Constituents of Cane Molasses

Part II. Separation and Identification of the Phenolic Compounds*

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By gas chromatography the following eight phenolic compounds and benzoic acid were identified from a sample of cane final molasses using both polar and non-polar stationary phases: anisole, phenetole, phenol, m-cresol, salicylic acid, resorcinol, vanillic acid, and syringic acid. The peaks corresponding to p-coumaric acid and vanillin were also found using non-polar phase. The structures of four or five unidentified components were inferred from the relation between retention temperature and functional group number of the phenolic compounds.

INTRODUCTION

In the course of investigation on so-called "sugary-flavor" inherent to crude cane-sugar, Takei and Imaki isolated acetic acid,1) valeric acid,1) benzoic acid,1) and syringic acid2) from an ether extract of cane molasses. They reported that odoriferous constituents may exist in the fraction corresponding to phenolic compounds. On the other hand, Obata and his co-workers3) detected paper chromatographically catechol, p-hydroxybenzoic acid, melilotic acid (dihydro-o-coumaric acid), salicylic acid, syringic acid, vanillin, vanillic acid, and three unknown compound in beet molasses. Recently the authors investigated the phenolic compound fraction in cane molasses employing gas-liquid chromatography and found the presence of at least eight phenolic compounds. Among these, the compounds newly found were anisole, phenetole, phenol, m-cresol, resorcinol, vanillic acid, and salicylic acid besides syringic and benzoic acids reported previously.1,2)

EXPERIMENTAL

Materials

Cane molasses. The cane final molasses used in this experiment was the product of Taiwan Sugar Corporation, Wushulin Factory, December 1963.

Phenolic compounds. All of the phenolic compounds including syringic acid were obtained from commercial sources.

Solid support of gas chromatographic column.4) Celite No. 545, 80-100 mesh, was washed with 6N hydrochloric acid as reported previously.5) Thirty grams of the Celite was suspended in 120g of n-hexane, and 7.2g of hexamethyldisilazane was added. The mixture was refluxed for 5hr, filtered and dried. The silanized Celite was washed with dry n-hexane.

Apparatus. Gas chromatographic analyses were conducted with a Model GCG-1 Yanagimoto gas chromatograph and a Model GC-1B Shimadzu gas chromatograph equipped with dual columns and a

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thermal conductivity detector.

Preparation of gas chromatographic columns. In Yanagimoto gas chromatograph, helical stainless-steel columns, 100 cm in length and 0.3 cm i. d. were packed with 30 w/w% DC 430 coated on pretreated Celite No. 545 and then equilibrated for about 15 hr at 240°C with helium before injection of the sample. In Shimadzu apparatus, U-shape stainless-steel columns, 75 cm in length and 0.6 cm i. d., were used.

As polar stationary phase, 10% neopentylglycol succinate (NGS), was used. The columns were packed by a combination of vibration with electric sander and gentle tapping.

Gas chromatographic conditions. With Yanagimoto apparatus

- Injector temperature .....................260°C
- Column temperature ..........initial 110°C, final 260°C
- Program rate ..................4~8°C/min
- Helium flow rate .............30 ml/min at 110°C

With Shimadzu apparatus

- Injector temperature .....................260°C
- Column temperature ..........initial 110°C, final 260°C
- Program rate ..................5°C/min
- Helium flow rate .............75 ml/min at 110°C

Fractionation of cane molasses. The molasses was fractionated into three fractions, bicarbonate-soluble, alkali-soluble, and neutral fractions, as follows. Fifty kilograms of the molasses was diluted with 50 l of deionized water. To the solution (pH 5.2~5.3), 50% trichloroacetic acid (TCA) was added until the final concentration of TCA reached to about 5%. The precipitate was removed by decantation and filtration, and the filtrate was then extracted with ether for about 180 hr employing continuous liquid-liquid extractor. The ether extract was dried over anhydrous magnesium sulfate and then fractionated in turn with saturated sodium bicarbonate, 1% sodium hydroxide solutions in similar manner as described previously.1)

The bicarbonate-soluble and the alkali-soluble fractions were evaporated to dryness under reduced pressure below 45°C and subjected to the following trimethylsilylation. The neutral fraction (yield 17 g) was stored for the investigation of its constituents.

Trimethylsilylation for the preparation of gas chromatographic samples. Five hundred milligrams of both bicarbonate-, and alkali-soluble fractions were dried over phosphoric anhydride in vacuo and dissolved in 0.4 ml of dry pyridine respectively. A mixture of 0.3 ml of trimethylchlororosilane and 0.3 ml of hexamethyldisilazane was added. The mixture was refluxed for 1 hr under anhydrous conditions6,7) and then centrifuged. An aliquot (20~30 µl) of the supernatant was injected into gas chromatograph.

Trimethylsilylation of the reference standards was carried out in the following manner.8) Ten milligrams of the standard compound was dissolved in 0.1 ml of dry pyridine and a mixture of 0.1 ml of trimethylchlororosilane and 0.1 ml of hexamethyldisilazane was added. After shaking for about 5 min, the reaction mixture was centrifuged to precipitate ammonium chloride. An aliquot (1~10 µl) of the supernatant was injected into gas chromatograph.

Identification. The components were identified by comparing the retention times and also by the addition of supposed constituents. The shape of each gas chromatographic peak was carefully examined to confirm symmetrical single peak after the addition of reference standard. Normally, such procedures were performed on both polar and non-polar phases.9)

RESULTS AND DISCUSSION

The representative gas chromatogram of the alkali-soluble fraction, obtained from a temperature-programmed operation on a 100 cm

Fig. 1. Chromatogram of the TMS Derivative of Alkali-Soluble Fraction on DC-430 Column.

8) A. Sato, K. Kitao, and M. Senda, Wood Research, No. 34, 94 (1965).
30% DC 430 column, is shown in Fig. 1. Main peaks are named 1, 2, 3, ... in the order of elution. At least eight different compounds appear to be present and of these compounds anisole (peak 1), phenetole (peak 2), phenol (peak 3), m-cresol (peak 5), and benzoic acid (peak 7) were identified in the manner described above. The peaks 4, 6 and 8 were not identified.

The typical gas chromatogram of the bicarbonate-soluble fraction, obtained under similar gas chromatographic conditions, is shown in Fig. 2. At least sixteen different compounds appear to be present and of these anisole (peak 1), phenetole (peak 2), phenol (peak 3), m-cresol (peak 4), benzoic acid (peak 6), salicylic acid (peak 8 and 11), resorcinol (peak 9), vanillin (peak 12), vanillic acid (peak 14), syringic acid (peak 15), and p-coumaric acid (peak 16) were identified. The occurrence of anisole and phenetol in these fractions seems to be surprising, but at present there is no definitive information to explain this phenomenon.

Salicylic acid exhibited two peaks and the pure authentic sample also showed double peaks. This phenomenon presumably is due to the presence of mono- and di-trimethylsilyl salicylates. The peaks 5, 7, 10 and 13 were not identified. The relative retention times (to phenol) of these peaks and the

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**TABLE I. RELATIVE RETENTION TIMES (tRR) OF THE INDIVIDUAL PEAKS OF BICARBONATE- AND ALKALI-SOLUBLE FRACTIONS**

<table>
<thead>
<tr>
<th>Bicarbonate-soluble fraction</th>
<th>Alkali-soluble fraction</th>
<th>Components identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak No.</td>
<td>tRR</td>
<td>Peak No.</td>
</tr>
<tr>
<td>1</td>
<td>0.62</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1.00*</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>1.26</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1.40</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1.58</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>1.81</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>1.94</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>2.06</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>2.22</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>2.42</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
<td>2.54</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td>2.90</td>
<td>13</td>
</tr>
<tr>
<td>14</td>
<td>3.15</td>
<td>14</td>
</tr>
<tr>
<td>15</td>
<td>3.55</td>
<td>15</td>
</tr>
<tr>
<td>16</td>
<td>3.69</td>
<td>16</td>
</tr>
</tbody>
</table>

* Retention time, 4.7 minutes; ** Retention time, 4.4 minutes.

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**TABLE II. RELATIVE RETENTION TIMES OF TRIMETHYLSILYL DERIVATIVES OF REFERENCE STANDARDS ON DC 430 COLUMN**

<table>
<thead>
<tr>
<th>Compound</th>
<th>tRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anisol</td>
<td>0.50</td>
</tr>
<tr>
<td>Phenetole</td>
<td>0.72</td>
</tr>
<tr>
<td>Phenol</td>
<td>1.00*</td>
</tr>
<tr>
<td>m-Cresol</td>
<td>1.41</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>1.70</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>1.71</td>
</tr>
<tr>
<td>Phenylacetic acid</td>
<td>1.85</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>2.04</td>
</tr>
<tr>
<td>o-Hydroxybenzyl alcohol</td>
<td>2.11</td>
</tr>
<tr>
<td>2, 6-Dimethoxyphenol</td>
<td>2.34</td>
</tr>
<tr>
<td>p-Hydroxybenzyl alcohol</td>
<td>2.44</td>
</tr>
<tr>
<td>Vanillin</td>
<td>2.57</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2.65, ** 1.93</td>
</tr>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>2.96</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>3.41</td>
</tr>
<tr>
<td>Syringic acid</td>
<td>3.90</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>4.03</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>4.51</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>4.60</td>
</tr>
</tbody>
</table>

* Retention time of TMS-phenol, 4.6 minutes; ** The TMS derivative showed double peaks. The smaller value presumably is due to the presence of mono-TMS derivative.
reference standards are listed in Tables I and II.

Much of the previously reported work\(^9\) on gas chromatography has indicated that successful identification of complex mixtures of closely related compounds requires analyses on both polar and non-polar phases. Thus, neopentylglycolsuccinate was chosen as a polar stationary phase. The gas chromatograms of the alkali- and bicarbonate-soluble fractions, obtained from a temperature-programmed operation on a 75 cm NGS column, are shown in Figs. 3 and 4 respectively. In both chromatograms it was elucidated that the peak 1 contained anisole and phenol, the peak 2 phenetole and m-cresol, and the peak 4 benzoic acid and resorcinol. The peaks 7, 10 (Fig. 4), and 11 (Fig. 4) were assigned to salicylic, vanillic, and syringic acids respectively. The peaks of vanillin and p-coumaric acid might be overlapped with other peaks which possess close retention times, presumably owing to incomplete separation.

It has been reported that a linear relationship exists between logarithm of the relative retention times and the structures in homologous series of phenolic compounds\(^8\) and alkylbenzenes.\(^{10,11}\) These investigators thought that the use of such relationship may provide structural information about the unknown components. In the experiment for this purpose, it is ideal that the relative retention

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times are determined at constant temperature operation. But the values obtained at a temperature-programed operation on DC430 column, were used tentatively. In semi-logarithmic paper phenol was plotted at unity in vertical axis, and the functional group numbers (for example, a methyl group arbitrarily set at 1, a methoxy group 2, a hydroxyl group 3, and a carboxyl group 5) were plotted in horizontal axis. A curve obtained in such manner is shown in Fig. 5. It is supposed that a straightline might be obtained when the values determined on an isothermal operations were used. However, when the retention temperature instead of logarithmic relative retention time was employed in the vertical axis, a straightline was obtained in the range between phenol and vanillic acid (see Fig. 6). On plotting the retention temperatures of unknown components on the line, the functional group numbers were obtained. From the result, the following structures were inferred for unidentified components, though considerations to the other functional groups familiar in such natural products (for example, aldehyde, isopropyl, and allyl groups, etc.) must be given preferably.

The peak 6 of alkali-soluble fraction:

\[
\text{HO-} \begin{array}{c} \text{CH}_3 \\
\end{array}
\]

The peak 8 of alkali-soluble fraction:

\[
\text{HO-} \begin{array}{c} \text{CH}_3 \\
\end{array}
\]

The peak 5 of bicarbonate-soluble fraction:

\[
\text{HO-} \begin{array}{c} \text{CH}_3 \\
\end{array}
\]

The peak 10 of bicarbonate-soluble fraction:

\[
\text{HO-} \begin{array}{c} \text{CH}_3 \\
\end{array}
\]

The peak 13 of bicarbonate-soluble fraction:

\[
\text{HO-} \begin{array}{c} \text{CH}_3 \\
\end{array}
\]

The occurrence of phenol\(^{12}\) and its ether\(^{13}\) in green tea and tobacco leaves had been recorded respectively. The presence of an ether of m-cresol in black tea has also been reported\(^{14}\).

Some of these compounds identified and inferred may relate to the flavor of molasses,

but more odoriferous constituents to which the molasses, the Wa-Sanbonjiro (white soft non-centrifugal sugar) and the rums owe mainly their characteristic flavor seemed to remain in the neutral fraction. The separation and characterization of the constituents are in progress.

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