These results confirm earlier reports (7, 17) that monocarboxylic acids are formed upon irradiation of fats under vacuum. Day and Papaioannou (7) obtained evidence for alk-2-enals and alk-2,4-dienals in irradiated milk fat. In the present study, traces of the C6, C8, C10, and C12 alk-2-enals but no alk-2,4-dienals were identified. Qualitatively, the methyl ketones did not show a significant change upon irradiation (Table IV); however, they are present in much larger quantities in irradiated milk fat. The origins of methyl ketones and long chain aldehydes have been discussed by Day and Papaioannou (7). They suggested that hydrolytic cleavage of ester and enol-ether linkages were responsible for these compounds. Work in our laboratory (14) has demonstrated that hydrolysis as well as cleavage of ester linkages occurs with irradiation. Langley and Day (15) have suggested that methyl ketones are formed in heated milk fat as a result of hydrolysis. Also, long chain aldehydes occur in milk fat as bound aldehydes (9, 24). Thus, irradiation-induced hydrolysis of ester and enol-ether linkages is probably a likely mechanism accounting for the production of ketones and long chain aldehydes in irradiated milk fat. The formation of short chain aldehydes may also be explained by a similar mechanism.

\[
\begin{align*}
O & \quad O \\
R-C-O-R' & \rightarrow R-C + R'O \quad (4) \\
O & \quad O \\
R-C + XH & \rightarrow R-C-H + X- \quad (5)
\end{align*}
\]

The finding of C6 and C8 methyl ketones is difficult to explain. The presence of these and many other even- and odd-numbered saturated and unsaturated ketones and the probable mechanism of their formation in irradiated fats have been reported by Kohn (16).

Compounds such as ethyl acetate, benzene, chloroform, and dichloro-
benzene were also detected in the control sample (Table III). Chloroform has been reported in milk fat by Wong and Patton (32). It was suggested that this compound arises from pesticide residues in milk. Dichlorobenzene, which has not been reported previously in milk, could also have its origin in pesticides.

Four compounds listed in Table I (peak numbers 31, 35, 37, and 39) had a characteristic candle-like odor. These compounds were present in relatively small quantities (Figure 2). The exact nature of these compounds could not be determined. Work is in progress in our laboratory to characterize and identify these components further.

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**ANALYTICAL METHOD**

**Determination of the Pungent Constituents of Piper nigrum**

Since spice oleoresins have come into the market in several forms, the need for good analytical methods for determination of the active constituents has grown. This holds especially true for pepper. For piperine (1-piperoylpyridine), the following methods have been described in the literature:

- **A.** Kjeldahl determination of nitrogen (7, 6, 7).
- **B.** Polarographic determination (6, 17).
- **C.** Iodometric procedure (5).
- **D.** Spectrophotometric method (ultraviolet-absorption) (6, 7).
- **E.** Colorimetric method using chromotropic acid (2, 7, 13).
- **F.** Colorimetric method using nitric acid (70).
- **G.** Colorimetric method using sulfuric acid and an aromatic aldehyde (8).
- **H.** Colorimetric method using phosphoric acid (9).
- **I.** Method presented in this paper. Figure 1 shows the part of the piperine

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Existing methods for the determination of piperine are briefly discussed. A new procedure is proposed, based upon alkaline hydrolysis at about 138°C, followed by titration of the liberated piperidine after distillation with water. The result of this method is a measure of the total pungency. A suggestion is made on the manner in which vinyl homologs of piperine are formed.

A-COC=CH=CH=CH-B

\[ \text{piperidine nucleus} \]

B = methylenedioxybenzene nucleus

pepper of a whole series of vinyl homologs of piperine and their stereoisomers would be imaginable. The investigations of Staudinger (20, 21) justify the supposition that these compounds, if present, contribute to the pungency of pepper. Possibly, some of these substances are determined by methods E, F, and G, but not, or not completely, by methods D and H, owing to the higher specificity for piperine of the latter methods.

The purpose of this paper is to introduce a new method, the "hydrolysis method," for the determination of piperine and other bite principles of pepper and pepper oleoresins (method I). This method attacks the amide bond of the piperidine ring, which is essential for the pungency in contrast with the methylenedioxy grouping and the aliphatic double bonds (12, 20, 21). The point of attack differs from that of all other methods. Possible interferences need not overlap those of existing procedures. For instance, piperonal, that may cause high results with both methods E and F, does not interfere. Piperic acid, which interferes with all colorimetric methods, does not influence the hydrolysis method. Thus methods D, E, F, H, and I can furnish complementary data, also in case of adulterations.

The investigation was started when the author encountered difficulties in applying method E. Some examples of these difficulties are given in Tables I and II. On heating piperine or piperine-containing products at 90°C, the author found that the piperine content, determined by method E, dropped with time (Table I). If, according to the directions of the American Spice Trade Association (2), ground pepper was extracted with alcohol on the steam bath for 3 hours, a lower piperine content was found than after an extraction of 24 hours. The fineness of grinding, however, did not affect the result of the analysis (Table II).

<table>
<thead>
<tr>
<th>No. of Hours</th>
<th>% Piperine by Chromotropic Acid Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>101.3 100.8</td>
</tr>
<tr>
<td>2</td>
<td>98.5 99.3</td>
</tr>
<tr>
<td>4</td>
<td>96.8 97.4</td>
</tr>
<tr>
<td>6</td>
<td>95.4 96.5</td>
</tr>
<tr>
<td>24</td>
<td>93.2 94.0</td>
</tr>
</tbody>
</table>

Table I. Piperine at 90°C C.
**Table II.** Determination of Piperine in Ground Pepper

<table>
<thead>
<tr>
<th>Preparation of Sample</th>
<th>3-hour Extraction</th>
<th>24-hour Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Coarsely ground in hammill</td>
<td>7.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Finely ground in electric mill</td>
<td>7.6</td>
<td>7.5</td>
</tr>
<tr>
<td>Extra finely ground in electric mill</td>
<td>7.5</td>
<td>7.6</td>
</tr>
</tbody>
</table>

**Experimental**

**Apparatus.** Special apparatus is not required. Flasks, condensers, and Soxhlet extraction apparatus should be provided with ground glass joints.

**Materials.** 2 N KOH in diethylene glycol is prepared by dissolving 112 grams of KOH in 80 grams of water and diluting to 1 liter with diethylene glycol. Boric acid, reagent grade.

**Diluting to 1 liter with diethylene glycol.** Required. Flasks, condensers, and Soxhlet extraction apparatus should be provided with ground glass joints.

**Oleoresin pepper 1.** was prepared from a commercial sample. Contains lactic acid. Oleoresin pepper II, was prepared from a natural oleoresin by adding lactic acid. Oleoresin pepper II, a commercial sample, contains lactic acid. Oleoresin pepper III is a natural oleoresin from black Lampong pepper.

**Procedures.** **CHROMOTROPIC ACID METHOD.** The directions for analysis were set down in the guise of the data of Table I. The conditions of the K.OH solution are listed in Table IV. The author obtained a conversion of about 60% by refluxing 8.8 meq. of pure piperine with 25 ml. of 1 N KOH in diethylene glycol for 4 hours.

**Table III.** Piperine Content by Hydrolysis Method

<table>
<thead>
<tr>
<th>Test No.</th>
<th>Solvent</th>
<th>KOH Solution</th>
<th>Reflux Temp.</th>
<th>Reflux Time</th>
<th>% Piperine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DEG</td>
<td>1</td>
<td>158</td>
<td>4</td>
<td>96.8</td>
</tr>
<tr>
<td>2</td>
<td>DEG</td>
<td>2</td>
<td>138</td>
<td>4</td>
<td>94.4</td>
</tr>
<tr>
<td>3</td>
<td>DEG</td>
<td>3</td>
<td>132</td>
<td>4</td>
<td>94.9</td>
</tr>
<tr>
<td>4</td>
<td>DEG</td>
<td>4</td>
<td>130</td>
<td>4</td>
<td>93.0</td>
</tr>
<tr>
<td>5</td>
<td>DEG</td>
<td>5</td>
<td>230</td>
<td>4</td>
<td>97.6</td>
</tr>
<tr>
<td>6</td>
<td>DEG</td>
<td>6</td>
<td>25</td>
<td>4</td>
<td>95.9</td>
</tr>
<tr>
<td>7</td>
<td>DEG</td>
<td>7</td>
<td>103</td>
<td>2</td>
<td>101.2</td>
</tr>
<tr>
<td>8</td>
<td>DEG</td>
<td>8</td>
<td>100</td>
<td>2</td>
<td>100.3</td>
</tr>
<tr>
<td>9</td>
<td>EA</td>
<td>2</td>
<td>82</td>
<td>2</td>
<td>48.9</td>
</tr>
<tr>
<td>10</td>
<td>EA</td>
<td>3</td>
<td>80</td>
<td>2</td>
<td>43.0</td>
</tr>
<tr>
<td>11</td>
<td>DEG</td>
<td>10</td>
<td>98.8</td>
<td>2</td>
<td>98.8</td>
</tr>
<tr>
<td>12</td>
<td>DEG</td>
<td>12</td>
<td>97.1</td>
<td>2</td>
<td>97.1</td>
</tr>
</tbody>
</table>

When the distillation is finished, titrate the liquid in the receiver with 0.1 N HCl to the original red color of 0.5 ml.

**Determination of Bitter Constituents in Pepper.** Extract 15 grams of ground pepper with acetone in a Soxhlet apparatus for about 16 hours, using a 500-ml. round-bottomed extraction flask. Distill the solvent over in a water bath through a descending Liebig’s condenser with splash head—first at normal pressure, then at the vacuum of a Geissler pump. Do not allow the temperature to rise above 85°C. It does not matter if part of the essential oil distills over. After removal of the solvent, hydrolyze the extract which should then be treated further in the 500-ml. flask the same way as oleoresin of pepper.

**Calculation.** Percentage bitter constituents, calculated as piperine:

\[ \rho = \frac{2.853 \times a \times t}{g} \]

where \( a \) = ml. of 0.1 N HCl

\( t \) = titer of HCl

\( g \) = mg. of oleoresin or pepper

**Results and Discussion.**

The use of KOH in diethylene glycol was borrowed from Critchfield (4). Taking the circumstances under which the ester content of an essential oil is normally determined (2-gram sample, 1-hour reflux with 25 ml. of 0.5 N alcoholic KOH), very little piperine is hydrolyzed. Even more vigorous conditions than those of Critchfield for the saponification of simple acid amides, 0.5 to 1.5 hours of reflux with 50 ml. of 1 N KOH in diethylene glycol—are insufficient for total hydrolysis. The author obtained a conversion of about 80% by refluxing 8.8 meq. of pure piperine with 25 ml. of 1 N KOH in diethylene glycol for 4 hours.

Table III gives the result of a series of tests intended to establish the optimal conditions for complete hydrolysis of piperine. The tests were generally carried out according to the directions given above. Deviating conditions are mentioned in Table III. In test 11, methyl red was used as an indicator; instead of saturated boric acid solution, water was placed in the receiver in test 12.

The directions for analysis were set down on the basis of the data of Table III. The conditions are those of test 7. The mixed indicator gives somewhat better results than methyl red (test 11). The use of saturated boric acid solution is a measure of precaution to prevent loss of piperidine in the initial stage of the distillation (test 12). Table III shows that a correct relation among reaction temperature, reaction time, and excess of reagent is important.

For various products the piperidine contents by both the hydrolysis and chromotropic acid methods are listed in Table IV. In the hydrolysis method, the standard conditions were generally followed. In experiments 5, 8, and 11, less piperidine was weighed than the directions call for; in tests 7 and 10, 120 ml. of KOH solution was used instead of 100 ml. Oleoresins I and II have a higher acid content than sample III and therefore were treated, as far as the hydrolysis method is concerned, with various proportions of oleoresin-KOH solution. The pepper of test 14 is a mixture of black Serrawak and black Lampong pepper. At present, no means are at the author’s disposal to ascertain the reliability of the hydrolysis method for determining the bitter constituents of pepper and oleoresins of pepper. Acid amides, ammonium compounds, and to a lesser degree proteins may cause interference. However, the author was not able to detect ammonia in the distillate after analysis of an oleoresin. Large amounts of alkali-consuming constituents interfere, and the quantity or
normality of the KOH solution may have to be varied from standard directions (Table IV, tests 5 to 10).

The four samples of piperine in Table IV have very different contents by the chromotropic acid method, whereas the hydrolysis method shows about 100% in all cases. In the author’s opinion, this fact favors the hydrolysis method. In Table IV, the colorimetric method gives much higher results than the hydrolysis method. In view of the uncertainty regarding the calibration factor, this is of minor importance. More significantly, the ratio between the results of the two methods is not constant.

Whether piperine might be directly determined by hydrolysis in oleoresins, distributed on dextrose, was examined in the experiments listed in Table V. These tests were performed before the hydrolysis method was subjected to an extensive examination, so that there are deviations from the standard directions. The oleoresins of pepper on dextrose II and on salt contain equal percentages of piperine, whereas the hydrolysis method has subjected to an large excess of alkali. Table V, test 2, indicates that dextrose severely interferes with the chromotropic acid procedure. The hydrolysis method can be used for oleoresins on a dextrose base. Experiments 4, 5, and 6 demonstrate that the presence of dextrose requires a large excess of alkali.

Acknowledgment

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