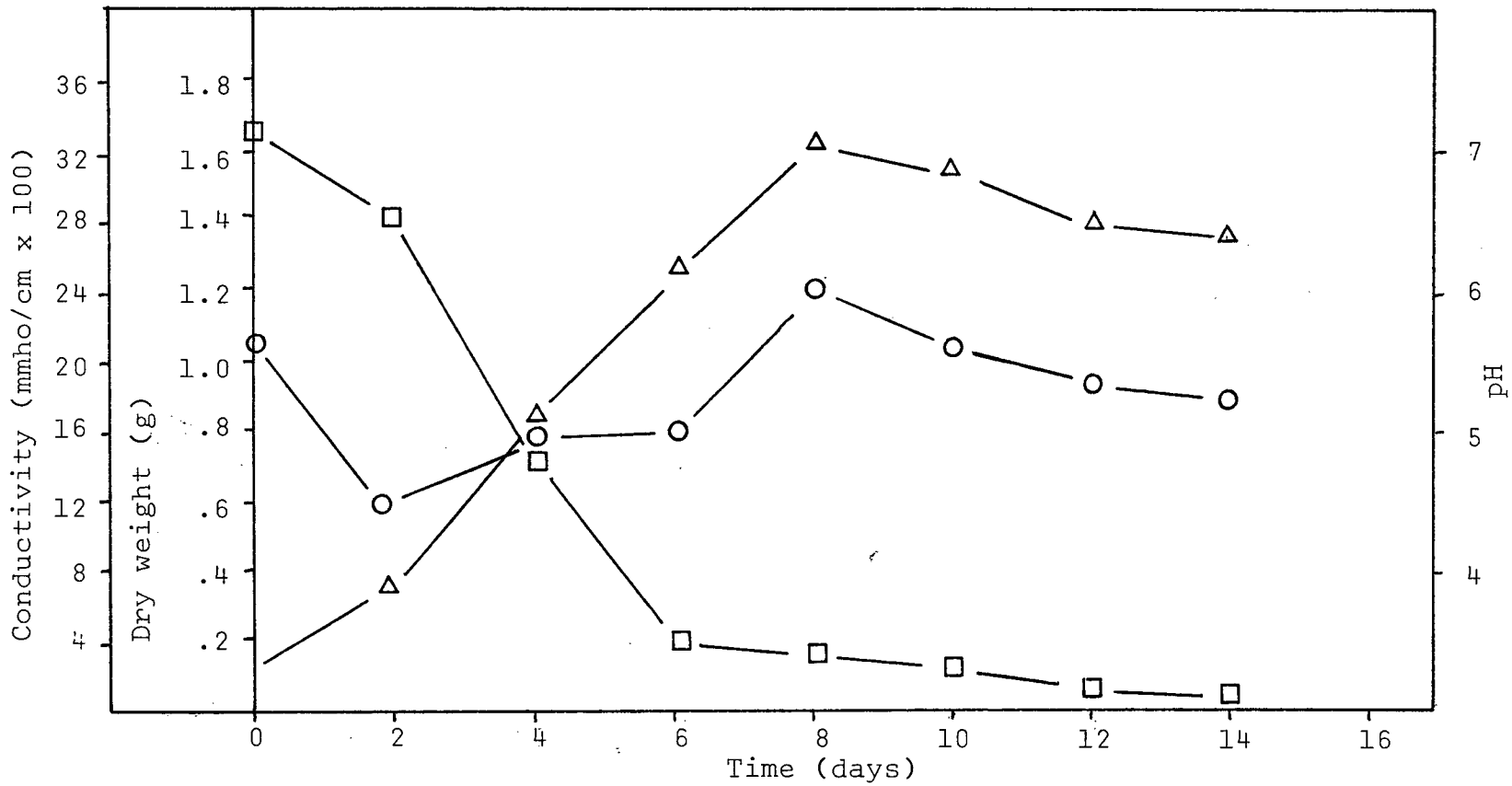

Figure 3. Growth curve of light grown coffee cultures

pH -○ Conductivity -□ Dry weight -△

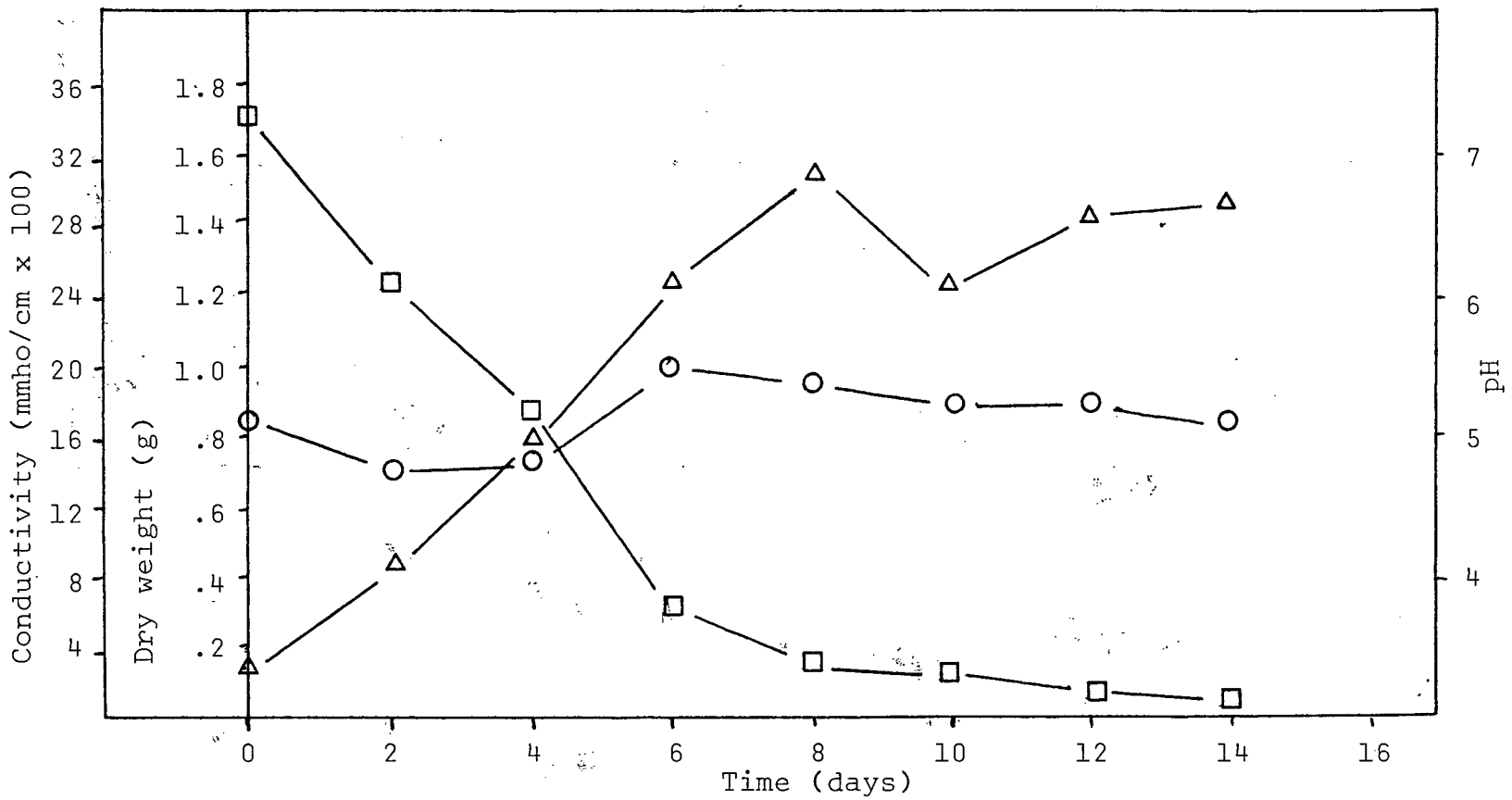

Figure 4. Growth curve of dark grown coffee cultures

pH -○ Conductivity -□ Dry weight -△

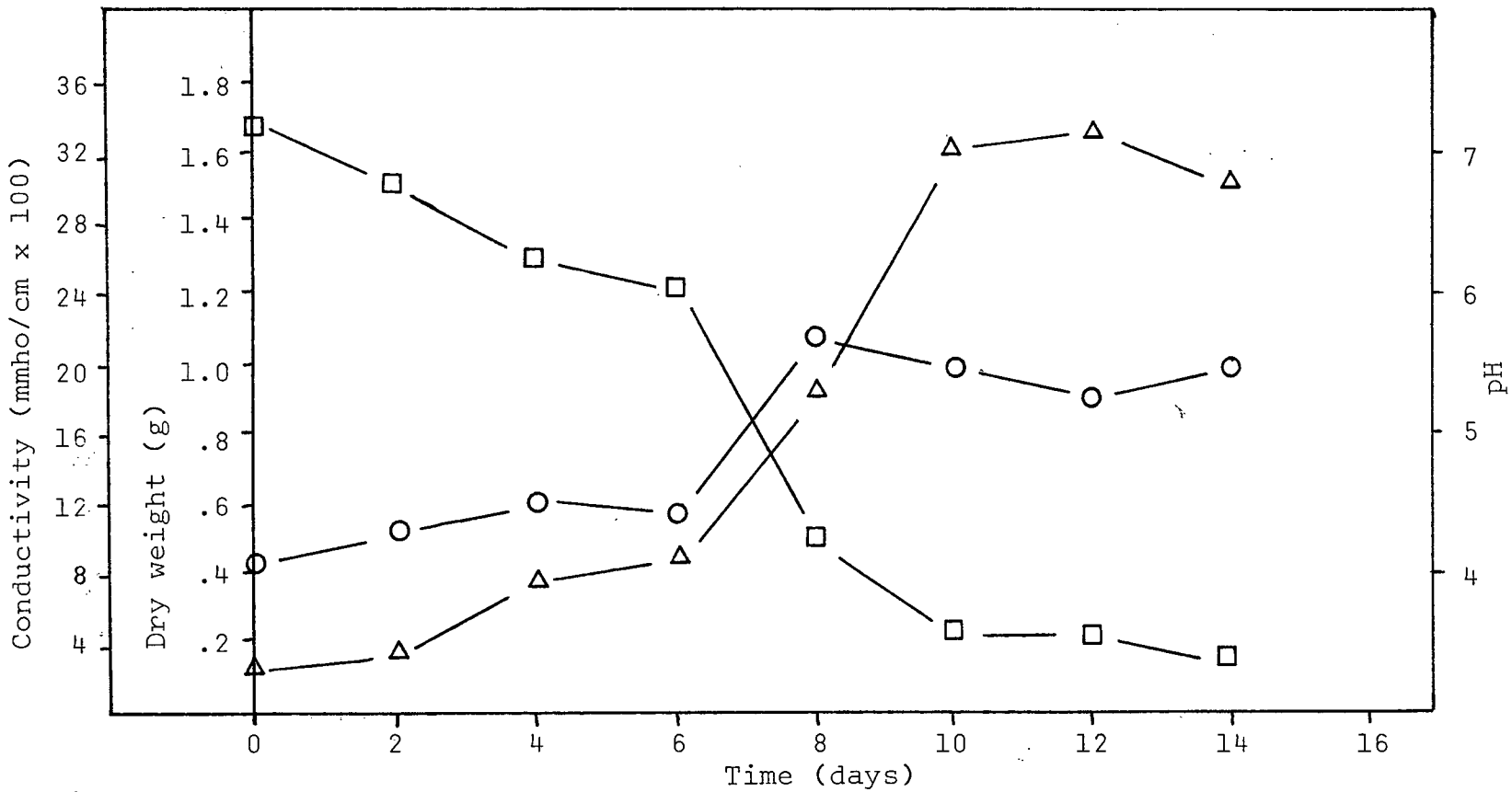

Figure 5. Growth curve of dark grown coffee cultures (low pH)

pH -○ Conductivity -□ Dry weight -△

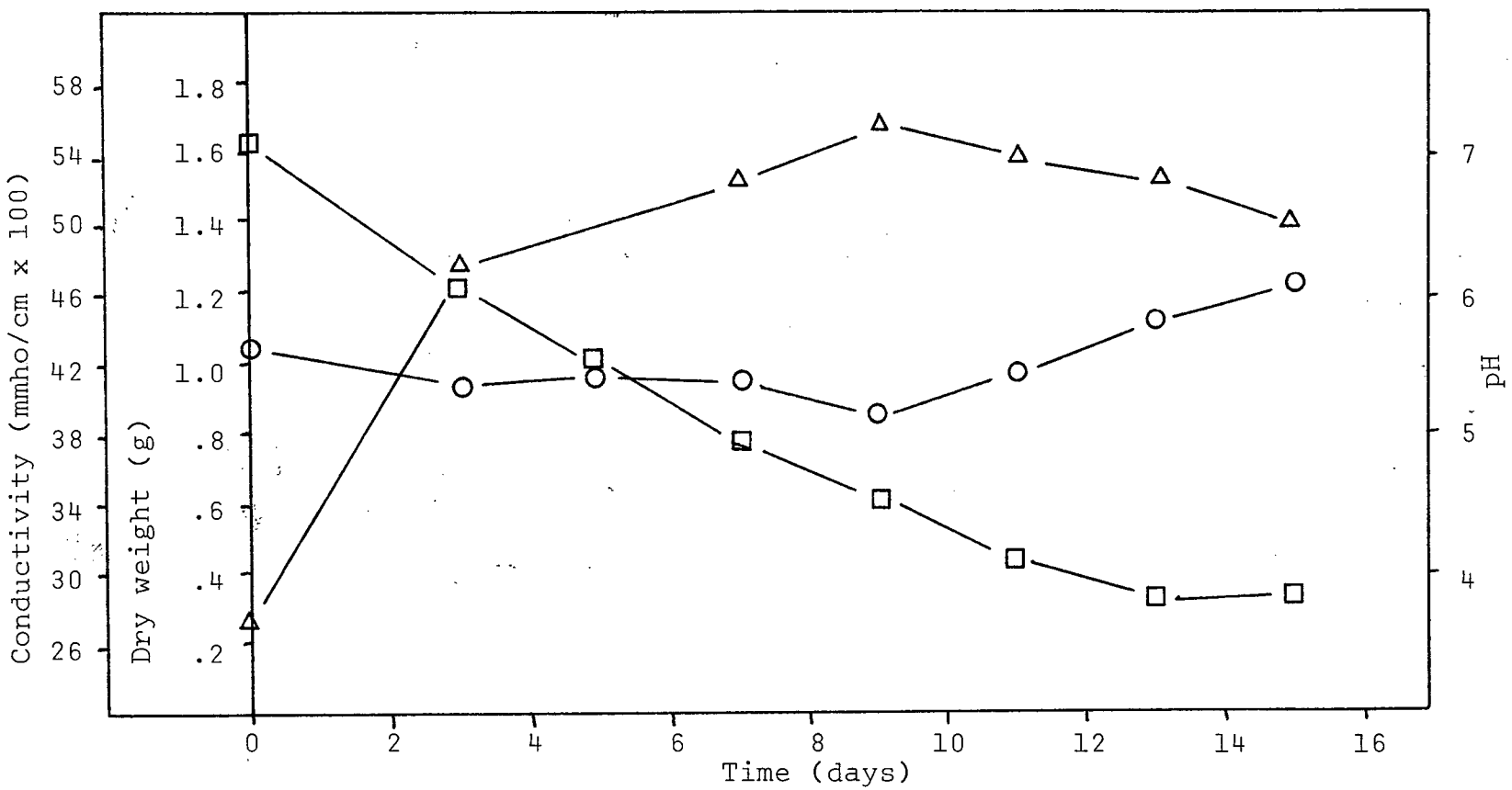

Figure 6. Growth curve of dark grown mint cultures

pH -○ Conductivity -□ Dry weight -△

C. Discussion

Although Buckland[6] grew coffee cells as a suspension culture in B-5 medium, the author's attempts to use that medium were unsuccessful. When PRL-4 and Medium #1 and #2 were employed, rate of growth and yield of cells were superior to Buckland's[6] results. All three media used produced similar results, indicating that the variations in composition did not influence growth of the culture. The fact that coffee cells grew continuously through successive transfers in Medium #2 indicated that 2,4-D was the major growth stimulator of the cultures, and that the other two hormones were superfluous. This postulate was verified when the culture was grown in the presence of indole-3-acetic acid and kinetin alone, or without any of the hormones. Under those conditions, growth ceased after the second transfer. The growth rate of both the coffee and mint cells was not affected by the presence of light. In both cultures the conductivity data indicated an inverse relationship between growth and conductivity, an observation noted by Hahlbrock et al.[18] in their study of parsley and soybean suspension cultures. A comparable rate of growth was observed in both cultures, even though the mint cells grew as granules rather than as "single cells", as in the case of coffee. The only major difference observed during the growth of coffee and mint cultures was the development of a strong green pigmentation in the mint cells when the culture was grown in the presence of light. Dobberstein and Staba[12] reported a similar pigmentation in Japanese mint suspension cultures grown in the presence of light.

II. The fatty acid composition and content of coffee and mint

A. The fatty acid composition and content of coffee tissues and coffee cell suspension cultures

After saponification and methylation of the coffee tissue lipid, the fatty acid composition of three tissues of the coffee plant (the bean, the stem and the leaf) was examined via gas chromatography in order to provide a comparative reference to the fatty acid composition of the coffee cell cultures.

The fatty acid compositions of coffee cell cultures grown in three different media (PRL-4, Medium #1 and #2) were investigated. Each of these cultures grown in different media was grown in both continuous light and in continuous dark, and analysed for fatty acid composition to see whether these two environmental conditions caused any changes in fatty acid constituents. Furthermore, the effect of culture incubation time on the fatty acid composition was studied by harvesting the cultures on day 7 (maximum cell yield) and on day 14 (declining cell yield).

The fatty acid contents of the tissues studied were calculated using Method A. The total weight of the fatty acids accounted for was derived from the standard curve.

i. Oil extraction from *Coffea arabica* tissues

Hexane extractions were performed on the coffee bean, the leaves and the stems, and on the coffee cell cultures grown in the three different media under the conditions of light or dark and harvested on day 7 or day 14. The oil

content of these various tissues is recorded in Table 5. The only difference which could be noted from the data was the decrease in oil content of the day 14 harvested cells. The data obtained for the oil contents of the cells grown in the three media and under conditions of light or dark was all very similar. In general, the oil content of the cells compared with the oil content of the leaf and stem of the coffee plant rather than the bean.

Table 5. Oil content of various Coffea arabica tissues

Coffee tissue	Percent oil extracted
Green bean	12.0
Leaf	3.0
Stem	1.9
Plant cell culture - day 7	2.4*
Plant cell culture - day 14	1.3*

*Average of separate analyses for cells grown in all three media under the conditions of light or dark

ii. The fatty acid composition of the green coffee bean, the leaf and the stem of the coffee plant

Gas chromatography of the methylated hexane extracts of the coffee bean, the leaves and the stems was performed to identify the component fatty acids, in order to compare their respective compositions to that of the plant cell cultures. The major fatty acids found to be present in the three coffee plant tissues are illustrated in Table 6. The fatty acid composition of the coffee bean was found to be palmitate (39%), stearate (6%), oleate (7%), linoleate (44%) and arachidate (3%), with traces of myristate and palmitoleate.

These results essentially followed the results obtained by Carisano *et al.*[7] in their study of the *Coffea arabica* coffee bean. The fatty acid composition of the leaf and stem tissues illustrated a generally similar fatty acid composition to the bean, with the notable exception of the presence of linolenate, which was absent from the bean, and the lack of arachidate, which was present in the bean.

Table 6. The fatty acid composition of the stems, leaves and beans of *Coffea arabica*

Component fatty acid	Stems	Leaves	Beans
≤C-14	--%	--%	Tr%
C-16	36	40	39
C-18	8	13	6
C-18:1	6	Tr	7
C-18:2	31	20	44
C-18:3	20	26	--
C-20	--	--	3

iii. The fatty acid content of the coffee bean, the leaf and the stem

Through the use of the standard curve (Method A), the weight of the fatty acids derived from the glycerides of the hexane extract, could be calculated. By considering the amount of oil methylated, an approximate percentage of the fatty acid content of the oil could be derived. On the basis of these calculations, the fatty acid contents of the coffee bean, the leaf and the stem of the coffee plant are recorded in Table 7. The fatty acid content of 70.0% (derived from glycerides) corresponds quite well with the value of 75.0% triglyceride reported in the literature[19] for the coffee

bean. The fatty acid content values obtained for the leaf and stem were much lower than those obtained for the coffee bean.

Table 7. The fatty acid content of the leaf, the stem and the bean of Coffea arabica

Tissue	Percent fatty acid
Green bean	70.0
Leaf	5.0
Stem	20.0

iv. The fatty acid composition of the coffee cell suspension cultures

Gas chromatography of the methylated hexane extracts of coffee cells grown in each of the three media, under the conditions of total light or total dark, revealed no differences in fatty acid composition. The relative fatty acid compositions of the coffee cell cultures grown in Medium #1, in light or dark, and harvested on day 7 or day 14 are presented in Table 8.

Table 8. The fatty acid composition of the coffee cell suspension cultures grown in Medium #1

Component fatty acid	Day 7 dark	Day 14 dark	Day 7 light	Day 14 light
≤C-14	--%	16%	--%	20%
C-16	42	36	39	30
C-18	8	5	6	4
C-18:1	Tr	2	Tr	3
C-18:2	33	22	33	20
C-18:3	15	18	22	22

The fatty acid composition of the culture grown in the dark and harvested on day 7, as illustrated in Table 8, was typical of all the coffee cell cultures, regardless of media composition. Variations in the relative percentages of individual fatty acids were present in each culture, but no consistent differences were observed. The only noticeable effect on the composition of the fatty acids of the cultures was an increase in short chain fatty acids (C-14 and less) in the cultures when they were harvested on day 14, as illustrated in Table 8. The fatty acid composition of the coffee cells essentially paralleled the fatty acid composition of the leaves and stems of the coffee plant, but differed from the coffee bean. The coffee cell cultures contained linolenate, which was not found in the coffee bean and lacked arachidate, which was present in the bean.

v. The fatty acid content of the coffee cell cultures

The fatty acid contents were determined for the cultures grown in the three different media, in the presence of light or in total darkness, and harvested on day 7 or day 14. The data obtained for the fatty acid contents of the cells grown in the different media and in the presence or absence of light was all very similar. However, a difference in the fatty acid content was observed between cultures harvested on day 7 and on day 14, as shown in Table 9. Although there was some variance in the amount of fatty acid present in each individual culture, a consistently higher amount of fatty acid was present in cultures harvested on

day 7. The results of the data indicated that a decrease in fatty acid levels occurred during the death phase of culture. Generally, fatty acid levels in the coffee cell cultures resembled the analytical values obtained for the stems of the coffee plant.

Table 9. The fatty acid content of Coffea arabica cell cultures

Day of Harvest	Percent Fatty Acid*
Day 7	30.0
Day 14	11.0

*Average of separate analyses for cells grown in all three media under conditions of light or dark

vi. Summary of fatty acid composition and content of coffee

The total quantity of oil found in the cell cultures was comparable to the oil content found in the leaves and stems of the coffee plant. Similarly, the component fatty acids of the coffee cell culture oil resembled the fatty acids found in the leaves and stems of the coffee plant. The coffee cell cultures and the leaves and stems of the coffee plant all contained linolenate, a fatty acid not found in the glycerides of the coffee bean. The fatty acid arachidate was found to be unique to the bean tissue. All other fatty acids found in the various tissues were common to all the tissues. No difference in the total fatty acid content or composition was observed between coffee cell cultures grown in the three media. Similarly, no changes in fatty acid content

or composition due to growth in the presence or absence of light were noted. The only apparent factor which affected both the fatty acid content and composition of the cultures was the time of harvest (or cell maturity). The appearance of short chain fatty acids and a decrease in the fatty acid level in the hexane extractable oil were noted in cells harvested in the dying phase of culture.

B. The fatty acid composition and content of mint tissues and mint cell suspension cultures

The fatty acid composition and content of the neutral lipids extracted from the mint seeds, plant material (foliage and stems) and mint cell cultures were investigated. Only two parameters, the effect of light and the time of harvest, were considered. The mint cell suspension cultures grown in Murashige and Skoog medium were compared to the parent seeds and plant material.

i. Oil extraction of Mentha tissues

The mint cell suspension cultures were extracted with hexane solvent, whereas the mint plant material and the mint seeds were extracted with petroleum ether:diethyl ether (50:50 v/v). The total extractable oil of the tissues is presented in Table 10. No difference was noted in the amount of oil extracted from the mint cells grown in the light or grown in the dark. Furthermore, no difference was observed in oil content between cells harvested on day 7 or day 15. The oil content found in the plant cell cultures was in the same range as that found in the mint plant foliage and stem material, contrasting sharply with the high oil content found

in the seed.

Table 10. Oil content of various Mentha tissues

Mint tissue	Percent oil extracted
Mint seed	19.0
Foliage and stem	1.2
Mint cell cultures	1.8*

*Average of separate analyses for cells grown in light or dark, and harvested on day 7 or day 14.

ii. The fatty acid composition of the mint seed and the mint plant tissues

Gas chromatography of the methylated neutral lipid extracts of the mint seed and mint plant material (foliage and stem) was performed in order to identify the component fatty acids of these mint tissues. Their respective compositions were compared to that of the plant cell culture. The major fatty acids found in the mint seed and mint plant tissues are shown in Table 11.

Table 11. The fatty acid composition of the mint seeds and mint foliage and stem tissues

Component fatty acid	Seed	Plant tissues (Foliage and Stem)
C-12	--%	10%
C-14	1	10
C-15	--	5
C-16	7	10
C-18	Tr	4
C-18:1	8	11
C-18:2	20	14
C-18:3	64	34

The fatty acid composition of the mint seed was found to be myristate (1%), palmitate (7%), stearate (Trace), oleate (8%), linoleate (20%) and linolenate (64%). Although both the seed and the foliage and stem tissues contained linolenate as the major fatty acid, a more complex fatty acid composition was apparent in the latter tissues. A major proportion of the total fatty acids found in the foliage and stem occurred as short chain fatty acids (C-16 and less).

iii. The fatty acid content of the mint seed and mint foliage and stem tissues

The fatty acid content of the mint seed and tissues was calculated using Method B, and the results are as shown in Table 12. The results indicated that a much lower content of fatty acid was present in the plant tissues than in the seed.

Table 12. The fatty acid content of the mint seeds and mint foliage and stem tissues

Tissue	Percent fatty acid
Seed	70.0
Foliage and stem	23.0

iv. The fatty acid composition of the mint cell suspension cultures

Gas chromatography of the methylated hexane extracts of the mint cells grown in light or in dark, and harvested on day 7 or day 15, revealed no changes in the constituent fatty acids. The relative fatty acid composition of the mint cell cultures is presented in Table 13.

Table 13. The fatty acid composition of mint cell cultures

Component fatty acid	Day 7 dark	Day 7 light	Day 15 dark	Day 15 light
C-16	34%	22%	23%	25%
C-18	4	2	2	1
C-18:1	7	5	6	3
C-18:2	19	17	18	15
C-18:3	34	51	49	54

The fatty acid composition of the mint plant cell cultures closely resembled the composition of the mint seed, rather than the foliage and stem. The foliage and stem tissues appeared to have a more complex fatty acid composition.

v. The fatty acid content of mint cell suspension cultures

The fatty acid content of the mint cell cultures, calculated via Method B, is presented in Table 14.

Table 14. The fatty acid content of mint cell cultures

Day of harvest and exposure to light	Percent fatty acid (w/w) %
Day 7 light	40.0
Day 7 dark	10.0
Day 15 light	25.0
Day 15 dark	5.0

The values presented in Table 14 illustrate a noticeable difference in the fatty acid content of the mint cell cultures grown in the presence of light, as compared to similar tissue grown in the dark. In both cases, a decrease in the fatty acid content is apparent when the cells were harvested on day 15.

vi. Summary of the fatty acid composition and content of the mint plant and tissue cultures

The oil content of the mint cell cultures was comparable in quantity to the content found in the mint foliage and stem. The mint cell cultures had a similar fatty acid composition to the mint seed. The mint foliage and stem differed from the mint cell cultures and mint seed by the presence of a substantial ammount of fatty acids which were of chain length equivalent to C-16 or less. The parameters of absence or presence of light and harvest time did not affect the oil content of the mint cell cultures. However, growth of the cultures in the light resulted in higher quantities of fatty acid in the cell culture oil. Exposure to light did not affect the quantity of fatty acid produced in the coffee cell cultures.

C. Discussion

The experimental studies have shown that the amount of neutral lipid found in both the mint and coffee cell suspension cultures was generally comparable in amount to that found in the parent plant leaf and stem tissues, rather than to that found in the seeds. The average amount of lipid recovered from the coffee cells (2.4%) and from the mint cells (1.8%) falls within the range reported in the literature(53,49,41,30,10) for various plant cell cultures. The fatty acid composition of the coffee cells resembled the composition of the coffee leaves and stems rather than that of the bean. The presence of linolenic acid and the absence of arachidic acid in the cells, leaves and stems distinguished

these tissues from the bean. The fatty acid composition of the mint cell cultures was found to resemble the mint seed rather than the foliage and stems. However, most investigators have stated that although minor differences in fatty acid composition may exist, the composition of plant cell cultures tends to resemble the composition characteristic to the parent plant. In this study a similar conclusion concerning fatty acid composition can be drawn for coffee and mint cell cultures.

Growth of the coffee cells in three different media did not affect the fatty acid composition of the cell. Therefore, it would appear that the fatty acid composition of the cultures was not a function of the ingredients present in the growth media. Both coffee and mint cell cultures were grown in the presence of light, and in both cases light did not affect the fatty acid composition.

In general, the fatty acid content of the plant cell cultures was found to be very low, an observation which has been reported by a number of other investigators(10,30,41,49,53). The fatty acid content of both coffee and mint cell cultures was shown to decrease in the later stages of cell culture (day 14 and 15). This decrease was probably due to the utilization of the glycerides as an energy source after the sugars of the medium had been exhausted. Light did not affect the fatty acid content found in the coffee cells. However, the culture of the mint cells in the presence of light resulted in an increase in the amount of fatty acid. A

similar phenomenon was reported by Davydova[10] who noted that flax tissue cultures grown in the presence of light contained larger amounts of fatty acid than the same cultures grown in the dark. This increase in fatty acid content was associated with the development of green pigmentation in the cultures. Similarly, in the case of the mint cell cultures, a strong green pigmentation developed which was lacking in the coffee cell cultures. Therefore, it would appear that the increase in fatty acid content of the cell cultures was related in some manner to the production of chlorophyll or the activation of chlorophyll synthesis. Analyses of the green pigment in the mint cell cultures exposed to light has been done by Plear (personal communication) using thin layer chromatography. His analyses revealed the presence of chlorophyll a and b in these cultures but not in similar cultures grown in the dark.

III. The sterol composition of coffee beans, mint seeds and coffee and mint cell cultures

The sterols present in the unsaponifiable fraction of the extracted lipid of the seeds and cell cultures of coffee and mint were analysed via the methods of thin layer chromatography and gas chromatography.

A. Thin layer chromatography (TLC)

All thin layer plates were developed using a chloroform:ethyl acetate mixture as the solvent. This solvent allowed the separation of the slightly polar sterols, in the order of their methyl substitution[25,42], from the

non-polar triglycerides and sterol esters. The separated compounds were indicated by exposure to iodine vapour. The pertinent data is reproduced to scale in Figures 7-12.

i. TLC of selected sterol standards

Three desmethyl sterols (cholesterol, stigmasterol and β-sitosterol) and one dimethyl sterol (lanosterol) were chosen as standards. The separation obtained for these sterols is presented in Figure 7. Although the desmethyl sterols could not be separated from each other, lanosterol, the dimethyl sterol, separated readily. A mixture of all four sterols did not interfere with the separation of the dimethyl sterol from the desmethyl sterols. The Rf values for the dimethyl sterol and the desmethyl sterols were 0.47 and 0.36 respectively.

It is known that the lipid fraction from plant tissues contains triglycerides and esterified sterols[22,45]. In order to characterize these lipid constituents, triolein and cholesterol acetate were run as representative compounds. The resulting thin layer analysis of these compounds is shown in Figure 8, samples 3 and 4. Both these compounds ran to the upper part of the thin layer plate and had essentially the same Rf values, i.e. 0.85.

ii. TLC of coffee bean oil and coffee cell culture oil

(a) Non-saponified oil analysis

Ten milligrams of coffee bean oil and coffee cell oil were dissolved separately in 1 ml of petroleum ether. Aliquots of 20 µl of the solution were spotted on a thin layer

plate for development. The resulting chromatograms are presented in Figure 8, samples 1 and 2. Both oils indicated substantial amounts of iodine positive material in the triglyceride:sterol ester region, Rf 0.85. In addition, the plant cell oil produced two spots in the sterol region, (Rf 0.44 and 0.35), which were absent in the bean oil sample at the concentration tested. Furthermore, the bean oil showed the presence of a spot not associated with the sterol region or the triglyceride:sterol ester region (Rf 0.61), which was absent from the cell oil at the concentration tested.

(b) Saponified oil analysis

A comparative analysis of coffee bean oil, before and after saponification, was performed using TLC. A desmethyl and a dimethyl standard were run along side the saponified and non-saponified coffee oils, in order to note possible changes in Rf values due to changes in solvent composition. The resulting thin layer chromatogram is presented in Figure 9. Saponification of the coffee bean oil resulted in the disappearance of spots 'a' and 'b' (sample 2) and the appearance of three spots as shown in sample 3, all of which have lower Rf values than the iodine positive material present in the non-saponified coffee bean oil. Two of the spots in the saponified sample have mobility similar to the sterols. One spot compares favorably with the desmethyl sterols. The other iodine positive spot is located between the desmethyl and dimethyl sterol standards. A third

spot, with an Rf of 0.03 (spot 'a') remains close to the origin.

A thin layer analysis was made of coffee cell oil and saponified coffee cell oil (Figure 10). The saponified cell oil produced similar iodine positive compounds as the saponified coffee bean oil. The two spots tentatively labelled as sterols in the unsaponified cell oil were intensified in the saponified cell oil chromatogram. A third spot (sample 3, spot 'a'), similar in Rf to that of the slow running component (Figure 9, sample 3, spot 'a'), which was present in saponified coffee bean oil, also occurred in saponified cell oil.

iii. TLC of mint seed oil and mint cell culture oil

(a) Non-saponified oil analysis

Ten milligrams of mint seed oil and 10 mg of mint cell oil were each dissolved in 1 ml of petroleum ether. Twenty μl of each sample were spotted on a thin layer plate and developed side by side with the sterol standards and with the coffee bean sample (Figure 11). The mint seed oil showed the presence of only one iodine positive area which was in the triglyceride:sterol ester region. The mint cell oil also contained this material, along with two spots running in the sterol region. Neither of the mint oils contained a component similar to the compound(s) which produced spot 'a' found in the coffee bean oil.

(b) Saponified oil analysis

The chromatograms of the saponified mint oils are shown in Figure 12. The saponified mint cell oil chromatogram

shows the presence of two sterol spots similar to those found in the unsaponified mint cell oil, one spot concurrent to the desmethyl sterol standard, and the other between the di and desmethyl standards. The saponified seed oil produced one large spot which almost encompasses the whole sterol region. No spots are present in the lower regions of the thin layer plate, such as are found in the saponified coffee bean oil.

iv. Summary of thin layer analysis

The non-saponified oil chromatograms of both the coffee and mint cell cultures indicated the presence of free sterol components, in detectable amounts, which were not located in the corresponding seed oil chromatograms. After saponification of the seed and cell culture oils, the resulting chromatograms indicated the presence of two sterol spots in all samples, with the exception of the oil of the mint seed. One of the sterols had an Rf value similar to the desmethyl sterols, and the other had an Rf value between the di and desmethyl sterol regions. The mint seed produced only one spot which encompassed both sterol regions. The coffee bean oil chromatogram showed a component between the sterol and triglyceride:sterol ester regions which was absent from the coffee cell oil at the concentration tested. This component was detectable in the saponified coffee cell oil when higher concentrations were analysed on a thin layer plate.

B. Gas chromatography (GLC)

GLC of the sterols was performed on a 5% OV-1 column. The sterols were derivatized to form their respective

trimethylsilyl ethers and injected into the gas chromatograph. A carrier nitrogen flow rate of 32.1 ml/min and a chart speed of 0.1 cm/min were used. The column temperature was kept at 240° C for 1.5 hours, to separate the more volatile fraction, and then increased to 280° C.

i. GLC of selected sterol standards

The four sterol standards, cholesterol, stigmasterol, campesterol and β-sitosterol, were derivatized separately in 1 ml of reactants, and 10 µl of each solution were injected into the gas chromatograph, which was set at an attenuation of x1000. The corresponding retention times of the sterol standards are recorded in Table 15. A mixture of the four sterol standards injected into the gas chromatograph did not result in any alteration of retention times.

Table 15. Retention times of sterol standards

Sterol	Retention time (minutes)
Cholesterol	109
Campesterol	123
Stigmasterol	126
β-Sitosterol	135

ii. GLC of the total unsaponifiables of coffee bean oil and cell culture oil

(a) Coffee bean oil unsaponifiables

Ten mg of coffee bean unsaponifiables were silylated in 1 ml reagent for five minutes, centrifuged, and the solvent volume reduced to 0.5 ml using a stream of dry nitrogen gas. Twenty µl of the reactant mixture were

injected into the gas chromatograph which was programmed in the manner described previously. The resulting chromatogram is presented in Figure 13. Two major peaks appeared in the non-steroidal region (prior to P) and are identified as peaks 'a' and 'b'. Eight peaks appeared in the steroidal region (after P) of the chromatogram. Three of these peaks, 'f', 'g' and 'h' were tentatively identified as campesterol, stigmasterol and β-sitosterol respectively, since they had retention times identical to the corresponding standards. The remaining peaks were not identified.

(b) Coffee cell culture oil unsaponifiables

In the same manner described for coffee bean oil unsaponifiables, 10 mg of coffee cell oil were silylated, and 10 µl were injected into the gas chromatograph. The resulting chromatogram, Figure 14, was quite similar to that of the coffee bean oil unsaponifiables. Two peaks, 'a' and 'b', which appeared prior to the steroidal region, had identical retention times to the peaks 'a' and 'b' found in the coffee oil unsaponifiables. Peaks 'c', 'd', 'e', 'f' and 'g' corresponded in retention times and relative ratios to the peaks 'f', 'g', 'h', 'i' and 'j' found in the coffee bean oil unsaponifiables.

C. Isolation and identification of a cafestol and kawheol mixture

i. Column chromatography

Hydrocarbons and sterols were eluted from a 100 mg sample of coffee bean unsaponifiables adsorbed on a 6 g alumina column, by passing petroleum ether followed by a

mixture of petroleum ether:diethyl ether (60:40 v/v) through the column. The diterpenoid alcohols, cafestol and kawheol, were eluted with chloroform:methanol (99:1 v/v), and the eluent was collected.

ii. TLC of the chloroform:methanol eluent

A sample of the eluent was spotted on a thin layer plate and developed using the standard solvent system. The resulting plate, when exposed to iodine vapour, revealed a single spot with an Rf value of 0.03, identical to spot 'a' (Figure 9, sample 3) produced by the development of the coffee bean unsaponifiables.

iii. Ultraviolet spectroscopy of the crystallized mixture suspected to be cafestol and kawheol

Upon removal of the chloroform:methanol solvent by rotoevaporation, the residual material was refluxed in petroleum ether and crystallized from the solvent. The crystals were dissolved in diethyl ether and the absorption maxima of the solution was determined on an ultraviolet spectrophotometer. Maximum absorption occurred at 2220 Å, identical to the value for cafestol[44].

iv. GLC of the crystallized mixture suspected to be cafestol and kawheol

The crystallized material was silylated in the usual manner and injected into the gas chromatograph, producing two peaks, ('a' and 'b' Figure 15) with the same retention times as peaks 'a' and 'b' present in the coffee bean unsaponifiables (Figure 13).

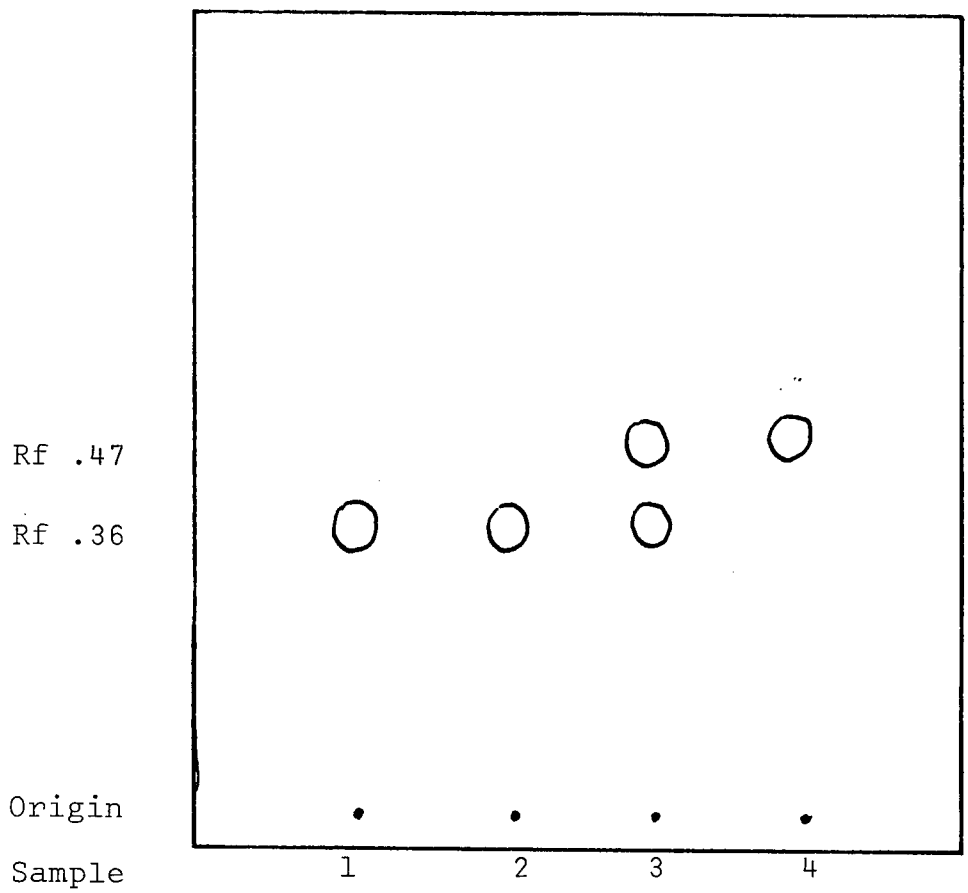

Figure 7. TLC of selected sterol standards

Sample: 1- Cholesterol

2- Cholesterol, stigmasterol and β-sitosterol

3- Cholesterol, stigmasterol, β-sitosterol and lanosterol

4- Lanosterol

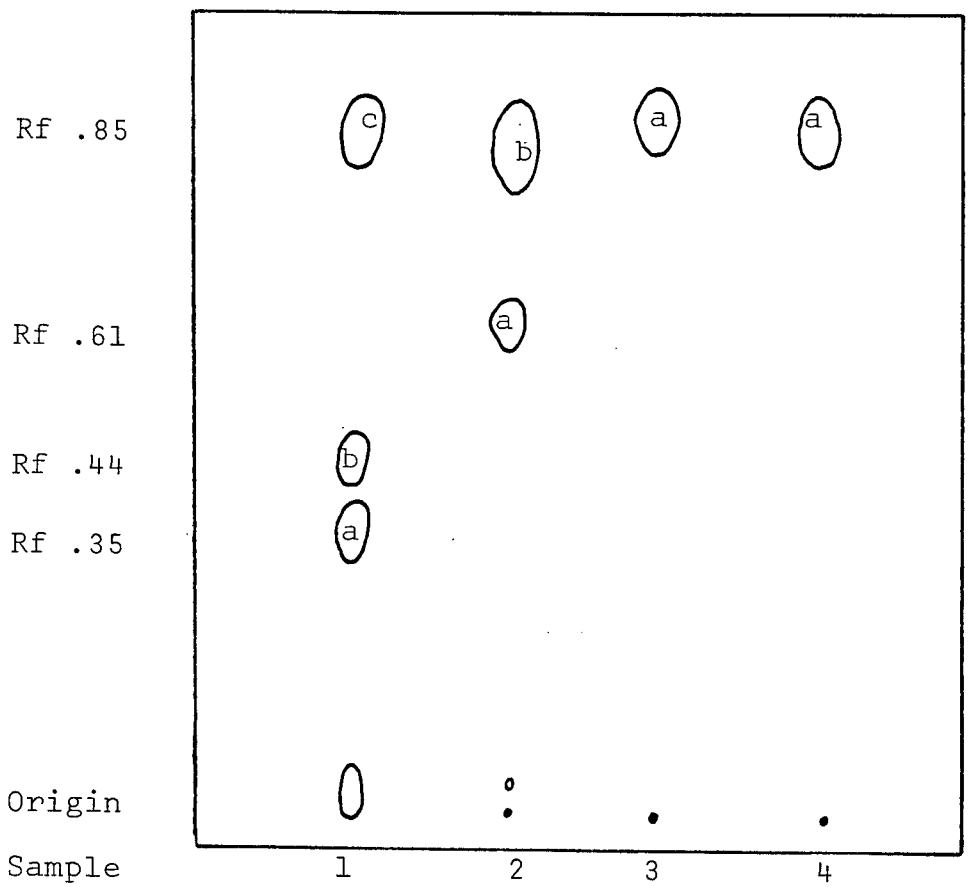

Figure 8. TLC of two standards and of samples of coffee bean oil and coffee cell culture oil

Sample: 1- Coffee cell culture oil

2- Coffee bean oil

3- Triolein

4- Cholesterol acetate

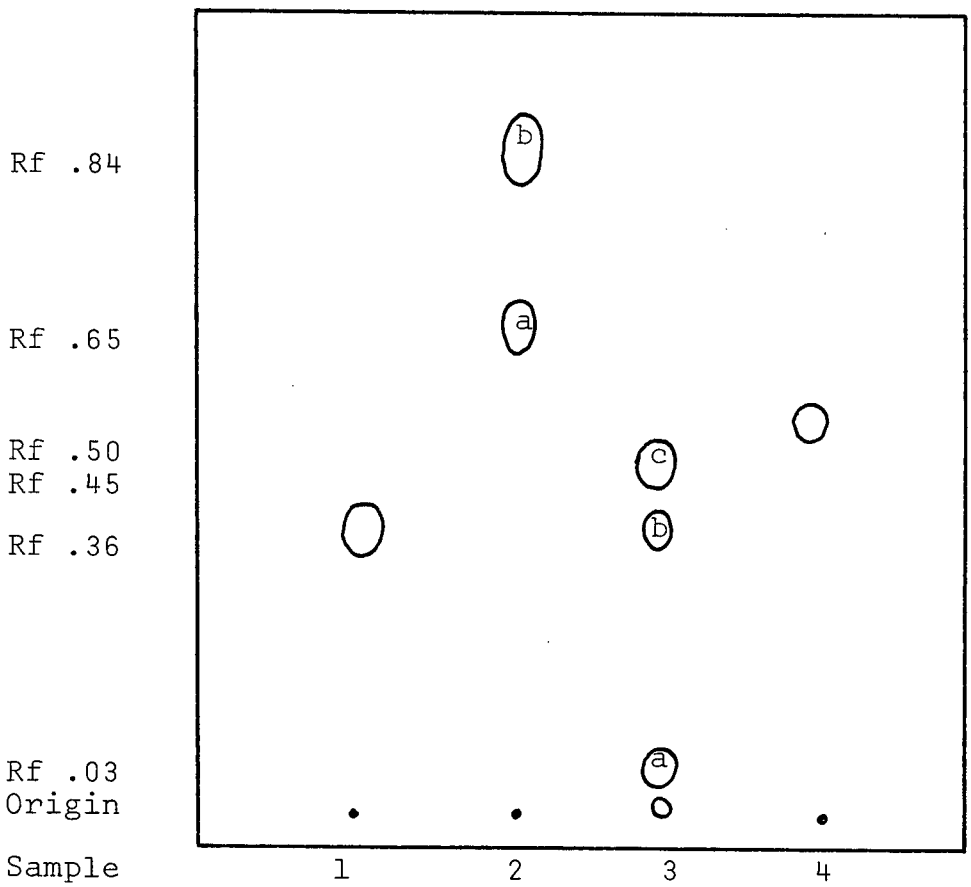

Figure 9. TLC comparison of saponified and non-saponified coffee bean oil

Sample: 1- Cholesterol

2- Coffee bean oil

3- Saponified coffee bean oil (ether solubles)

4- Lanosterol

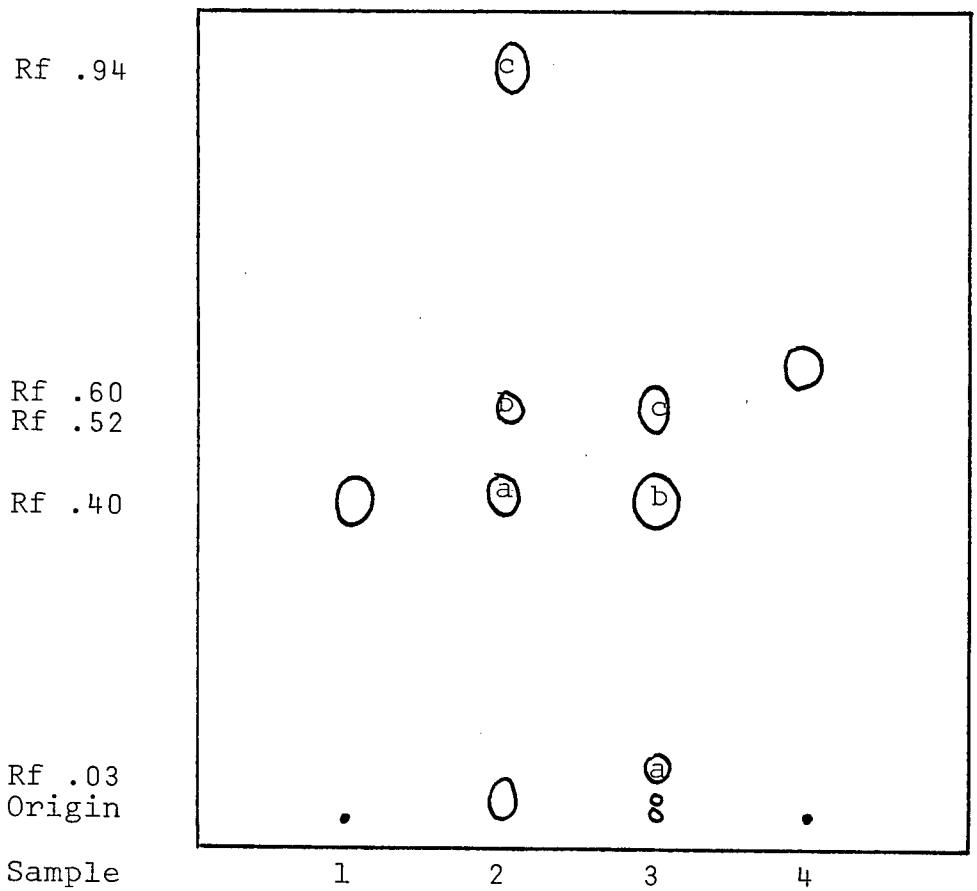

Figure 10. TLC comparison of saponified and non saponified coffee cell culture oil

Sample: 1- Cholesterol

2- Coffee cell culture oil

3- Saponified coffee culture oil (ether solubles)

4- Lanosterol

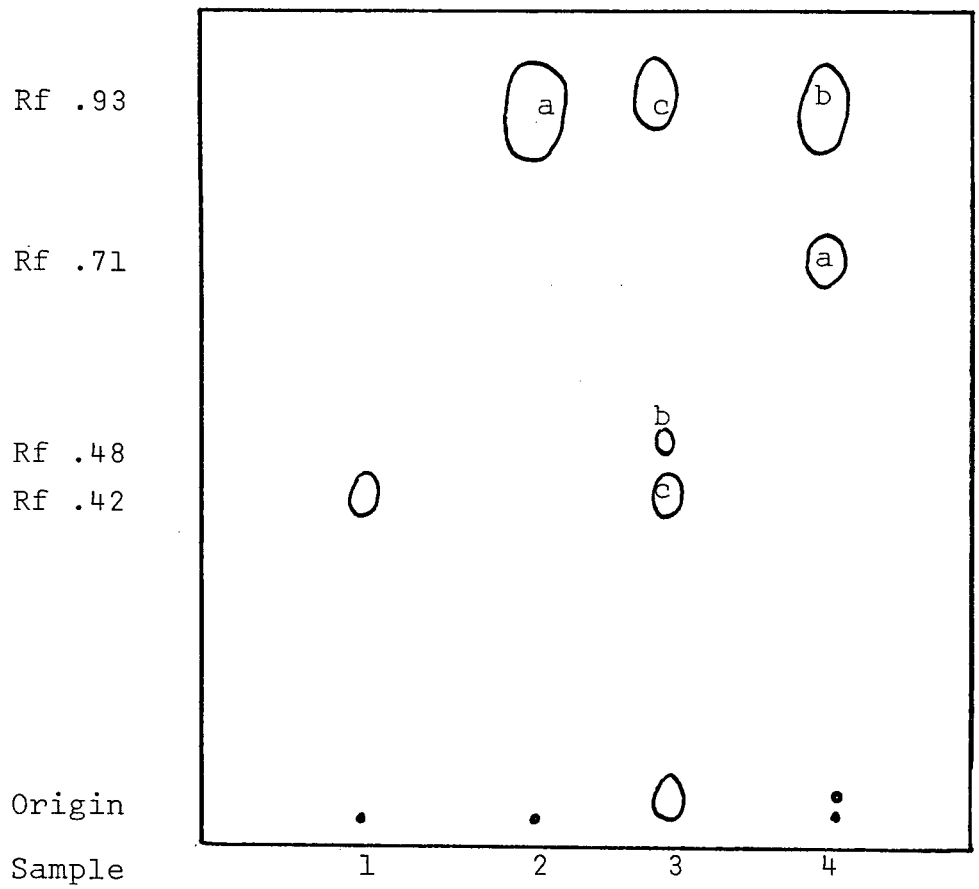

Figure 11. TLC comparison of mint seed oil and mint cell culture oil

Sample: 1- Cholesterol

2- Mint seed oil

3- Mint cell culture oil

4- Coffee bean oil

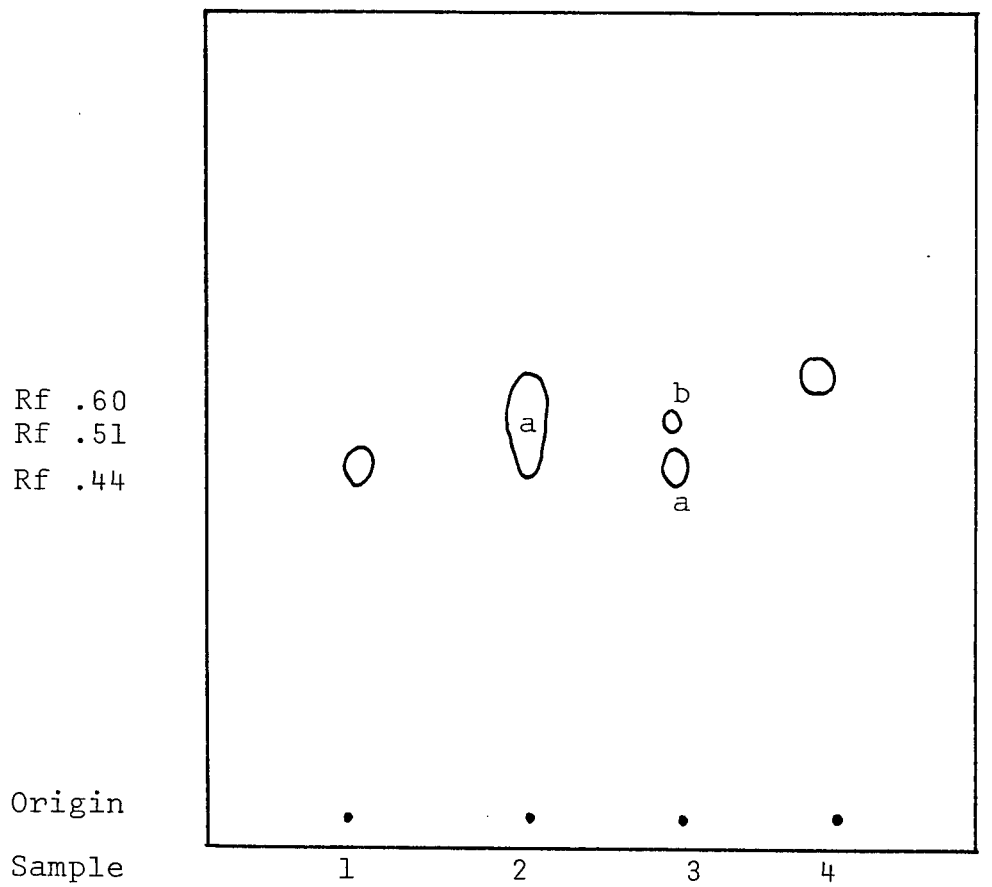

Figure 12. TLC of saponified mint seed and mint cell culture oil

Sample: 1- Cholesterol

2- Saponified mint seed oil (ether solubles)

3- Saponified mint cell culture oil (ether solubles)

4- Lanosterol

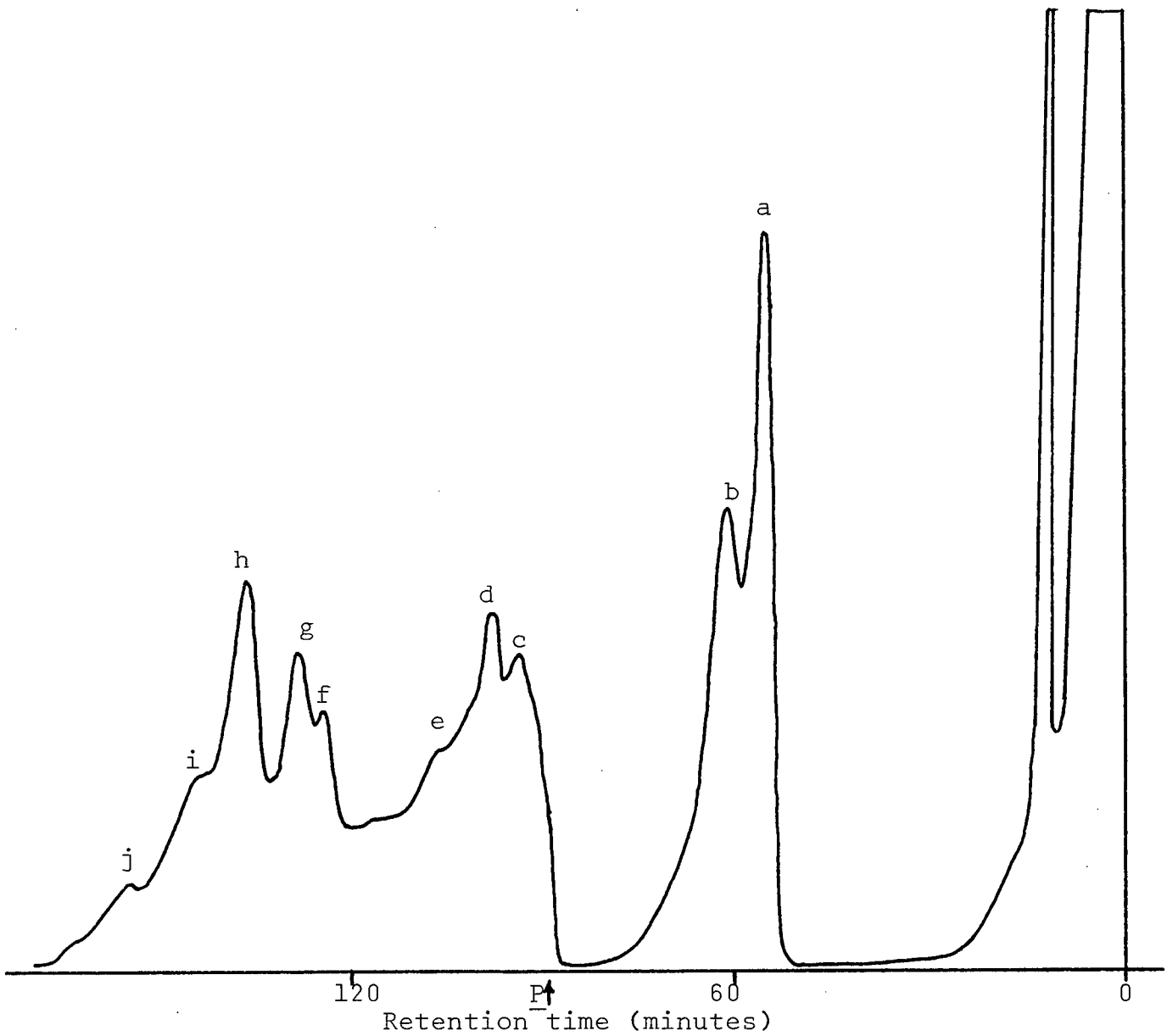

Figure 13. Gas chromatogram of the coffee bean unsaponifiables

Individual peak retention times (minutes)

(a) - 56	(c) - 93	(e) - 106	(g) - 126	(i) - 143
(b) - 61	(d) - 98	(f) - 123	(h) - 135	(j) - 154

Attenuation x2000 until program temperature change (P), then increase sensitivity to x100

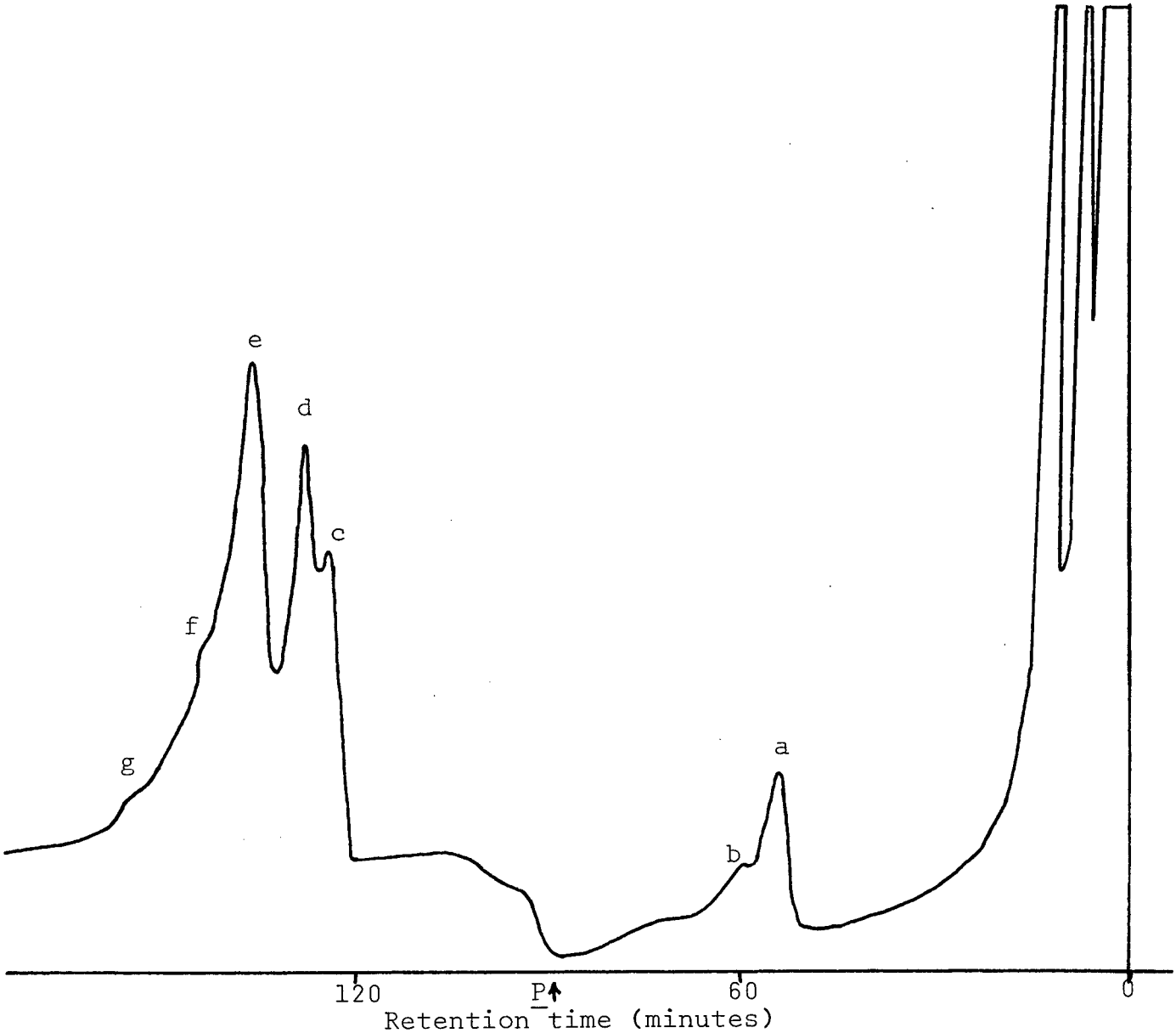

Figure 14. Gas chromatogram of coffee cell culture unsaponifiables

Individual peak retention times (minutes)

(a) - 56	(c) - 123	(e) - 135	(g) - 154
(b) - 61	(d) - 126	(f) - 143	

Attenuation x500

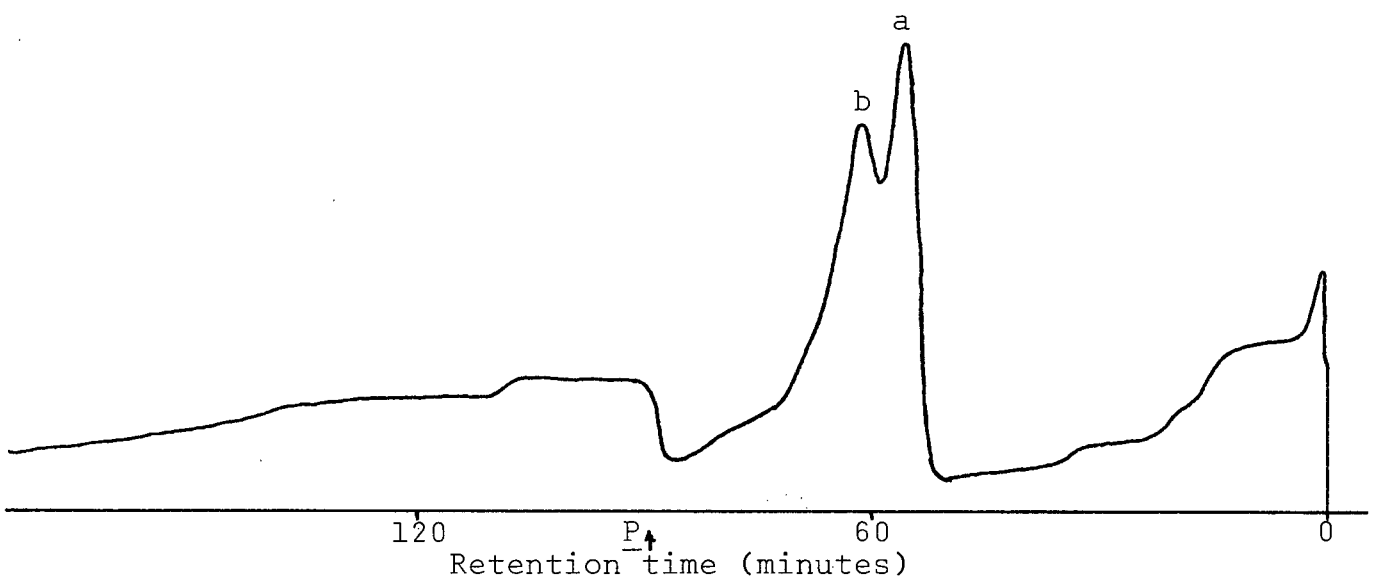

Figure 15. Gas chromatogram of the crystallized mixture of cafestol and kawheol isolated from saponified coffee bean oil

Individual peak retention times (minutes)

(a) - 56
(b) - 61

Attenuation x1000

Cafestol and kawheol are known from the literature to be the major constituents of the unsaponifiables of the coffee bean oil. In this study, gas chromatography of the unsaponifiables of coffee bean oil indicated the presence of two major components which appeared prior to the sterol region on the gas chromatogram. Thin layer chromatography also indicated the presence of a component not associated with the sterol constituents. The procedure followed for the isolation of cafestol and kawheol was essentially the same as that used by Nagasampagi et al(35). The isolation procedure produced a crystalline material which had an absorption maxima at 2220 Å, the characteristic absorption of cafestol. GLC of the crystalline material produced two peaks with retention times matching the two component peaks 'a' and 'b' of the coffee bean unsaponifiables. From this data it was concluded that the material producing peaks 'a' and 'b' was most probably cafestol and kawheol.

D. Gas chromatography of the individual iodine positive spots isolated from the preparative thin layer plates of the coffee bean and the coffee cell oil unsaponifiables

Preparative thin layer plates were developed for all the saponified oil samples. The separated constituents along with the silica gel were scraped off the plates and were eluted from the gel with an appropriate solvent. The solvent was removed via a stream of nitrogen gas, and the residual material silylated in the standard manner. In some cases pyridine was removed after silylation and replaced with

iso-octane or carbon disulfide, in order to reduce the solvent peak produced on the gas chromatogram.

i. GLC of the suspected diterpenoid alcohol spot

GLC of the suspected diterpenoid alcohol spot (Rf 0.03), present in thin layer chromatograms of coffee bean and coffee cell oil unsaponifiables, resulted in the chromatograms shown in Figures 16 and 17. As was expected, only two peaks were present in the chromatograms, and they were the peaks previously identified as cafestol and kawheol. The only difference between the two chromatograms is the ratio of the two components.

ii. GLC of the sterol spot having the same Rf value as the desmethyl standards

Figures 18 and 19 illustrate the chromatograms produced by GLC of the sterol spot present on bean and cell thin layers. The cell sterol spot showed three components, 'a', 'b' and 'c', with retention times identical to those of campesterol, stigmasterol and β-sitosterol. The bean sterol spot contained the same sterols as the cell sterol spot, plus an additional two unknowns. In both cases, the relative ratios of the concentrations of the identified sterols were the same.

iii. GLC of the sterol spot which is located between the di and desmethyl standards

The resulting chromatograms for the bean and cell are given in Figures 20 and 21. In the case of the bean sterols, two major components were dominant. In contrast, a more even distribution of components was apparent in the

cell sterols. None of the peaks were identified, but are presented for comparative purposes, to show the differences in composition between the bean and cell sterols which appeared on the thin layer plate at the same Rf value.

E. GLC of the total unsaponifiables of mint seed oil and mint cell culture oil

i. Mint seed unsaponifiables

In the usual manner 12.3 mg of mint seed unsaponifiables were derivatized, the solvent was reduced to 0.5 ml and 20 μl were injected into the gas chromatograph. The resulting chromatogram is presented in Figure 22. Five minor peaks were produced ('a','b','c','d' and 'e'), plus one major peak ('f') which had a retention time corresponding to β-sitosterol.

ii. Mint cell unsaponifiables

After derivatization of 11.4 mg of the mint cell unsaponifiables, the solvent was reduced to 0.5 ml, and 25 μl were injected into the gas chromatograph. The resulting chromatogram, presented in Figure 23, shows only two peaks, a minor unidentified peak and a major peak with a retention time corresponding to β-sitosterol.

F. GLC of individual iodine positive spots isolated from preparative thin layer plates of saponified mint seed and mint cell oil

i. GLC of the mint cell sterol spot having the same Rf value as the desmethyl standards

The resulting chromatogram (not presented) showed only one peak with a retention time identical to the β-sitosterol standard.

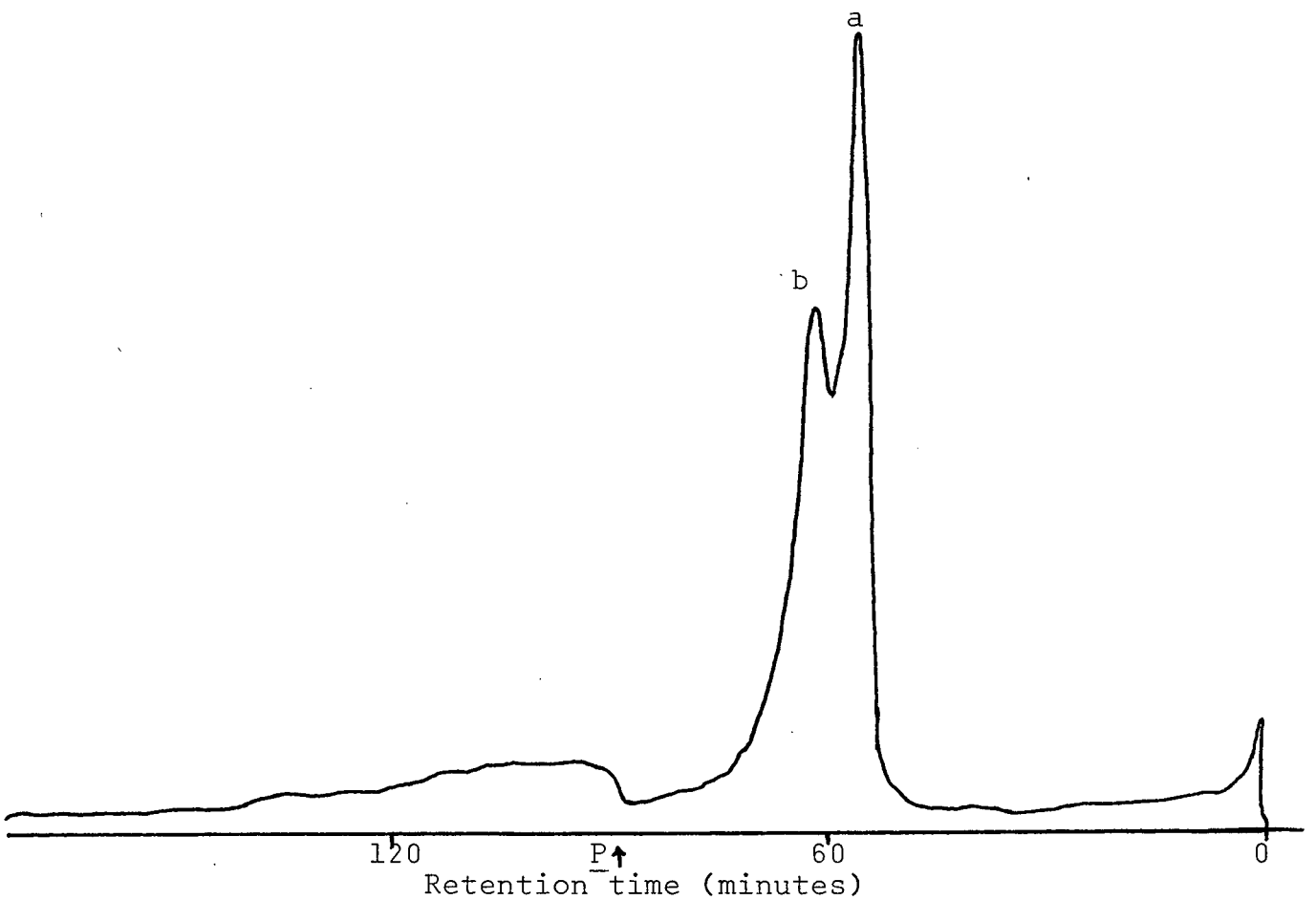

Figure 16. Gas chromatogram of cafestol and kawheol isolated by preparative TLC from the coffee bean

Individual peak retention times (minutes)

(a) - 56
(b) - 61

Attenuation x1000

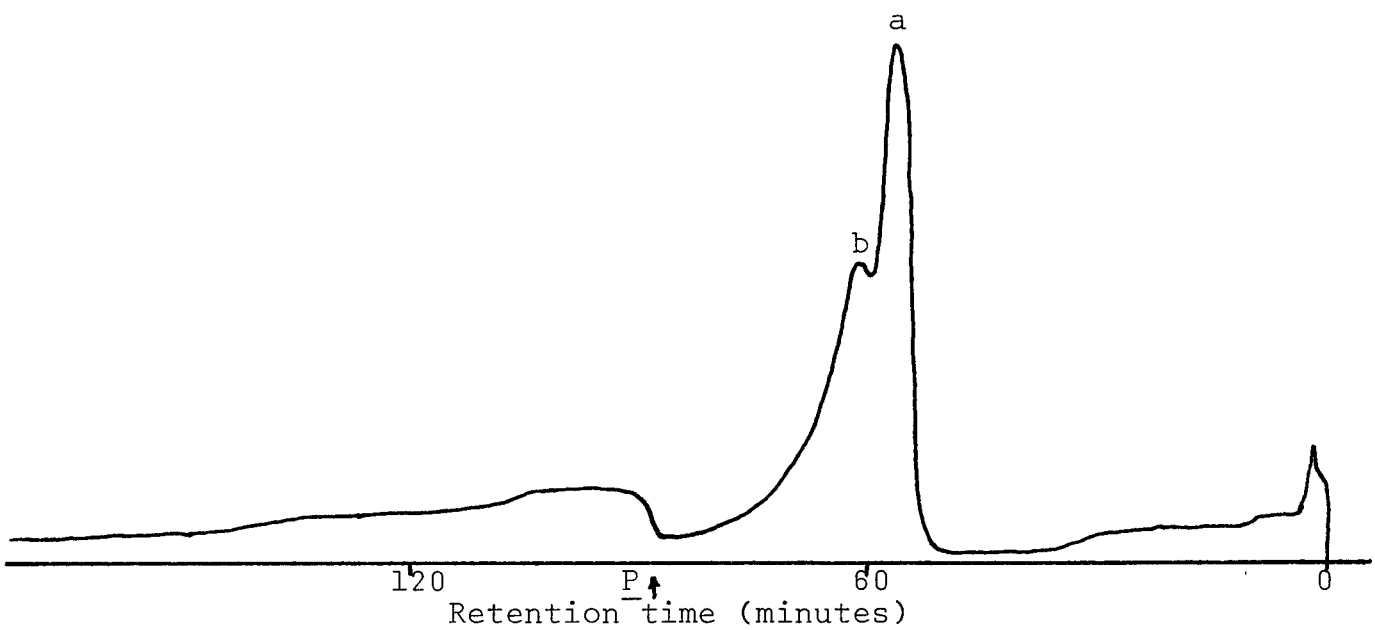

Figure 17. Gas chromatogram of cafestol and kawheol isolated by preparative TLC from the coffee cell culture

Individual peak retention times (minutes)

(a) - 56
(b) - 61

Attenuation x1000

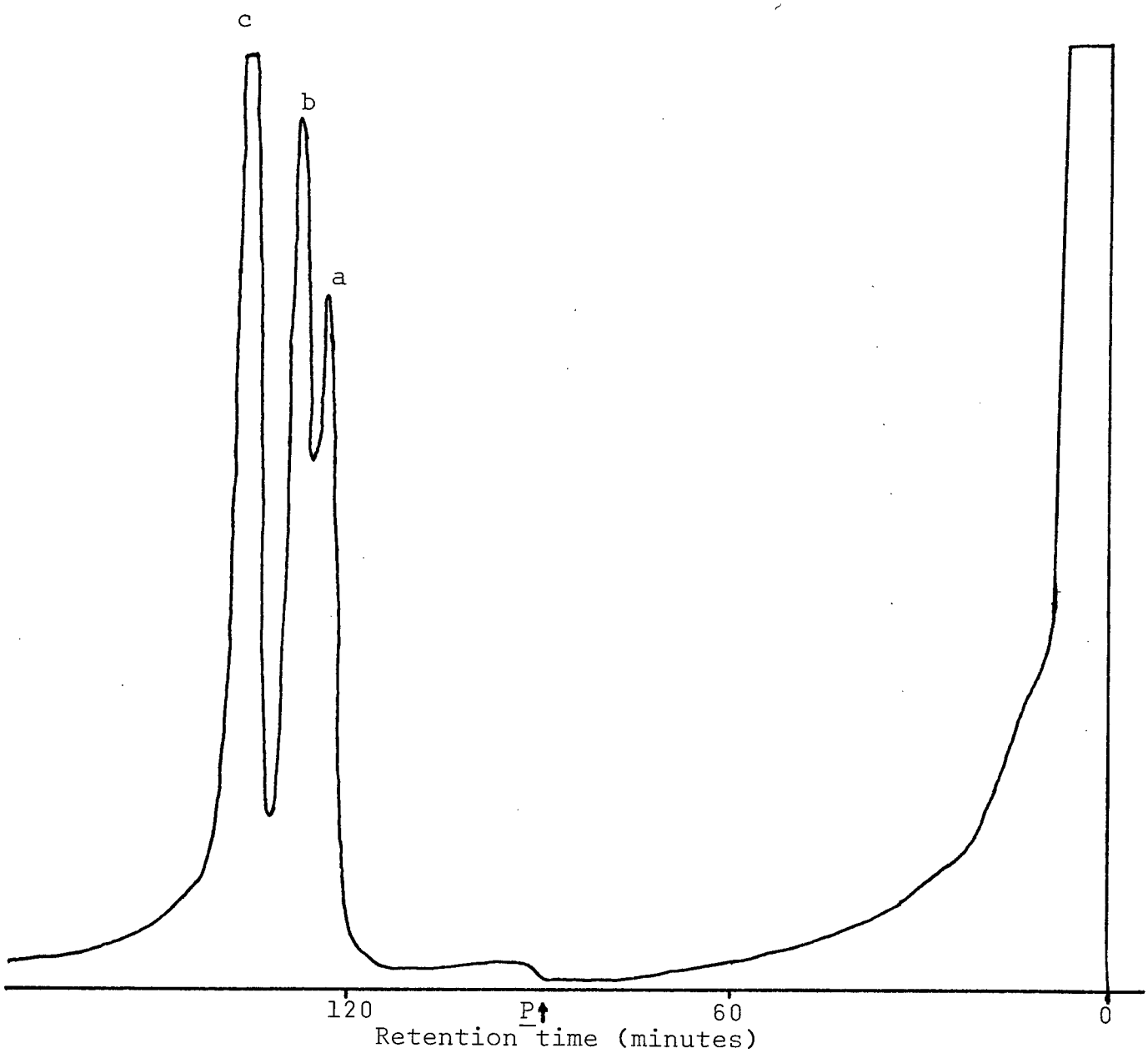

Figure 18. Gas chromatogram of desmethyl sterols isolated by preparative TLC from the coffee cell culture

Individual peak retention times (minutes)

(a) - 123 (c) - 135
(b) - 126

Attenuation x1000

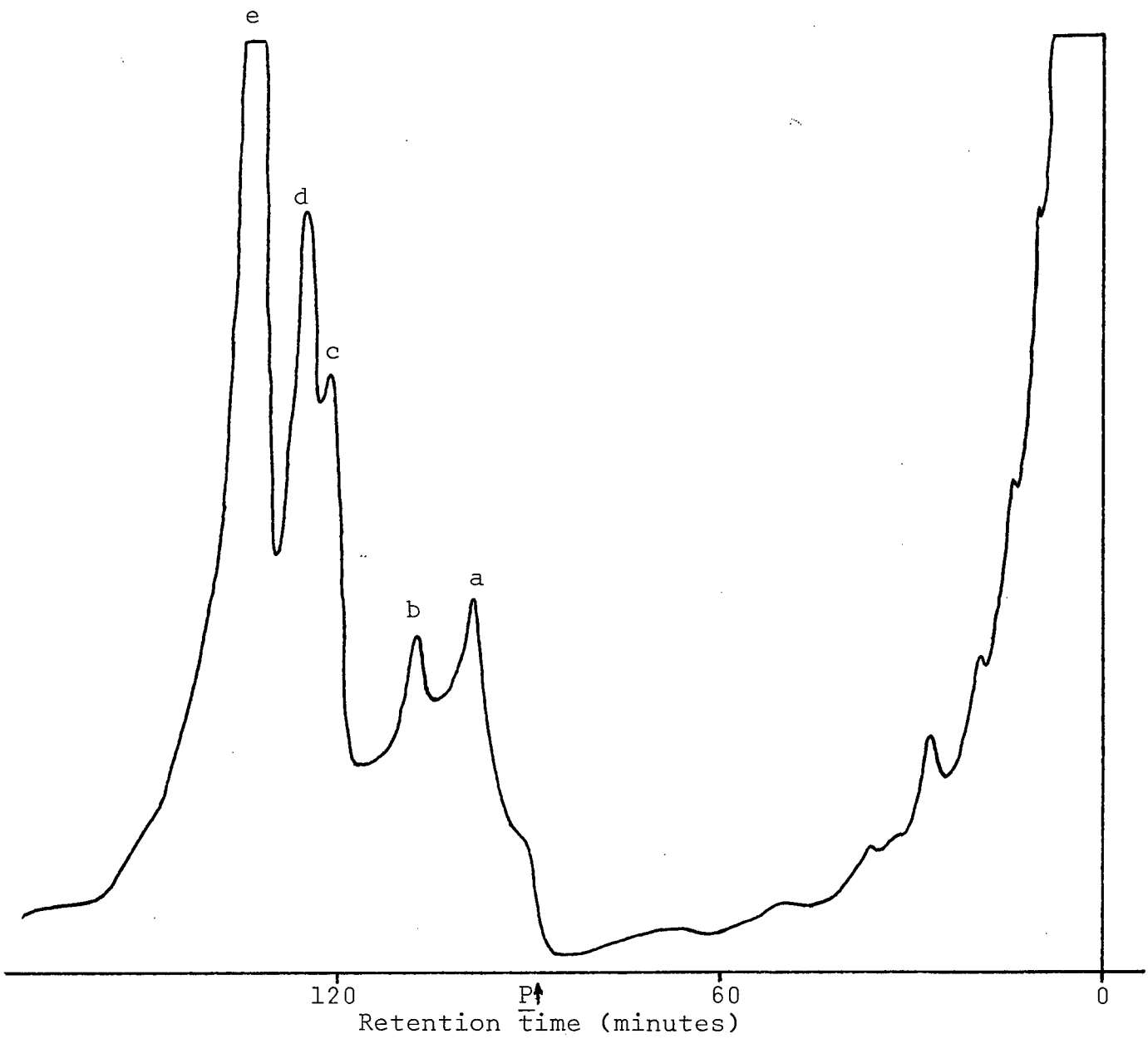

Figure 19. Gas chromatogram of the desmethyl sterols isolated by preparative TLC from the coffee bean

Individual peak retention times (minutes)

(a) - 100 (c) - 123 (e) - 135
(b) - 109 (d) - 126

Attenuation x200

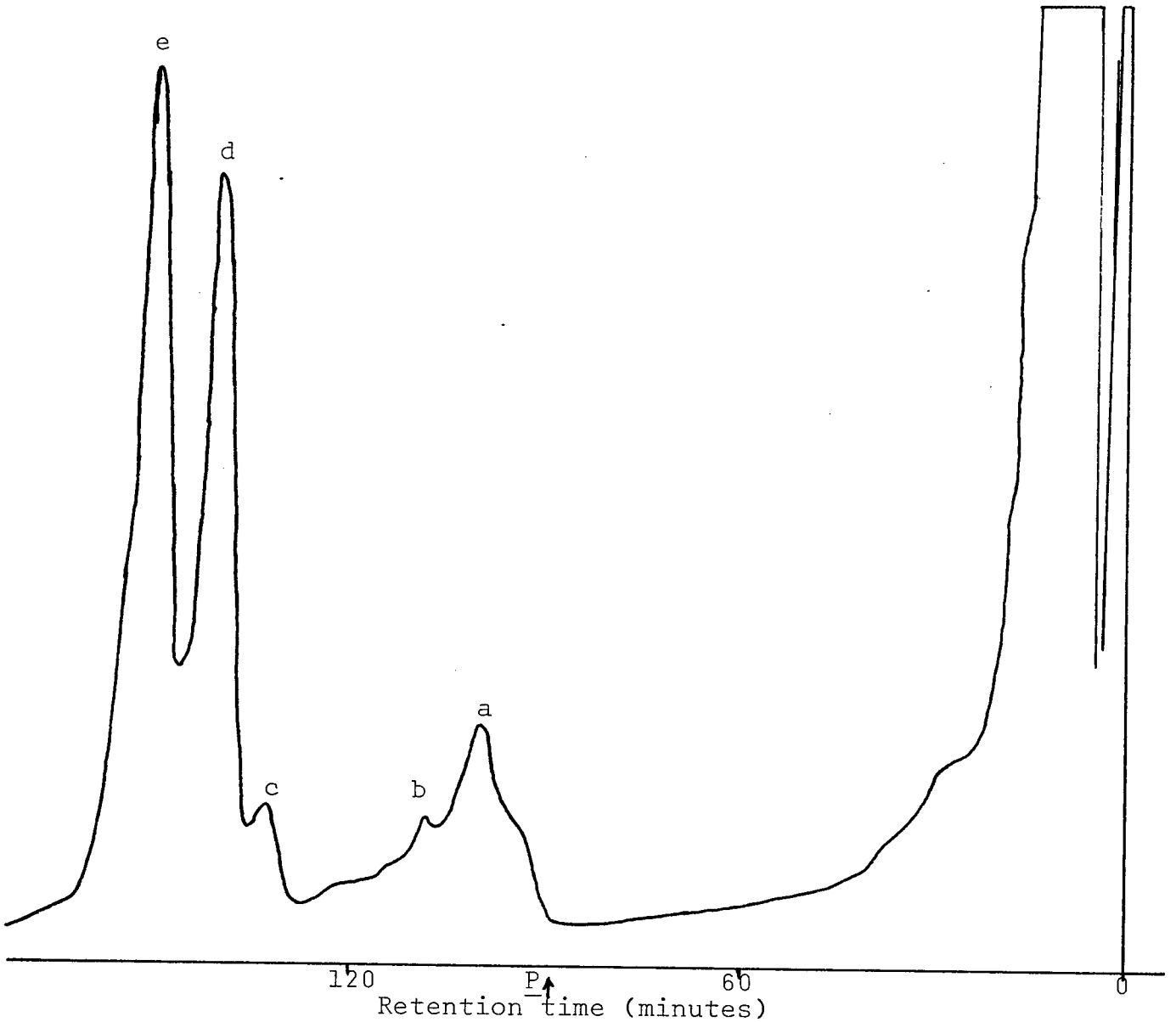

Figure 20. Gas chromatogram of sterols (Rf between di and desmethyl sterol standards) isolated by preparative TLC from the coffee bean

Individual peak retention times (minutes)

(a) - 100 (c) - 132 (e) - 152
(b) - 109 (d) - 141

Attenuation x 100

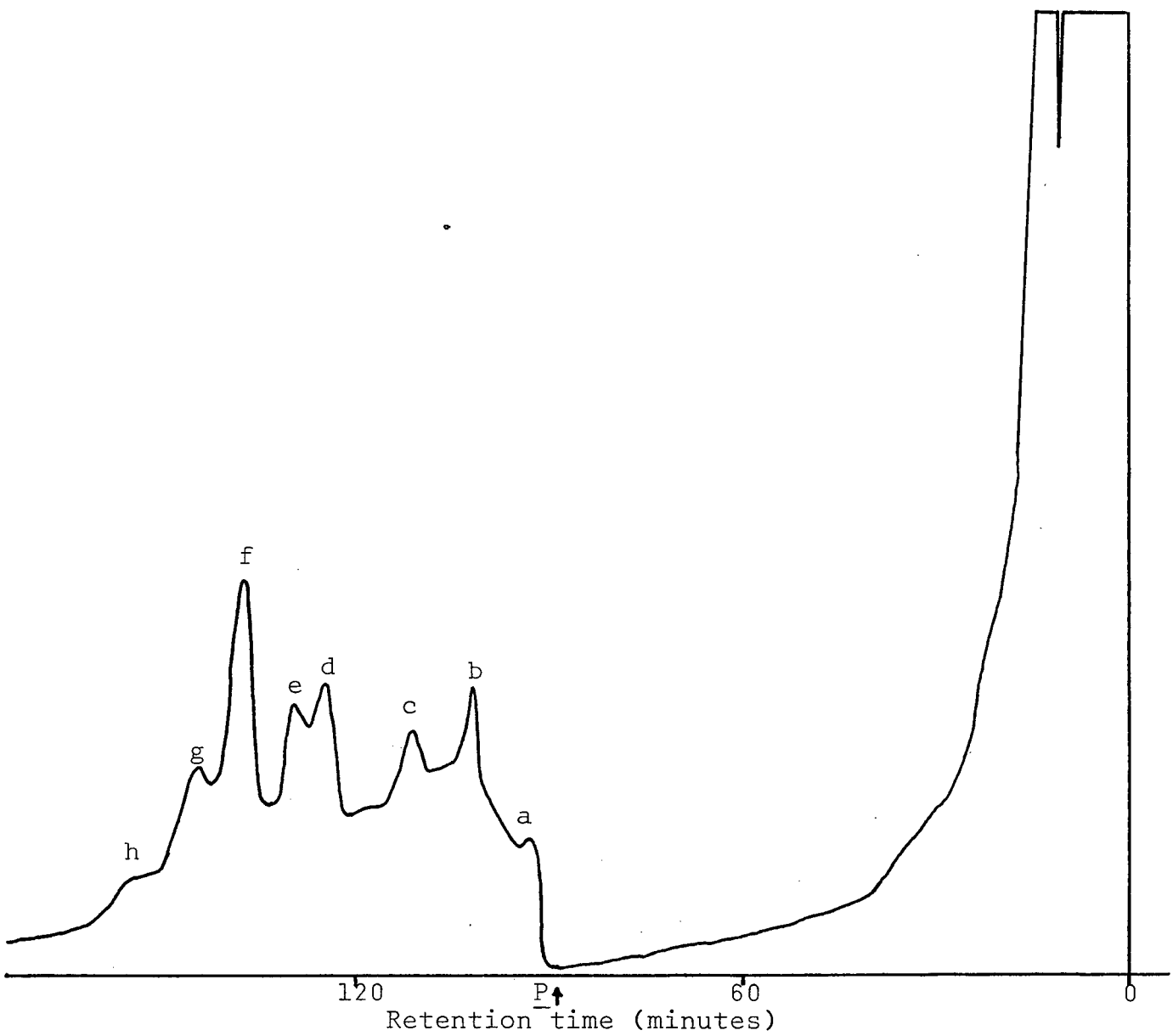

Figure 21. Gas chromatogram of sterols (Rf between di and desmethyl sterol standards) isolated by preparative TLC from the coffee cell culture

Individual peak retention times (minutes)

(a) - 93	(c) - 111	(e) - 130	(g) - 144
(b) - 102	(d) - 124	(f) - 137	(h) - 153

Attenuation x100

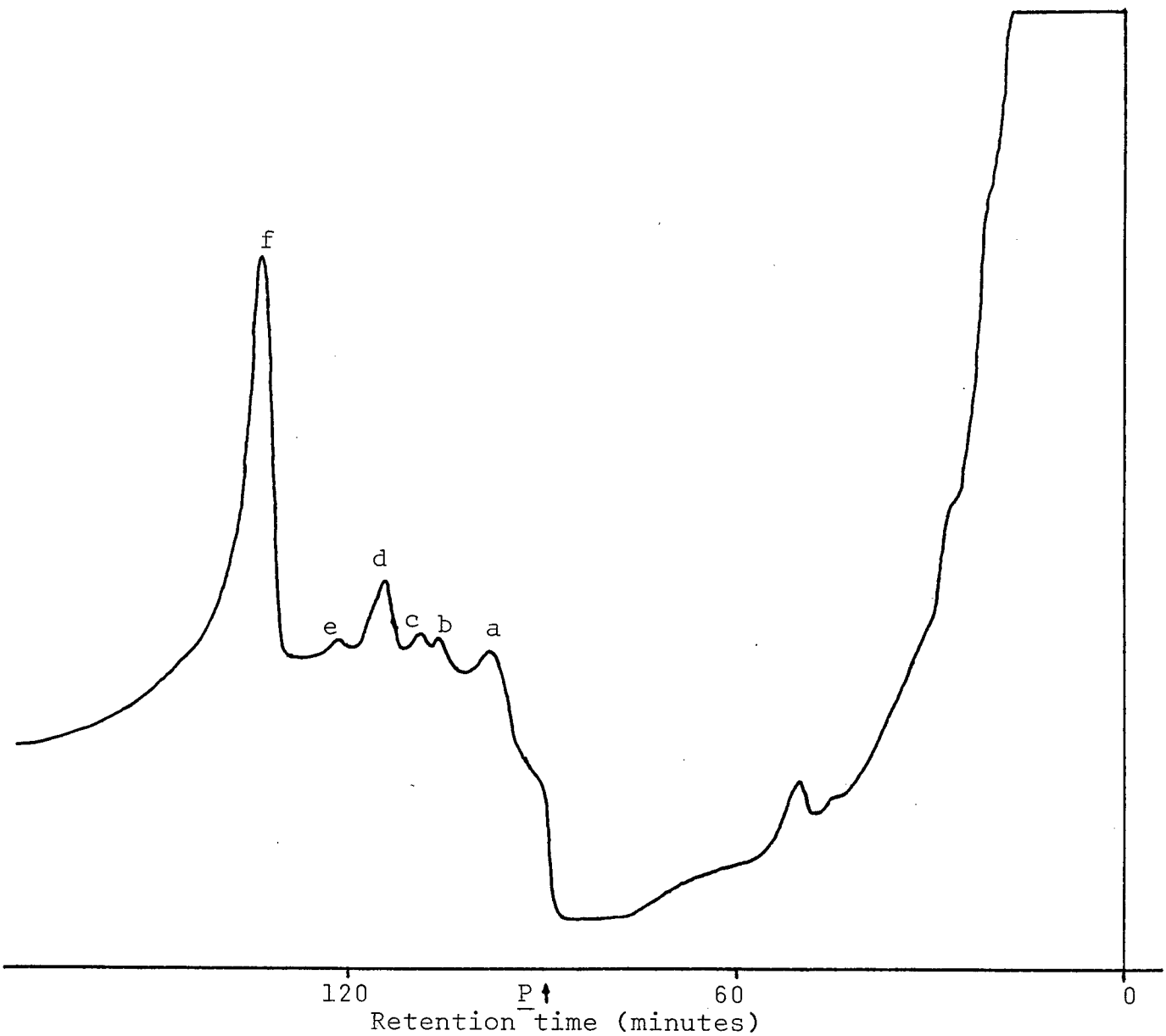

Figure 22. Gas chromatogram of the mint cell culture unsaponifiables

Individual peak retention times(minutes)

(a) - 98	(c) - 110	(e) - 122
(b) - 106	(d) - 115	(f) - 135

Attenuation x200

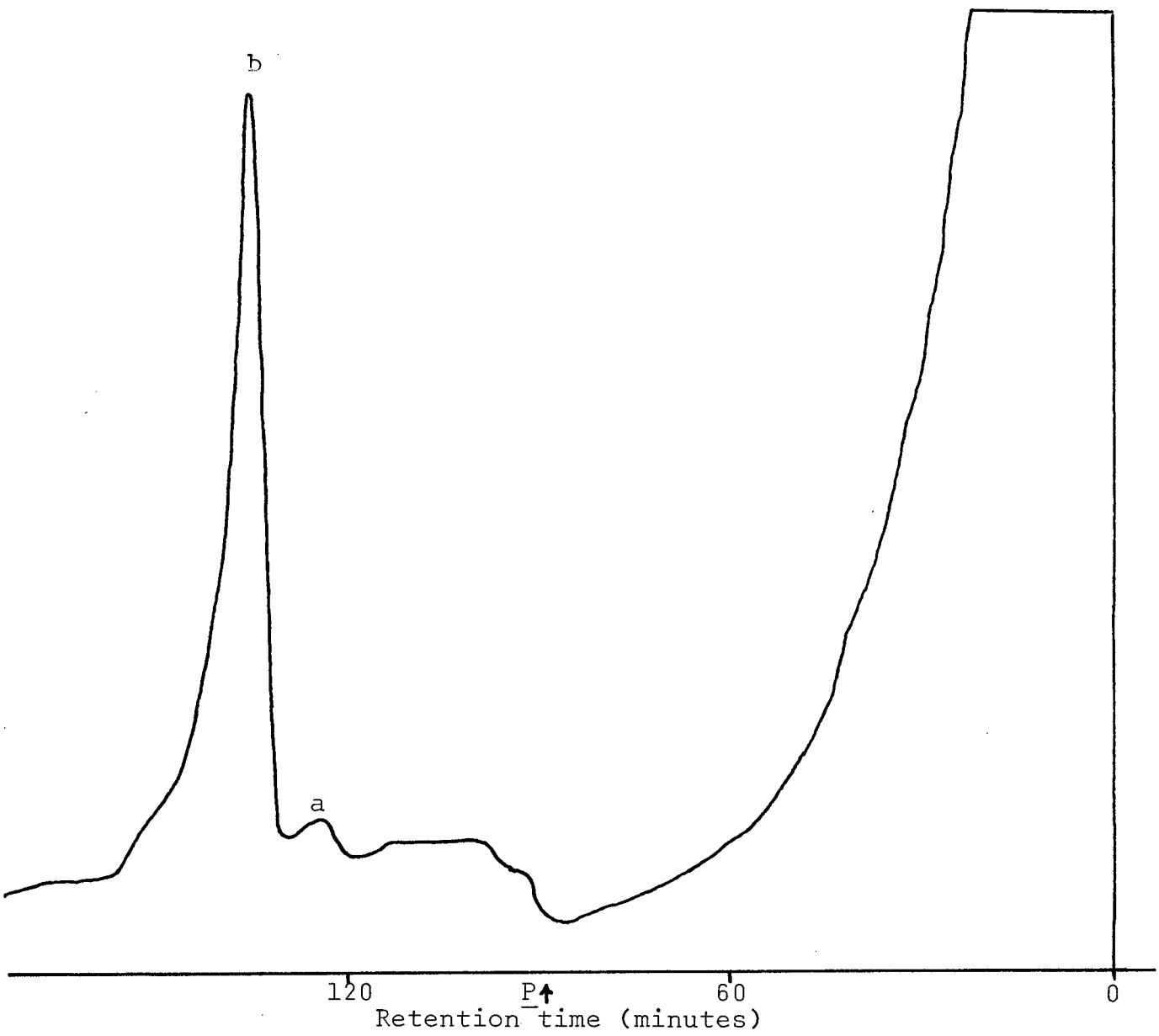

Figure 23. Gas chromatogram of the mint cell culture unsaponifiables

Individual peak retention times (minutes)

(a) - 124
(b) - 135

Attenuation x1000

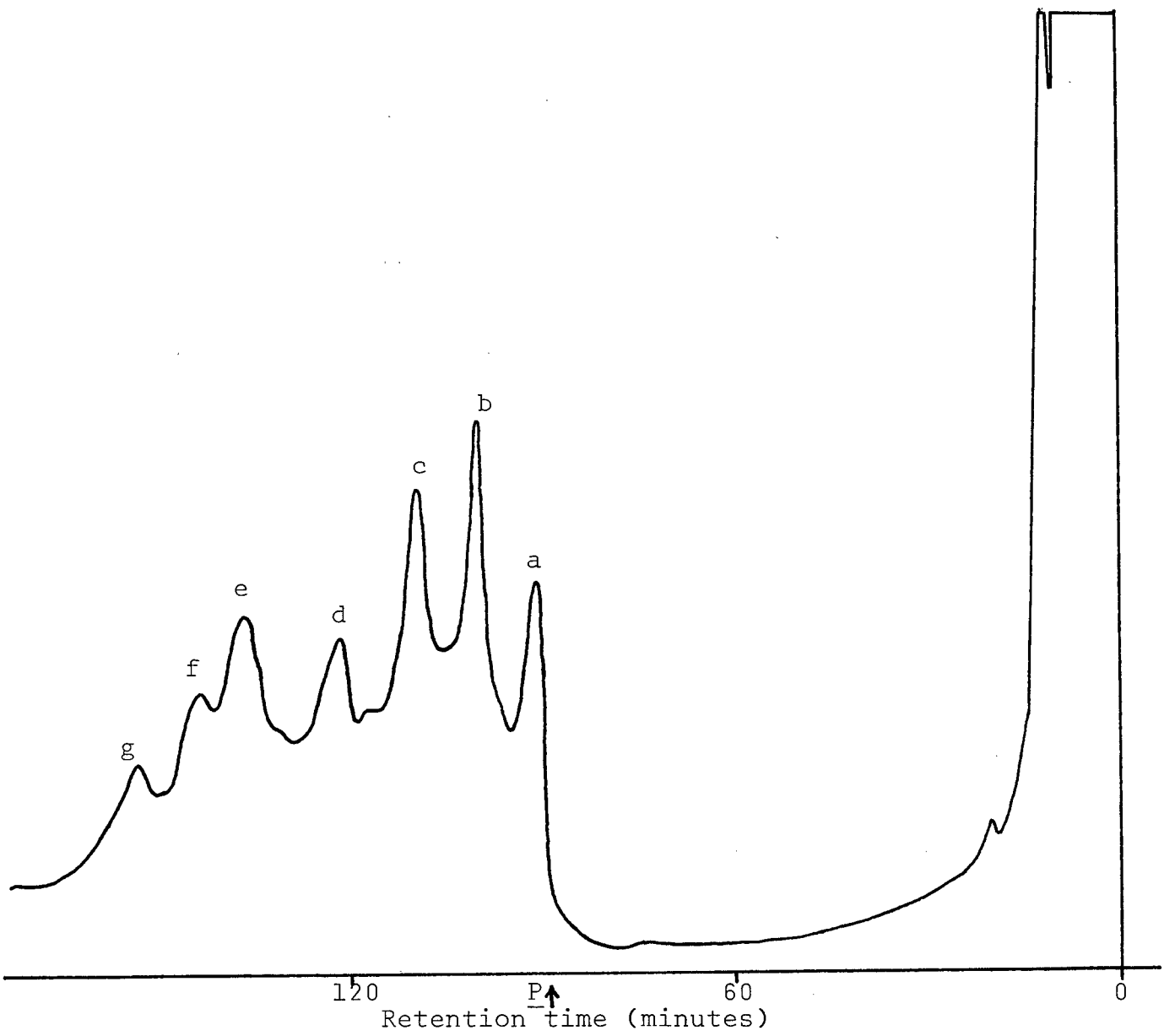

Figure 24. Gas chromatogram of sterols (Rf between di and desmethyl sterol standards) isolated by preparative TLC from the mint cell culture

Individual peak retention times (minutes)

(a) - 91	(c) - 110	(e) - 136	(g) - 153
(b) - 100	(d) - 121	(f) - 142	

Attenuation x100

ii. GLC of the mint cell sterol spot located between the di and desmethyl sterol standards

The resulting chromatogram is presented in Figure 24. Seven unidentified peaks appeared on the chromatogram, indicating that a complex mixture of sterol components exists.

iii. GLC of the mint seed sterol spot which covers the regions occupied by the di and desmethyl sterol standards

As expected, the resulting chromatogram (not presented) was exactly the same as that of the total mint seed unsaponifiables (Figure 22).

G. Summary of gas chromatography of the total unsaponifiables of coffee and mint and of gas chromatography of TLC isolated sterol spots of coffee and mint

Although no quantitation of the gas chromatographic data was performed, some general statements can be made with regard to the relative amounts of steroidal and non-steroidal material present in the total coffee bean and cell unsaponifiables. A relative estimation was possible through consideration of the amount of sample injected and of the recorder attenuation required to produce peaks on the chromatogram. In the case of the coffee bean oil unsaponifiables, the non-steroidal material (peaks 'a' and 'b') accounted for the majority of the sample. The coffee cell unsaponifiables contained relatively more steroidal material. Both the coffee bean and the coffee cell unsaponifiables contained three sterols, in the same relative ratios, tentatively identified as β-sitosterol, stigmasterol and

campesterol in order of decreasing concentration.

A general comparison of the relative quantities of sterols present in the total mint seed and mint cell oil unsaponifiables was possible through the use of the same semi-quantitative method described above for coffee. Both sources of mint oils contained β-sitosterol as the major sterol component, but the plant cells contained substantially more of this sterol.

Gas chromatography of sterol components of the coffee bean and coffee cell oil isolated by preparative TLC revealed that the desmethyl sterol spot was composed of the desmethyl sterols, β-sitosterol, campesterol and stigmasterol. This result verified the initial assumption, based on GLC of the total unsaponifiables, that these sterols were present. The coffee bean sterols located between the di and desmethyl sterol standards were different in composition and relative concentration than the similarly located coffee cell sterols. Sterols found in this region of the TLC plate are probably monomethyl sterols because of the order of separation in the chloroform:ethyl acetate solvent system (des, mono, dimethyl sterols)[48].

Gas chromatographic analysis of the TLC isolated sterol spots of mint confirmed the premise that β-sitosterol was the major sterol of the mint seed and mint cell oil unsaponifiables. Although a number of minor sterols were present in the mint seed, β-sitosterol was overwhelmingly dominant in the mint cell. Analysis of the cell sterol

material which had an Rf value between the di and desmethyl sterol standards indicated a complex mixture of components.

H. Discussion

The sterols and diterpenoid alcohols of the coffee bean and the coffee cell cultures were studied in a qualitative and semi-quantitative manner. The major sterols of the coffee bean were shown to be β-sitosterol, stigmasterol and campesterol in order of decreasing concentration. These results agree with those obtained by Nagasampagi et al.(35) in their study of the coffee bean. The coffee cell cultures were found to contain the above mentioned sterols in the same relative concentrations present in the coffee bean. The sterol content of the coffee cell cultures was found to be considerably higher than that found in the coffee bean, although no absolute quantitative values were obtained. Furthermore, comparative thin layer chromatographic studies of the non-saponified oils of the bean and of the cell illustrated the presence of more free steroidal material in the cell culture oil than in the bean oil. Both the coffee bean and coffee cell unsaponifiables contained minor amounts of sterols other than the desmethyl sterols. Gas chromatographic examination of these minor sterols illustrated differences in composition and concentration between the bean and the cell.

Two diterpenoid alcohols, cafestol and kawheol, were isolated from coffee bean oil unsaponifiables, and identified by U.V. spectroscopy, TLC and GLC. Gas

chromatography of the total unsaponifiable coffee bean oil revealed that the two diterpenoid alcohols were the major constituents, a result in agreement with the literature[19,35]. Cafestol and kawheol were also found to be present in the coffee cell unsaponifiable matter, but in lower concentrations.

As stated previously, studies of the sterols isolated from the seeds and plant cell cultures of the unknown Mentha species showed β-sitosterol to be the major and commonly occurring sterol in both tissue sources. This data is in agreement with the literature[3,40], as β-sitosterol has been noted to be a major sterol in the Mentha piperita plant. The mint cell cultures contained more free steroidal lipid components than found in a corresponding analysis of the seeds. Gas chromatography of the sterols which were located between the di and desmethyl sterol regions on the thin layer plate of the mint cell unsaponifiables, revealed the presence of a complex unidentified mixture of sterols. Thin layer chromatography of the mint seed unsaponifiables revealed only one large iodine positive spot which covered almost the complete sterol region of the plate. This encompassing spot indicated the possible presence of sterols of all three degrees of methyl substitution within the mint seed. The total sterol content of the mint cell cultures was found to be considerably higher than that found in the mint seed.

Various investigators[4,20,21,29,49,50,55] have found sterols in a wide variety of plant cell cultures.

When the sterol composition of each of the cell cultures was compared to that of the respective parent plant tissues, it has been stated that, in general, no major qualitative differences were discerned. In this study, a similar conclusion was drawn for coffee and mint cell cultures, i.e. the major sterols found in coffee and mint cell cultures were similar to those found in the parent plants.

CONCLUSION

Cell suspensions of Coffea arabica and an unknown Mentha species were cultured in liquid media, and the sterols and fatty acids were analysed. These lipid constituents were also investigated in the parent plant tissues, in order to provide comparative data which could be of use to other workers investigating lipid metabolism in plant cell cultures.

The oil content of the plant cell cultures fell within the range found in the leaves and stems of the parent plants. In contrast, the seed had a higher oil content.

The fatty acid composition of the coffee cell cultures resembled the composition of the leaves and stems. In the study of mint, the pattern of fatty acids found in the cell cultures was similar to that found in the seed. However, although differences in fatty acid composition between plant cell cultures and various parent plant tissues were noted, it can be stated that the major fatty acids characteristic of the parent plant were found in the plant cells.

Growth of the coffee cell cultures in three different media did not result in changes in the fatty acid composition or content. Exposure to light did not affect the fatty acid composition or content of coffee cell cultures, but did cause the development of a green pigmentation and a concurrent increase in the fatty acid content in the mint cell cultures.

The major sterols of the plant cell cultures were found to be the same as those present in the parent seeds. The free sterol content of the extracted lipid from the plant cell cultures and the sterol content of the unsaponifiables of the lipid were found to be higher than the corresponding contents found in the seed.

In general, species specific metabolites, which are characteristically found in a differentiated parent plant tissue, are present in only very minor amounts, if at all, in the undifferentiated plant cell cultures. In this study, two constituents of the coffee bean, cafestol and kawheol, were found to be present in the coffee cell cultures. This is the first report of these constituents being present in the plant cell cultures of coffee.

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