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

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ABSTRAK

Kaedah High Performance Liquid Chromatography untuk menganalisa asid tidak teruap (oksalik, sitrik, tartarik, suksinik, malik dan laktik) di dalam biji koko yang difermentasi dan dikeringkan diterangkan. Sampel koko dihancurkan di dalam air yang telah dinyahion dengan menggunakan Polytron Homogenizer (Brinkman) selama 20 saat dan diemparkan pada 14000 ppm selama 45 min pada 25 °C. Extrak kemudian dialkalikan kepada pH 8-9 dan dilalukan melalui resin penukaran anion bes sederhana; fraksi asid dielusikan selepas pencampuran 10% asid sulfurik ke dalam turus. Kandungan poliphenol di dalam fraksi tersebut dibuang dengan melalukan fraksi asidik ke dalam fasa terbalik SEP-PAK yang sudah dibasahkan dengan methanol. Eluat tersebut dianaliskan untuk asid-asid tidak terwap dengan menggunakan Turus Asid Organik (Bio-Rad) dengan 0.1N H_2SO_4 sebagai fasa bergerak pada 65 °C. Asis-asid dikesan pada 214 nm dan dikira dengan membandingkan tinggi puncak bagi sampel dan piawai. Kaedah yang digunakan memberi kebolehulangan dan pemulihan yang baik.

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_2SO_4 as a mobile phase at 65 °C. The acids were detected at 214nm 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, Mildford, 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 H_2SO_4, 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) C_{18} 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, 1.00 g/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.

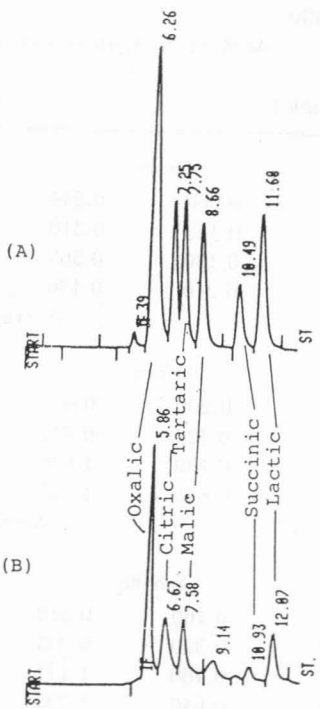

Fig. 1. Typical electronic chromatogram of standard non-volatile acid solution (A) and of cocoa bean sample (B).

TABLE 1
Calibration curve data for different concentrations of standard non-volatile acids[a]

g/100 g injected	Peak height (unit) at 0.05 AUFS[b] (X)
Oxalic	
0.06	10.73
0.12	19.97
0.18	31.45
0.24	42.07
0.30	54.75
$y = -1.25 + 55.07x$[c]; r = 0.999[d]	
Citric	
0.16	6.64
0.32	13.43
0.48	19.43
0.64	25.97
0.80	32.39
$y = 0.36 + 32.02x$; r = 0.999	
Tartaric	
0.08	4.41
0.16	9.05
0.24	13.04
0.32	17.04
0.40	21.76
$y = 0.22 + 21.52x$; r = 0.999	
Succinic	
0.16	500.76
0.32	1023.59
0.48	1465.54
0.64	1946.32
0.80	2429.21
$y = 39.20 + 2389x$; r = 0.999	
Malic	
0.16	255.90
0.32	577.07
0.48	838.43
0.64	1101.31
0.80	1377.41
$y = 1383.63x$; r = 0.999	
Lactic	
0.20	528.54
0.40	1077.59
0.60	1550.49
0.80	2044.73
1.00	2570.36
$y = 39.11 + 2525.39x$; r = 0.999	

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

TABLE 2
Reproducibility of non-volatile acids determination in cocoa beans[a]

Lots of same cocoa sample extracted	Oxalic	Citric	Succinic	Malic	Lactic	Tartaric
	g/100g cocoa bean sample					
1	0.183[a]	0.525	0.631	0.620	0.113	0.070
2	0.181	0.526	0.642	0.637	0.118	0.060
3	0.180	0.528	0.637	0.627	0.117	0.070
4	0.181	0.527	0.638	0.625	0.114	0.060
5	0.183	0.525	0.635	0.630	0.118	0.070
Average (s.d.)	0.182 (0.001)	0.526 (0.001)	0.637 (0.004)	0.628 (0.006)	0.116 (0.002)	0.066 (0.005)
Relative Standard Deviation	0.66	0.22	0.57	0.90	1.81	7.42

a All data are the average of duplicate extractions and duplicate injections.

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 C_{18} reverse phase SEP-PAK. The use of the guard column helped remove possible contaminants in the mobile phase. The SEP-PAK C_{18} retained polyphenol(s) ([-]-epicatechin, caffeine) and other contaminants having affinities for the C_{18} stationary phase.

For recovery tests, the procedure yields high rates of recovery; an average of 101.8%, 100.1%, 101.0%, 99.6%, 100.9% and 105.2% were obtained for oxalic, citric, succinic, malic, lactic and tartaric acids, respectively (Table 3).

TABLE 3
Recovery of non-volatile acids added to cocoa beans

Acid naturally present in cocoa bean (g/100g sample)	Acid added	Acid recovery	Recovery[b]
	Oxalic		
0.182[a]	0.060	0.244	100.8
0.182	0.120	0.310	102.7
0.182	0.180	0.363	100.3
0.182	0.240	0.436	100.3
		Average	101.8
	Citric		
0.526	0.260	0.691	100.7
0.526	0.320	0.841	99.4
0.526	0.480	1.026	102.0
0.526	0.640	1.145	98.2
		Average	100.1
	Succinic		
0.637	0.160	0.819	102.8
0.637	0.320	0.933	97.5
0.637	0.480	1.141	102.2
0.637	0.640	1.295	101.4
		Average	101.0

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)

Acid naturally present in cocoa bean (g/100g sample)	Acid added	Acid recovery	Recovery[b]
	Malic		
0.628	0.160	0.776	98.5
0.628	0.320	0.950	100.2
0.628	0.480	1.111	100.3
0.628	0.640	1.262	99.5
		Average	99.6
	Lactic		
0.116	0.100	0.222	102.8
0.116	0.200	0.311	98.4
0.116	0.300	0.417	100.2
0.116	0.400	0.527	102.1
		Average	100.9
	Tartaric		
0.066	0.030	0.099	103.1
0.066	0.060	0.130	103.2
0.066	0.090	0.160	102.6
0.066	0.120	0.208	111.8
			105.2

a All data are the average of five lots, duplicate extractions and duplicate injections.

b % Recovery = $\frac{\text{Acid recovered}}{\text{Sample acid + added acid}} \times 100$

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%

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