Analysis of Non-volatile Organic Acids in Fermented and Dried Cocoa Beans by High Performance Liquid Chromatography.

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Key words: Cocoa beans, non-volatile acids, HPLC.

ABSTRACT

A high performance liquid chromatographic method for analysis of non-volatile acids (oxalic, citric, tartaric, succinic, malic and lactic) in fermented and dried cocoa beans is described. Bean samples were pulverized in dionized water using a Polytron Homogenizer (Brinkman) for 20 sec and centrifuged at 14000 rpm for 45 min at 25°C. The extract was alkalized to pH between 8-9 and passed through intermediate base anion exchange resin; the acidic fraction was eluted after adding 10% sulphuric acid to the column. Polyphenols in the fraction were then eliminated by passing the acidic fraction through a reverse phase SEP-PAK that had been pre-wet with methanol. The eluate was analyzed for non-volatile acids using Organic Acid Column (Bio-Rad) with 0.1N H\textsubscript{2}SO\textsubscript{4} as a mobile phase at 65°C. The acids were detected at 214 nm and quantified by comparing peak height of sample to those of standards. The method demonstrated excellent reproducibility and recoveries of the added acids.

INTRODUCTION

Non-volatile acids are widely present in many fruits, and processed food. Freshly harvested cocoa beans contain about 0.2-0.3% citric acid (Duncan 1969) but only traces occur in the cotyledons. However, when the beans undergo fermentation, other acids are developed through the metabolism of reducing sugar in the pulp by microorganisms. The acids from the pulp then diffuse into the cotyledons, resulting in an increase in the acidity of the cotyledons. The acidic environment provides an optimum pH for the enzyme reactions leading to the formation of flavour precursors. Acids could also be produced from a metabolic process within the cotyledon. HPLC has been widely used in the measurement of organic acids.
in many foods viz. dairy products (Marsili et al. 1981), beef (Nassos et al. 1984), guava (Wilson et al. 1982), potatoes (Bushway et al. 1984; Augustin et al. 1981), tomato juice (Gaucedo and Luh 1986), sweet potatoes (Picha 1985), grape mustard wine (Frayne 1986). Most investigations of nonvolatile acids in cocoa have utilized gas chromatography (Weissberger et al. 1971) and paper chromatography (Bonar et al. 1968; Rohan and Stewart 1964). Paper chromatography requires large samples, lengthy analysis time and gives only semi-quantitative results. Gas chromatography requires lengthy sample preparation time. Furthermore, the gas chromatography technique requires that the non-volatile acids be converted to volatile components such as TMS ethers and methyl esters; in the process some of the acids are lost. The TMS ether method cannot be used for quantitative measurements due to incomplete precipitation and loss of lead acids during sample preparation. The methyl ester method is suitable for quantification purposes for most acids. However, tartaric acid was not detected using the method (Weissberger et al. 1971). The objectives of this investigation were to develop a suitable extraction method for HPLC analysis of non-volatile acids (oxalic, citric, tartaric, succinic, malic and lactic) in fermented and dried cocoa beans, and to evaluate the accuracy and precision of the procedure.

**MATERIALS AND METHODS**

**HPLC Apparatus and Operation Conditions**

Analytical column: Organic Acid 300 x 7.8 mm i.d. (Bio-Rad lab., Richmond, CA); guard column: cation H⁺, 40 x 4.6mm (Bio-Rad). The column was immersed in the water bath at 65 ± 2 °C and the temperature was maintained by controlling the flow rate of the circulated hot water. Pump: Model 6000A (Waters Associates, Milford, MA); injector: Model U6K (Waters); detector: Model 441 fixed wavelength (Waters) set at 214 nm; integrator: Model 3392A (Hewlett-Packard, Avondale, PA); mobile phase: 0.01N H₂SO₄, 0.7 mL/min.

**Standards and Solvents**

Oxalic, tartaric, citric, malic, succinic, lactic: Sigma Chemical Company, St. Louis, MO; sulphuric acid: Fisher Scientific Company, Pittsburgh, PA.

**Sample Preparation**

The fermented and dried bean was deshelled and degermed. The nibs were ground using a micro jet — 10J (Quartz Technology Inc., New York, NY) attached with a 50 mesh — size screen; small pieces of solid carbon dioxide were added to prevent any frictional heat caused by grinding from melting the cocoa lipids. The ground sample was pulverized in 25 mL deionized water using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) for 20 sec. The extract was then centrifuged at 14,000 rpm for 45 min at 25 °C. The extract was alkalized to a pH between 8-9 with 5 N ammonium hydroxide. The alkalized extract was pipetted into a disposable chromatography column (0.8 x 4 cm) containing intermediate base anion exchanger Bio-Rex 5 of 200 mesh, Cl form (Bio-Rad lab., Richmond, CA) that had already been washed with deionized water. Using a 10 mL high pressure Micromate syringe (Popper and Sons, Inc., New Hyde Park, NY), a 5 mL neutral fraction was eluted from the column. One mL of 10% sulphuric acid was pipetted into the column, and 25 mL of the acidic fraction was eluted. The acidic fraction was filtered through a 0.45 um, 25 mm GA-6 Metrical membrane filter (Gelman Science Inc., Ann Arbor, MI). The phenolic compounds were removed from the fraction by passing the fraction through a Waters Associates (Milford, MA) C₁₈ reverse phase SEP-PAK which had been pre-wet with 2 mL methanol and 5 mL distilled water.

**Quantitative Analysis**

Preparation of standard curves. Standard non-volatile acid solutions were prepared in deionized water in triplicate at the following concentrations, oxalic (0.06, 0.12, 0.18, 0.24, 0.30 g/100g); tartaric (0.08, 0.16, 0.24, 0.32, 0.40 g/100g); lactic (0.20, 0.40, 0.62, 0.80, 1.00g/100g); citric, malic, succinic (0.16, 0.32, 0.48, 0.64, 0.80 g/100g). The acid solutions were analyzed on the HPLC, and the average peak height response from two injections of each triplicate sample were measured at 0.05 AUFS and were recorded. Linear regression was determined by the least squares method and the correlation coefficient (r) of peak height versus concentration was calculated.

Reproducibility and recovery studies. Reproducibility of the entire method was determined by measuring the acids from five different lots of...
homogenous ground cocoa beans. Duplicate extractions of duplicate injections of each extract were analyzed on the HPLC.

Standard deviation and elective standard deviation were calculated to assess the reliability of the procedure. For recovery tests, duplicate samples of cocoa extracts were 'spiked' with known concentrations of authentic acids - oxalic (0.06, 0.12, 0.18, 0.24 g); citric (0.26, 0.32, 0.48, 0.64 g); succinic, malic (0.16, 0.32, 0.48, 0.04 g); lactic (0.10, 0.20, 0.30, 0.40 g); tartaric (0.03, 0.06, 0.09, 0.12 g). The samples were analyzed as previously described.

RESULTS AND DISCUSSION
Quantitative Analysis of Non-volatile Acids
Typical chromatograms of both the cocoa extract and the standard acids are presented in Figure 1. Peak height response for each acid in cocoa extracts was compared to standard curves for quantification purposes. Curves for each standard acid at 0.05 AUFS were obtained (Table 1). Correlation coefficients in excess of 0.999 were obtained for all the acid calibration curves.

<table>
<thead>
<tr>
<th>g/100 g injected</th>
<th>Peak height (unit) at 0.05 AUFSb (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalic</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>10.73</td>
</tr>
<tr>
<td>0.12</td>
<td>19.97</td>
</tr>
<tr>
<td>0.18</td>
<td>31.45</td>
</tr>
<tr>
<td>0.24</td>
<td>42.07</td>
</tr>
<tr>
<td>0.30</td>
<td>54.75</td>
</tr>
</tbody>
</table>

\[ y = -1.25 + 55.07x; r = 0.999^a \]

\[ Citric \]
\[ 0.08 \]
\[ 0.16 \]
\[ 0.24 \]
\[ 0.32 \]
\[ 0.40 \]

\[ y = 0.36 + 32.02x; r = 0.999 \]

\[ Tartaric \]
\[ 0.08 \]
\[ 0.16 \]
\[ 0.24 \]
\[ 0.32 \]
\[ 0.40 \]

\[ y = 0.22 + 21.52x; r = 0.999 \]

\[ Succinic \]
\[ 0.16 \]
\[ 0.32 \]
\[ 0.48 \]
\[ 0.64 \]
\[ 0.80 \]

\[ y = 39.20 + 2389x; r = 0.999 \]

\[ Malic \]
\[ 0.16 \]
\[ 0.32 \]
\[ 0.48 \]
\[ 0.64 \]
\[ 0.80 \]

\[ y = 1383.63x; r = 0.999 \]

\[ Lactic \]
\[ 0.20 \]
\[ 0.40 \]
\[ 0.60 \]
\[ 0.80 \]
\[ 1.00 \]

\[ y = 39.11 + 2525.39x; r = 0.999 \]

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a All data are the average of triplicate standard solutions and duplicate injections.

b AUFS = absorbance units full scale

c Linear regression equation

d Correlation coefficient

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Reproducibility and Recovery Studies

A relative standard deviation range of 0.22 - 7.42% was obtained (Table 2) in estimating the overall error in the final results. The error is attributed in part to the extraction procedure carried out before the HPLC analysis. The narrow range of standard deviation (0.001 - 0.006) demonstrates the precision of the entire method. Reliability of the method was enhanced by the use of guard column and a C18 reverse phase SEP-PAK. The use of the guard column helped remove possible contaminants in the mobile phase. The SEP-PAK C18 retained polyphenol(s) ([-]-epicatechin, caffeine) and other contaminants having affinities for the C18 stationary phase.

For recovery tests, the procedure yields high rates of recovery; an average of 101.8%, 101.1%, 101.0%, 99.6%, 100.9% and 105.2% were obtained for oxalic, citric, succinic, malic, lactic and tartaric acids, respectively (Table 3).

**CONCLUSIONS**

This study was conducted to develop a rapid HPLC method for determination of non-volatile acids (oxalic, citric, tartaric, succinic, malic and lactic acids) in fermented and dried cocoa beans. Excellent linearity for detector response was shown...
### TABLE 3

Recovery of non-volatile acids added to cocoa beans (continued)

<table>
<thead>
<tr>
<th>Acid naturally present in cocoa bean (g/100g sample)</th>
<th>Acid added</th>
<th>Acid recovery</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.628</td>
<td>0.160</td>
<td>0.776</td>
<td>98.5</td>
</tr>
<tr>
<td>0.628</td>
<td>0.320</td>
<td>0.950</td>
<td>100.2</td>
</tr>
<tr>
<td>0.628</td>
<td>0.480</td>
<td>1.111</td>
<td>100.3</td>
</tr>
<tr>
<td>0.628</td>
<td>0.640</td>
<td>1.262</td>
<td>99.5</td>
</tr>
<tr>
<td>Lactic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.116</td>
<td>0.100</td>
<td>0.222</td>
<td>102.8</td>
</tr>
<tr>
<td>0.116</td>
<td>0.200</td>
<td>0.311</td>
<td>98.4</td>
</tr>
<tr>
<td>0.116</td>
<td>0.300</td>
<td>0.417</td>
<td>100.2</td>
</tr>
<tr>
<td>0.116</td>
<td>0.400</td>
<td>0.527</td>
<td>102.1</td>
</tr>
<tr>
<td>Tartaric</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.066</td>
<td>0.030</td>
<td>0.099</td>
<td>103.1</td>
</tr>
<tr>
<td>0.066</td>
<td>0.060</td>
<td>0.130</td>
<td>103.2</td>
</tr>
<tr>
<td>0.066</td>
<td>0.090</td>
<td>0.160</td>
<td>102.6</td>
</tr>
<tr>
<td>0.066</td>
<td>0.120</td>
<td>0.208</td>
<td>111.8</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
<td><strong>Average</strong></td>
<td><strong>99.6</strong></td>
</tr>
</tbody>
</table>

\( a \) All data are the average of five lots, duplicate extractions and duplicate injections.  
\( b \) \( \% \) Recovery = \( \frac{\text{Acid recovered}}{\text{Sample acid} + \text{added acid}} \times 100 \)

by a correlation coefficient of more than 0.999 for the acid calibration curve. The relative standard deviation of 0.22 - 7.42\% and a narrow range of standard deviation (0.001 - 0.006) were obtained in the reproducibility test(s). Recoveries of each standard acid added prior to the extraction procedure have an average range of 99.62 - 101.72\%.

### ACKNOWLEDGEMENTS

The authors thank Universiti Pertanian Malaysia and the Pennsylvania State University for funding the research.

### REFERENCES


(Received 19 July, 1989)