The Determination of Tocopherols and Isoprenoid Quinones in the Grain and Seedlings of Wheat (Triticum vulgare)

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1. A comparison is made of several procedures for the extraction of tocopherols and isoprenoid quinones from plant tissues. 2. Gradient-elution column chromatography on acid-washed alumina efficiently separates the isoprenoid quinones and tocopherols into groups that can then be assayed spectrophotometrically or, with the tocopherols, separated into their individual components and determined by gas–liquid chromatography. 3. This improved analytical procedure was used to study the distribution of the tocopherols and of ubiquinone in the ungerminated wheat grain.

To study the changes in the amounts of the various tocopherols and related quinones during the process of germination in wheat (Hall & Laidman, 1966) it was found necessary to reassess some of the existing methods of analysis used for plant tissues. The accurate determination of isoprenoid quinones and tocopherols in biological materials involves three stages: the extraction of the tissue lipids, the purification and fractionation of the crude lipid extract and finally the determination of the individual compounds.

Though Soxhlet extraction of freeze-dried samples with acetone has been shown to be superior to other methods for the extraction of tocopherols and isoprenoid quinones from animal tissues (Edwin, Diplock, Bunyan & Green, 1960), no critical assessment of the various methods of extraction has hitherto been reported for plant tissues. We have compared some of the more established methods of extraction with a new procedure using hot propan-2-ol (Gaunt & Stowe, 1967). This comparison became essential when it was demonstrated that the activity of a tocopherol-destroying enzyme in the pea plant increased in the presence of certain organic solvents, but that the enzyme was rapidly deactivated by propan-2-ol (S. M. Barlow & J. K. Gaunt, personal communication).

Since the isoprenoid quinones and the tocopherols are minor components of the lipid fraction, some form of chromatographic separation is necessary before their individual assay. A variety of adsorbents have been used for the column chromatography of isoprenoid lipids (Kofler, Sommer, Bolliger, Schmidli & Vecchi, 1962). In the present work the well-tried procedure of Morton and his school (Laidman, Morton, Paterson & Pennock, 1960), with columns of acid-washed alumina, was retained and developed. Methods involving chromatographic separations on paper or on thin layers of adsorbent cannot be applied to the separation of lipid mixtures from cereal grains because excessive amounts of the triglycerides, which comprise the major constituent of most seed lipids, interfere with both these forms of chromatography.

Spectrophotometric methods with a borohydride reduction procedure have been adopted for the determination of isoprenoid quinones (Crane, Lester, Widmer & Hatefi, 1959). The spectrophotometric method for the assay of tocopherols, devised by Dilley & Crane (1963), has replaced the older and less specific colorimetric methods. In the present work we used a modification of the Dilley & Crane (1963) method, which utilizes ferric chloride in place of the more expensive gold chloride of the original procedure.

After the initial demonstration that tocopherols can be separated by gas–liquid chromatography (Nicholaides, 1960), the separation of the mono-, dimethyl- and trimethyl-toccols as groups was achieved on an Apiezon N stationary phase. (Kofler et al. 1962). Wilson, Kodicek & Booth (1962) demonstrated that the toccols could be separated from their corresponding tocotrienols on an SE–30 column. Libby & Sheppard (1964) and Ishikawa & Katsui (1966) investigated the quantitative analysis of tocotocols by gas–liquid chromatography and Bieri & Andrews (1963) used the method for the determination of tococols in animal tissues. In the present work the routine application of gas–liquid chromatography to the quantitative study of tocopherols in plant materials was investigated.

By using these improved procedures of extraction
and analysis, a study was made of the distribution of ubiquinone and the various tocopherols in the ungerminated wheat grain.

**METHODS**

*Extraction of lipids.* All extraction procedures and analyses were carried out in diffuse daylight to avoid the photodestruction of tocopherols and quinones. All solvents were redistilled before use. The extracted lipids were stored at $-20^\circ$ and fractionated by gradient-elution alumina-column chromatography. The appropriate fractions were assayed for tocopherols by a modified version of the method of Dilley & Crane (1963) (see below) and for ubiquinone and plastoquinone by the spectrophotometric borohydride method of Crane et al. (1959).

Initially three solvents, acetone, ethanol and propan-2-ol-chloroform (1:1, v/v), were compared as extractants of freeze-dried plant material. Each solvent was tested in each of two extraction procedures: direct extraction of the dried material at room temperature and extraction at higher temperatures in a Soxhlet apparatus. Samples (5g.) of wheat grain (var. Atle) were ground in a mortar and pestle and were then freeze-dried. In the first extraction procedure the freeze-dried powders were extracted at room temperature with three successive 50ml. volumes of solvent for 30min., 60min. and overnight respectively. The extracts were filtered, combined and concentrated to a small volume in a rotary evaporator (bath temp. 50°). The last traces of solvent were removed under a stream of N$_2$. In this and in all other experiments a small volume of ethanol was added during the later stages of solvent removal to remove any trace of water that might have been present. In the Soxhlet extraction the freeze-dried powders were extracted with three 50ml. volumes of solvent for one 30min. and two 60min. periods successively. This experiment was repeated on 5g. of grain that had been allowed to germinate for 5 days in the dark at 24°.

In view of the superior results obtained with the propan-2-ol-chloroform mixture in these experiments (see Table I), the use of this solvent was further examined, especially with regard to the extraction of tocopherols from seedlings. A procedure similar to that described by Gaunt & Stowe (1967) was used. Samples (5g.) of grain were allowed to germinate in the dark for either 18 or 72hr. The whole seedlings were directly homogenized for 4min. in 100–150ml. of hot propan-2-ol in a top-drive homogenizer (Silverson Machines Ltd.). The homogenized material was filtered off and further homogenized and extracted successively with 100–150ml. of hot propan-2-ol-chloroform (1:1, v/v) and 100–150ml. of hot chloroform. The three extracts were combined, about 100ml. of ethanol was added and the whole was concentrated to a small volume in a rotary evaporator (bath temp. 50°). It is essential that all the chloroform be removed at this stage. Then 75ml. of light petroleum (b.p. 40–60°) and 40ml. of water were added to the extract in a separating funnel. The mixture was shaken and, after separation of the phases, the upper phase was removed. The aqueous phase was then extracted with two further lots of light petroleum. The light-petroleum extracts were combined, washed twice with water and evaporated to dryness.

Other samples of grain were extracted with the same sequence of solvents (each for 6min.) in a stainless-steel analytical micro-pulverizer (Glen Creston Ltd., Stanmore, Middlx.) fitted with a cooling jacket maintained at 0°. The combined extracts were centrifuged before being concentrated and partitioned in light petroleum as described above. Further samples of seedlings were freeze-dried and extracted in a Soxhlet apparatus with the same sequence of solvents. The first two extractions were for periods of 30min. and the final chloroform extraction was for 60min. The combined extracts were partitioned and evaporated to dryness as described above.

**Chromatography on columns of alumina.** Known amounts of authentic compounds, or the lipid from 5g. samples of grain or seedlings, were chromatographed on 5g. of acid-washed alumina (Brookman grade III) in small glass columns (1 cm. internal diam.). The alumina was grade 9 (Peter Spence and Co., Widnes, Lancs.) that had been acid-treated in the manner described by Laidman et al. (1960). The lipid samples, dissolved in a minimum of light petroleum (b.p. 40–60°), were placed on the alumina columns and the columns were developed with light petroleum containing a linearly increasing percentage of diethyl ether as provided by the apparatus of Parr (1964). Two cylindrical glass containers of equal internal diameter (3.7 cm.) were connected at their base by a short length of narrow-bore nylon tubing and the base of one of them, the mixing chamber, was connected to the top of the column by small-bore nylon tubing. The contents of the mixing chamber were continuously mixed by a magnetic stirrer. At the commencement of the chromatography, the mixing chamber contained 90ml. of light petroleum and the other chamber contained 90ml. of 35% (v/v) ether in light petroleum. Fractions of volume 4ml. (250–300 drops) were collected automatically, evaporated to dryness under a stream of N$_2$ and stored at $-20°$ until required for analysis.

The fractions from the column may be monitored by u.v. spectrophotometry or by absorption thin-layer chromatography, but once the gradient-elution system had been established, it was accurately reproducible. Saponification was necessary before the trimethyl tocopherol and ubiquinone fractions could be chromatographed on thin layers of silica gel. [The IUPAC-IUB nomenclature of quinones with isoprenoid side chains [Biochem. J. (1967) 102, 15] has been used throughout this paper. In addition the following trivial names have been used: trimethyl tocopherol, $\alpha$-tocopherol+$\alpha$-tocotrienol; dimethyl tocopherols, any mixture of two or more from $\beta$-tocopherol, $\beta$-tocotrienol, $\gamma$-tocopherol, $\gamma$-tocotrienol.] After saponification, the ubiquinone isoprenologue was identified by reversed-phase paper chromatography (Linn et al. 1959) or by reversed-phase thin-layer chromatography (Bolliger, 1962). Tocopherols were identified by the reversed-phase chromatographic system of Eggitt & Ward (1953) and the thin-layer chromatographic procedure of Pennock, Hemming & Kerr (1964).

**Spectrophotometric methods of assay.** The fractions from alumina chromatography were dissolved in 1ml. of spectroscopically pure ethanol, and quartz micro-cells with an optical path of 1 cm. and a capacity of 0-4ml. were used. In the assays of ubiquinone and plastoquinone with the borohydride reduction technique of Crane et al. (1959), the following $\Delta\epsilon_{275}$ values were used: ubiquinone-9, $\Delta\epsilon_{275}^\text{quin} = 275\,\mu M = 158$ (Hatafi, 1963); plastoquinone-9, $\Delta\epsilon_{275}^\text{quin} = 254\,\mu M = 198$ (Crane, 1958). Phyloquinone was assayed spectrophotometrically at 249 $\mu M$ ($\epsilon_{249} = 249\,\mu M = 300$).

The appropriate fractions from alumina chromatography
were assayed for tocopherols by using a modified version of
the method of Dilley & Crane (1963) in which the eq. 20% 
(w/v) AuCl₃ soln. was replaced as oxidizing agent by a
similar 20% (w/v) solution of FeCl₃. In both methods the
tocopherol is oxidized to tocopherolquinone, which
may subsequently be assayed by a spectrophotometric boro-
hydride reduction technique. However, the oxidation of
α-tocopherol by FeCl₃ differs from that by AuCl₃ in that
the reaction is not complete in the former (Karrer & Geiger,
1940; Eggitt & Norris, 1956). α-Tocopherolquinone is the
major product of the FeCl₃ oxidation, but other products
may also be formed (Green & McHale, 1965). In the modified
procedure it was therefore necessary to obtain empirical
ΔE₁₀⁺ values at 262 mμ for the borohydride reduction of
each tocopherolquinone in terms of its parent tocopherol.
Further, since ubiquinone cannot be separated from α-
tocopherol and α-tocotrienol by the column chromato-
graphy, and since ubiquinone also shows an extinction
change at 262 mμ on reduction by borohydride, corrections
were made for the contribution of ubiquinone to the tocoph-
erol assays. This contribution was determined by carry-
ing out a borohydride reduction of the tocopherol–
ubiquinone mixture before treatment with FeCl₃ at the
same time as the determination of the ΔE value at 275 mμ
for the assay of ubiquinone.
Saponification and methanolation of triglycerides. Saponifi-
cation was carried out according to the procedure recom-
manded by the Analytical Methods Committee (1959).
Though this method of removing triglycerides before the
gas-liquid chromatography of the trimethyl tocopherols
was used throughout the work described in this and the next
paper (Hall & Laidman, 1968), it was not entirely suitable for
the purpose (see the Results section). Subsequently
methanolation in a non-aqueous medium was investigated as
a possible alternative method. Methanolysis with either
anhydrous HCl (Stoffel, Chu & Ahrens, 1959) or BF₃
(Morrison & Smith, 1964) as catalyst converts triglycerides
quantitatively into glycerol and fatty acid methyl esters,
which do not interfere with the gas-liquid chromatography
of tocopherols. These methods were only partly successful
(see the Results section).

Gas-liquid chromatography of tocopherols. Analyses were
carried out with a Pye series 104 gas chromatograph fitted
with flame ionization detectors and a single-channel pen
recorder. A single-glass-column system was employed iso-
thermally at 250° with O₂-free N₂ at 40 ml/min. as the
carrier gas. The glass columns (5 ft. × 4 mm.) were silicone-
treated and packed with 4% SE-30 (G. Pye and Sons Ltd.,
Cambridge) or 4% SE-301 (Imperial Chemical Industries
Ltd., Stevenston, Scotland) on silicone-treated Gas-Chrom
Z (100-120 mesh) (Applied Science Laboratories Inc., State
University, Pa., U.S.A.). The packing material was pre-
pared according to the procedure described by Wilson et al.
(1962). The columns and their packings were conditioned
for 24 hr. at 300° before use. Systems with polyethylene
glycol adipate as the liquid stationary phase (Wilson et al.
1962) deteriorated rapidly at 250° and proved unsuitable
for the routine gas-liquid chromatography of tocopher-
ols.

The retention times of the tocopherols, tocotrienols and
their acetates relative to that of α-tocopherol were similar
to those reported by Wilson et al. (1962). For quantitative
work, the instrument was calibrated with standard tocoph-
erol solutions and the standardization was checked at
least daily. Each tocopherol exhibited an individual non-
linear calibration curve (see Libby & Sheppard, 1964) and
the values were reproducible within ±4%. At an amplifier
attenuation of x 100 (ionization current 10⁻¹²A) quantities
of tocopherol ranging from 0-2 to 1-0 μg. could be conven-
tiently determined. Peak areas were calculated by multiply-
ing the peak height by the peak width at half peak height.

RESULTS

Comparison of extraction procedures. The results of the first series of experiments are shown in
Table 1. Acetone extracted more tocopherols from
powdered grain in the Soxhlet apparatus than it did
at room temperature and was superior to ethanol,
thus confirming the results of Edwin et al. (1960)
with animal tissues. Though the extraction of
ubiquinone was relatively independent of tempera-
ture, acetone was again superior to ethanol. In the
Soxhlet extraction, the propan-2-ol-chloroform
mixture extracted more ubiquinone and tocopherol
from the dried grain than did acetone, but the two
solvents gave equivalent results in the extraction of
ubiquinone from 5-day seedlings.

Table 2 shows the results of the second series of
experiments, which compare different extracting
procedures when they are used with propan-2-ol-
chloroform. The micro-pulverizer and top-drive
homogenizer gave comparable results and were
superior to Soxhlet extraction for the extraction of
both ubiquinone and tocopherols. Because of the
greater manipulative ease of the procedure that
used the top-drive homogenizer, this method was
adopted as the standard procedure in all subsequent
work.

Chromatography on columns of alumina. The
order of elution of compounds from the column is

<table>
<thead>
<tr>
<th>Table 1. Comparison of extraction procedures</th>
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<tbody>
<tr>
<td>Experimental details are given in the text.</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Ubiquinone-9 tocopherol</td>
</tr>
<tr>
<td>(μg./g. dry wt.)</td>
</tr>
<tr>
<td>Extraction of dry grain at room temperature</td>
</tr>
<tr>
<td>Acetone</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Propan-2-ol-chloroform</td>
</tr>
<tr>
<td>Soxhlet extraction of dry grain</td>
</tr>
<tr>
<td>Acetone</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Propan-2-ol-chloroform</td>
</tr>
<tr>
<td>Soxhlet extraction of 5-day seedlings</td>
</tr>
<tr>
<td>Acetone</td>
</tr>
<tr>
<td>Propan-2-ol-chloroform</td>
</tr>
</tbody>
</table>
Table 2. Comparison of extraction procedures with propan-2-ol–chloroform as the extraction solvent

Experimental details are given in the text.

<table>
<thead>
<tr>
<th></th>
<th>Plastoquinone-9 (µg./150 seedlings)</th>
<th>Ubiquinone-9 (µg./150 seedlings)</th>
<th>Total tocopherols (µg./150 seedlings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction of 18hr. seedlings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soxhlet extraction</td>
<td>-</td>
<td>32</td>
<td>76</td>
</tr>
<tr>
<td>Micro-pulverizer</td>
<td>-</td>
<td>44</td>
<td>160</td>
</tr>
<tr>
<td>Homogenizer</td>
<td>-</td>
<td>46</td>
<td>170</td>
</tr>
<tr>
<td>Extraction of 72hr. seedlings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soxhlet extraction</td>
<td>19</td>
<td>73</td>
<td>160</td>
</tr>
<tr>
<td>Micro-pulverizer</td>
<td>18</td>
<td>92</td>
<td>208</td>
</tr>
<tr>
<td>Homogenizer</td>
<td>20</td>
<td>85</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatography of isoprenoid quinones and tocopherols on columns of acid-washed alumina with gradient elution. Experimental details are given in the text. The values in parentheses are the percentage recoveries of the various compounds. Abbreviations: K, phylloquinone; PQ-9, plastoquinone-9; Q-9, ubiquinone-9; α-TQ, α-tocopherolquinone; β-TQ-3, β-tocopherolquinone-3; α-T, β-T, γ-T and δ-T, α-, β-, γ- and δ-tocopherol; α-T3, β-T3 and γ-T3, α-, β- and γ-tocotrienol.

shown in Fig. 1. Tocopherols and tocopherol-quinones are separated according to the number of methyl groups that they possess on their aromatic ring, though the tocotrienols could be only partly separated from the corresponding tocols. Attempts to improve the separation by altering the gradient elution pattern were not successful. Ubiquinone-9 ran with α-tocopherol and α-tocotrienol. Plastoquinone-9 tended to merge with phylloquinone. These two compounds could, however, be separated by developing the column with 10 ml. of light petroleum before commencing the gradient elution.

Modified Dilley & Crane (1963) procedure. The modification of the Dilley & Crane (1963) procedure was found to be very reproducible and tocopherols could be assayed accurately in the presence of ubiquinone. Ubiquinone was unaffected by the ferric chloride treatment.
The experimental values of $\Delta E_{\text{1cm}}^{1\%, \text{at } 262\mu m}$ for the various tocopherolquinones in terms of their parent tocopherols are given in Table 3. Each value is the average of three determinations. The value of 370 for $\alpha$-tocotrienol was chosen arbitrarily; unexpectedly high values were obtained with our ‘authentic’ sample of $\alpha$-tocotrienol and it was assumed that it had deteriorated on storage. When $\alpha$-tocopherol and $\alpha$-tocotrienol or $\beta$-tocopherol and $\beta$-tocotrienol were collectively determined, $\Delta E_{\text{1cm}}^{1\%, \text{at } 262\mu m}$ values at 262$\mu m$ of 390 and 375 respectively were used.

**Table 3. $\Delta E_{\text{1cm}}^{1\%, \text{at } 262\mu m}$ values at 262$\mu m$ for the tocopherol-quinones in terms of their parent tocopherols**

Experimental details are given in the text. Each value is the average of three determinations.

- $\alpha$-Tocopherol: 390
- $\alpha$-Tocotrienol: 370
- $\beta$-Tocopherol: 395
- $\beta$-Tocotrienol: 368
- $\alpha$-Tocopherol + $\alpha$-tocotrienol: 390
- $\beta$-Tocopherol + $\beta$-tocotrienol: 375

The prior removal of triglycerides by saponification made the separation of $\alpha$-tocopherol and $\alpha$-tocotrienol possible, but resulted in high background signals, and it was then necessary to use higher attenuations, i.e. lower sensitivities. In contrast, the removal of triglycerides by methanolysis afforded gas chromatograms with very low background signals. However, though the recovery of $\alpha$-tocopherol from the methanolysis procedure was quantitative, the recovery of $\alpha$-tocotrienol was very low. This loss of $\alpha$-tocotrienol is almost certainly a result of an acid-catalysed cyclization of the unsaturated isoprenoid side chain (Shunk, Trenner, Hoffman, Wolf & Folkers, 1960).

In the analysis of shoots from 4-, 6- and 8-day seedlings (Hall & Laidman, 1968) the gas–liquid chromatograms of the alumina-column fractions corresponding to the dimethyl tocopherols contained a large peak, which had a retention time identical with that of $\beta$-tocotrienol. When this material was acetylated, it had a retention time identical with that of $\beta$-tocotrienol acetate. However, thin-layer chromatography showed that the fractions contained neither $\beta$-tocotrienol nor any other Emmerie–Engel-positive compound. It was concluded that the material was not a tocopherol but that it contained a single free hydroxyl group. A small amount of wax-like material was present in this fraction and this was examined as the possible contaminant. The bulk of this wax, which was eluted just before the dimethyl tocopherols from the alumina column, was crystallized three times from hot ethanol and yielded a white solid, m.p. 80°C. The material had a low solubility in light petroleum and was not saponifiable. It did not react with Liebermann–Burchard reagent and did not reduce permanganate. These observations were consistent with the material being a long-chain aliphatic alcohol and this was confirmed by infrared spectroscopy.

The wax was further investigated by gas–liquid chromatography on a dual-column 15%–SE–52 system with a linear temperature programme over the range 230–290°C (8°C increase/min.) and the retention times were compared with authentic samples of $C_{23}$, $C_{24}$ and $C_{25}$ n-alcohols. With the authentic compounds a straight line was obtained...
by plotting chain length against log (retention time). By extrapolation the natural wax sample was shown to consist predominantly of the C_{28} alcohol, n-octacosanol (m.p. 83°), accompanied by smaller amounts of n-hexacosanol and n-tetracosanol. These findings were confirmed by applying the authentic alcohols and the recrystallized wax to the 4%-SE-30 column. This stationary phase, in addition to separating the tocopherols, also separated the aliphatic alcohols and the plot of chain length against log (retention time) was again a straight line. The retention time of the n-octacosanol was identical with that of β-tocotrienol.

Since α-tocopherol also has the same retention time as n-octacosanol and β-tocotrienol on the 4%-SE-30 column it was necessary to ensure that the gas-liquid chromatography of the trimethyl-tocopherols did not include some of the wax material also. The results obtained for α-tocopherol by gas-liquid chromatography were therefore checked on duplicate samples by the Dilley & Crane (1963) assay procedure. The results obtained from the two methods of assay were in good agreement and the alumina-column chromatography procedure quantitatively separates the alcohols from the trimethyl tocopherols. When this work had been completed, it was reported (Eisner, Iverson & Firestone, 1966) that α-tocopherol was eluted together with n-octacosanol from a 1.5%-SE-52 column. Other aliphatic alcohols did not interfere with the separation of the tocopherols.

**Distribution of tocopherols and quinones in wheat grains.** The wheat grain contains a single ubiquinone homologue, ubiquinone-9 (Page, Gale & Folkers, 1959), and four tocopherols, α-tocopherol, α-tocotrienol, β-tocopherol and β-tocotrienol (Green, Marcinkiewics & Watt, 1955; Green, 1958) (for revised nomenclature see Pennock et al., 1964). These compounds can be separated and determined by the sequence of alumina column and gas-liquid chromatography described in the Methods section.

Hand dissection of the wheat grain into embryo and endosperm, followed by separate analysis of the parts, gave the results shown in Table 4. Ubiquinone-9 was present in both the embryo and the endosperm in approximately equivalent amounts. The tocopherols were also equally distributed between the embryo and endosperm, though the quantitative pattern was distinct in the two parts. β-Tocotrienol was found mainly, and α-tocotrienol entirely, in the endosperm, whereas α- and β-tocopherols were concentrated in the embryo. The relative percentages of each tocopherol are indicated in Table 5, and are compared with the results of the other workers on commercial samples. The percentage of α-tocopherol was higher, and that of β-tocopherol was lower, in our hand-dissected embryo compared with the published values for commercial wheat-germ oil. Though commercial wheat-bran samples do not contain significant amounts of starchy endosperm and are therefore not strictly comparable with our whole-endosperm fraction, a greater proportion of the total tocopherols in the hand-dissected endosperm could be accounted for as α-tocopherol. Since almost all of this α-tocopherol is in the bran, and very little of it is in the starchy endosperm (see Table 6), there is clearly a higher proportion of α-tocopherol present in the bran of hand-dissected endosperm than there is in the commercial preparation of bran.

In another experiment, the distributions of ubiquinone-9 and the tocopherols within the endosperm and embryo were investigated. The endosperm was further dissected into starchy endosperm and bran and the embryo was further dissected into embryo axis and scutellum. Though these analyses were performed on grain that had been soaked for 24 hr. to facilitate dissection, there are no changes in the total amounts of tocopherol during this period. In this experiment, after the alumina chromatography the trimethyl tocopherols were determined together and the dimethyl tocopherols were determined together by the modified Dilley & Crane (1963) method. Though gas–liquid chromatography was not used on this occasion, thin-layer chromatography was used to indicate whether the tocot was the predominant component within each group. The results of these experiments are shown in Table 6. Within the embryo, ubiquinone and α-tocopherol were found

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**Table 4. Distribution of ubiquinone and tocopherols within the ungerminated wheat grain**

Experimental details are given in the text. All values refer to 150 grains or grain parts. Abbreviations: α-T and β-T, α- and β-tocopherol; α-T3 and β-T3, α- and β-tocotrienol.

<table>
<thead>
<tr>
<th></th>
<th>Dry wt. (g.)</th>
<th>Total lipid (mg.)</th>
<th>Ubiquinone-9 (μg.)</th>
<th>Tocopherols (μg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td>0.13</td>
<td>37</td>
<td>16.5</td>
<td>α-T 73 β-T 16.4  α-T3 0 β-T3 6.7 Total 96.0</td>
</tr>
<tr>
<td>Endosperm</td>
<td>4.25</td>
<td>56</td>
<td>14.5</td>
<td>α-T 31 β-T 7.0  α-T3 15.5 β-T3 48.0 Total 102.0</td>
</tr>
<tr>
<td>Whole grain</td>
<td>4.4</td>
<td>94</td>
<td>32</td>
<td>Total by addition 104 23.4 15.5 54.7 198</td>
</tr>
</tbody>
</table>

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Table 5. Tocopherol distribution in the wheat grain compared with previously published values

All values are given as percentages of the total tocopherol content of the appropriate grain part.

<table>
<thead>
<tr>
<th>Percentage distribution of tocopherols</th>
<th>Present results</th>
<th>Bacharach &amp; Green (1961)</th>
<th>Mason &amp; Jones (1958)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dissected embryo)</td>
<td>(wheat-germ oil)</td>
<td>(wheat-germ oil)</td>
</tr>
<tr>
<td><strong>Embryo</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>76</td>
<td>56</td>
<td>57</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>17</td>
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<td>40</td>
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<tr>
<td>α-Tocotrienol</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-Tocotrienol</td>
<td>7</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td><strong>Endosperm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>30</td>
<td>38</td>
<td>11</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>α-Tocotrienol</td>
<td>47</td>
<td>36</td>
<td>68</td>
</tr>
<tr>
<td>β-Tocotrienol</td>
<td>15</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total by addition</strong></td>
<td>111</td>
<td>111</td>
<td>111</td>
</tr>
</tbody>
</table>

Table 6. Distribution of ubiquinone and tocopherols within the embryo and endosperm of the wheat grain

The grain was steeped in water for 24 hr. before dissection. All values refer to 150 grains or grain parts. Abbreviations: α-T and β-T, α- and β-tocopherol; α-T3 and β-T3, α- and β-tocotrienol.

<table>
<thead>
<tr>
<th>Total lipid (mg.)</th>
<th>Ubiquinone-9 (µg.)</th>
<th>α-T (µg.)</th>
<th>β-T + β-T3 (µg.)</th>
<th>Total (µg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryonic axis</td>
<td>14</td>
<td>12.9</td>
<td>21.3</td>
<td>26.8</td>
</tr>
<tr>
<td>Scutellum</td>
<td>21</td>
<td>10.9</td>
<td>29.5</td>
<td>42.5</td>
</tr>
<tr>
<td>Total by addition</td>
<td>35</td>
<td>23.8</td>
<td>50.8</td>
<td>68.3</td>
</tr>
<tr>
<td>Endosperm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bran</td>
<td>—</td>
<td>21.7</td>
<td>28</td>
<td>58</td>
</tr>
<tr>
<td>Starchy endosperm</td>
<td>—</td>
<td>2.6</td>
<td>5.0</td>
<td>30</td>
</tr>
<tr>
<td>Total by addition</td>
<td>—</td>
<td>24.3</td>
<td>33</td>
<td>88</td>
</tr>
</tbody>
</table>

DISCUSSION

The extraction of wheat grain and seedlings by hot propan-2-ol and chloroform, with a top-drive homogenizer, has been shown to be the best procedure for the extraction of the isoprenoid lipids from these materials. This may reflect the superiority of propan-2-ol in penetrating the starchy material of the grain. It is known that some lipids are difficult to remove from the starch of wheat (Wren & Szczepanowska, 1965). The results may also indicate the efficiency of propan-2-ol in inhibiting the activity of the tocopherol-destroying enzymes described by Booth (1962, 1965).

The application of a simple gradient-elution system to the chromatography of neutral lipids on alumina columns provides a system with considerable resolving power and substantially decreases the tailing of peaks, which occurs in procedures with stepwise gradient-elution patterns. Though the system efficiently separates the tocopherols into three groups according to the number of methyl groups on their aromatic ring, it is unable to separate fully the two geometric isomers of the dimethyl...
tocopherols, i.e. the $\beta$-tocopherol–$\beta$-tocotrienol group and the $\gamma$-tocopherol–$\gamma$-tocotrienol group. However, these two groups do not appear to occur together in significant quantities in Nature (Green, 1963), so that the problem of separating them is unlikely to occur.

Provided that precautions are taken, gas–liquid chromatography is a rapid and accurate means of separating and determining the individual tocopherols in the groups of tocopherols eluted from the aluminia column. Before the trimethyl tocopherol fraction can be subjected to gas–liquid chromatography, excess of triglyceride must be removed. Though methanolation provides a cleaner way of doing this than does saponification, application of the former method must be restricted to lipid extracts that contain $\alpha$-tocopherol but no $\alpha$-tocotrienol. Because $n$-octacosanol might be present in plant lipids and because it interferes with the gas–liquid chromatography of the dimethyl tocopherol fraction, it is essential, when the first analysis of a new tissue is being made, to check the gas–liquid-chromatographic results with an assay by the Dilley & Crane (1963) spectrophotometric method and by thin-layer chromatography. $n$-Octacosanol, though it is found in some plants, does not appear to be widely distributed (Deuel, 1951; Kreger, 1958), but it is possible that the alcohol may be a minor component of many plant waxes. Pollard, Chibnall & Piper (1933) have shown that the wheat-seedling wax is virtually pure octacosanol.

The value obtained for the total tocopherol content of the wheat grain (45 $\mu$g./g. dry wt.) is in good agreement with that (42-7 $\mu$g./g. dry wt.) given by Green (1958). In any case, varietal differences are to be expected, and we have found that amounts of tocopherol in grain can fall markedly during storage. Previous attempts to determine the tocopherol contents of parts of the wheat grain have been made on commercial preparations of bran, germ and germ oil. Such samples are often far from pure (Johnson & Stern, 1957) and, as a result of the milling process, tocopherols may have been destroyed to a considerable degree. All indications (Moore, Sharman & Ward, 1957; Mason & Jones, 1958) point to the fact that $\alpha$-tocopherol is less stable than the tocotrienols both in vitro and in the grain. The relatively low amounts of $\alpha$-tocopherol, compared with other tocopherols, that were found in commercial bran, flour and germ by previous workers probably reflects this fact. However, even in the hand dissection of the grain there is some loss of tocopherol, particularly of $\alpha$-tocopherol. There is no equivalent loss of ubiquinone on dissection. Despite these differences from the earlier published values, the qualitative aspects of the data are in agreement. In particular, the absence of $\alpha$-tocotrienol from the embryo has been confirmed. No evidence could be obtained for the existence of tocopherol esters in the grain or seedling, despite recent reports of their occurrence in other species (Dunphy, Wittle, Pennock & Morton, 1965). The finding that, within the endosperm, ubiquinone is concentrated in the bran is in good agreement with the knowledge that in the cereal grain the aleurone layer is the only living tissue outside the embryo (Varner, 1964; Ingle, Beever & Hageman, 1964). For the same reason, the tocopherols of the bran are probably also located in the aleurone tissue. The small amount of ubiquinone in the starchy endosperm is probably either non-functional or reflects a very low rate of respiration (Barnell, 1937). $\alpha$-Tocopherol, like ubiquinone, was found only in those parts of the grain that are capable of active metabolism. Both are virtually absent from the starchy endosperm, in contrast with the tocotrienols.

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REFERENCES


