

Comparison of Flavonoids in Cocoa From Different Countries Using LC-TOFMS

LECO Corporation; Saint Joseph, Michigan USA

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1. Introduction

Cocoa contains over 500 compounds and is rich in flavonoids, particularly catechins (flavan-3-ols) and procyanidins. Cocoa is grown principally in West Africa, Central and South America, and Asia. The six largest cocoa producing countries are Ivory Coast, Ghana, Indonesia, Nigeria, Brazil, and Cameroon. These countries represent about 90% of world production. As cocoa makes its way from fresh bean to finished products, the flavonoid content can be affected by a variety of growing and processing conditions. Cocoa bean countries such as Madagascar and Ghana can have as much as a two-fold difference in total procyanidin concentration.¹ Fermentation and drying, although important for contributing to developing cocoa flavor, tends to decrease the flavanol content. The chocolate flavonoids can also be lost by the roasting of cocoa beans, and a number of other conditions such as dutching, which is common to cocoa and chocolate processing.

This report describes methodology and instrumentation used to rapidly screen and assess the variation of flavonoid content in commercially available cocoa products from four different countries. Due to the complexity of the compounds found in cocoa, LECO's Unique[®] Time-of-Flight Mass Spectrometer (TOFMS) is an ideal tool for fast separation methods due to its fast acquisition rates of up to 100 spectra/second. Additional features including accurate mass, high full mass range sensitivity, and advanced data processing software provide valuable tools for routine identification of components in complex mixtures.

2. Experimental Conditions

Sample Preparation

Cocoa samples (64% cacao) were obtained from a local chocolatier. The samples were prepared, with some modifications, as described by Andres-Lacueva et al.² The cacao solids were crushed, mixed with liquid nitrogen, and powdered. A 500 mg sample was weighed into a 50 mL beaker. Approximately 20 mL of methanol and 5 mL of deionized water were added into the beaker and stirred for 5 minutes. The contents of the beaker were transferred to a 50 mL centrifuge tube. The extract was manually shaken for one minute and then centrifuged for 15 minutes at 2000 g. The supernatant was then concentrated to 10 mL using a stream of N₂ and filtered through a 4 mm nylon-glass 0.45 μm filter. A 100 μL aliquot of the extract was combined with 900 μL of 70% water/methanol in an amber HPLC vial to give a final volume ready for LC-TOFMS analysis.

Instrumentation

LC: Agilent 1100
Column: Restek Ultra Aqueous C18
2.1 x 100 mm, 3 μm
Column Temp: 30°C
Flow Rate: 400 μL/minute
Run Time: 15.00 minutes
Autosampler Temp: 10°C
Injection Volume: 5.0 μL
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: Acetonitrile/methanol (50:50)
Gradient: 0 minutes, 10% B; 10 minutes, 60% B; 15 minutes, 60% B

MS: Unique[®] TOFMS
Source: High Flow ESI
ESI Voltage: (-) 3500 V
Nozzle: (-) 160 V
Skimmer: (-) 62 V
Nebulizer Pressure: 375 kPa
Desolvation Gas (N₂): 7 L/minute
Desolvation Temp: 300°C
Interface Temp: 100°C
Data Acquisition Rate: 4 spectra/second

Mass Calibration: 4-point external mass axis calibration
(m/z = 112.9855, 431.9827,
601.9789, and 1033.9882) from
Agilent tune mix

3. Results and Discussion

Cocoa samples containing 64% cacao from four different countries (Venezuela, Ivory Coast, Bolivia, and Madagascar) were compared for flavanoid content. Based upon the Total Ion Chromatogram overlay (Figure 1), the samples appear quite similar in composition. However, a peak-to-peak comparison of all analytes in each sample provides the best opportunity to compare varieties. Usually a labor intensive manual process, this peak-to-peak comparison step is offered as one of many automated features available in LECO's ChromaTOF[®] data processing software. The Compare algorithm allows acquired data to be processed against a reference chromatogram. First, the peak information from a 'reference' chromatogram is saved to a reference table. Tolerances for mass error, retention time deviation, signal-to-noise ratio, and peak area are then input. Lastly, the sample being compared to the reference is processed and a peak-to-peak comparison is made between the sample and the reference. Based upon the tolerance settings in the reference table, each peak found is designated as a Match (within retention time, mass, mass error, and concentration tolerance), Out of Tolerance (within retention time, mass, and mass error, but outside

concentration tolerance), Not Found (a compound in the reference but not in the sample), or an Unknown (a compound in the sample but not in the reference). Through the aid of peak table filters, similarities between samples, differences, and even relative changes in peak concentrations can be immediately identified.

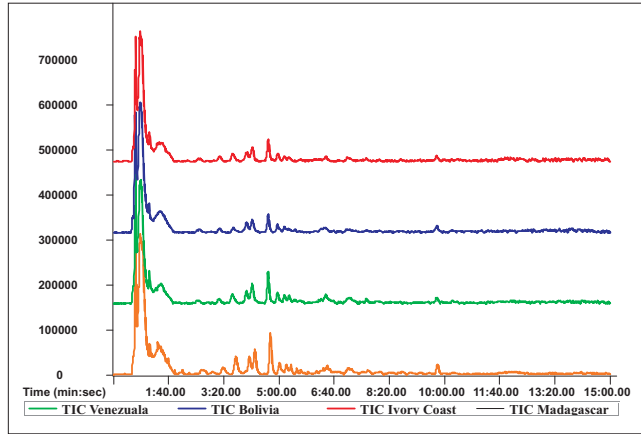


Figure 1. Overlaid TIC chromatograms of cocoa samples from Venezuela, Ivory Coast, Bolivia, and Madagascar.

Cocoa from Venezuela is considered by some chocolate manufacturers to be the best and was therefore chosen as the reference. Sixteen compounds were selected and transformed into a reference list which was then automatically compared against the other cocoa samples. The reference list is shown in Table 1. Peak detection tolerances, including mass, mass error, and retention time deviation are used to filter non-matching peaks. Reference compound concentrations were arbitrarily defined as 100% in order to compare relative peak areas. As shown in Tables 2 through 4, the Compare feature allows the analyst to easily and automatically locate similarities between samples without extensive data analysis. The peak table lists peaks of interest as Match, Out of Tolerance, Not Found, or Unknown. Based on the results, the comparison between the samples and the reference were different.

Table 1. Peak table for the reference sample (Venezuela).

Peak #	Name	R.T. (min:sec)	Unique Mass	Area	Concentration	Units
1	Catechin	03:42	289.0716	123144	100	%
2	Unidentified Peak A	04:07	305.0697	168146	100	%
3	Procyanidin B2	04:16	577.1432	136262	100	%
4	Epicatechin	04:44	289.0735	261628	100	%
5	Procyanidin C1	05:01	865.2169	42478	100	%
6	Procyanidin (Tetramer)	05:13	1153.3114	4615	100	%
7	Clovamide	05:22	358.1008	6221	100	%
8	Procyanidin II-G	06:19	737.1912	6433	100	%
9	Procyanidin B5	06:28	577.1423	16886	100	%
10	Procyanidin II-A	06:33	707.1816	6696	100	%
11	Dideoxyclovamide	07:06	326.1072	6767	100	%
12	Quercetin-Galactoside	07:12	463.0911	13638	100	%
13	Quercetin-Arabinoside	07:41	433.0804	12502	100	%
14	Unidentified Peak B	08:13	497.3418	46500	100	%
15	Quercetin	09:31	301.0965	1443	100	%
16	Unidentified Peak C	09:47	723.5212	145569	100	%

Table 2. Comparison of Bolivia sample vs. reference chromatogram (Venezuela sample). Tolerance level of reference sample was set to 30%.

Peak #	Name	Type	R.T. (min:sec)	Unique Mass	Area	Concentration (%)
1	Catechin	Out of Tolerance	03:36	289.096	79859	64.9
2	Unidentified Peak A	Match	04:01	305.096	147553	85.6
3	Procyanidin B2	Match	04:11	577.1877	123146	90.4
4	Epicatechin	Match	04:40	289.0972	205785	77.9
5	Procyanidin C1	Match	04:57	865.2912	42886	114.2
6	Procyanidin (Tetramer)	Out of Tolerance	05:10	1153.2792	6452.2	151.6
7	Clovamide	Out of Tolerance	05:19	358.1218	9692.5	180.2
-	Unknown	Unknown	06:13	415.1478	3443.4	-
8	Procyanidin II-G	Out of Tolerance	06:16	737.2478	9668.3	164.6
-	Unknown	Unknown	06:22	393.212	21830	-
9	Procyanidin B5	Match	06:25	577.1878	19227	125.4
10	Procyanidin II-A	Match	06:29	707.2475	8404.7	125.5
11	Dideoxyclovamide	Out of Tolerance	07:03	326.1265	6671.1	137.9
12	Quercetin-Galactoside	Out of Tolerance	07:10	463.1259	21051	172.4
13	Quercetin-Arabinoside	Out of Tolerance	07:39	433.1132	16695	133.5
14	Unidentified Peak B	Out of Tolerance	08:11	497.3731	22203	45.3
15	Quercetin	Not Found	-	-	-	-
16	Unidentified Peak C	Out of Tolerance	09:46	723.5746	75469	50.1

Table 3. Comparison of Madagascar sample vs. reference chromatogram (Venezuela sample). Tolerance level of reference sample was set to 30%.

Peak #	Name	Type	R.T. (min:sec)	Unique Mass	Area	Concentration (%)
1	Catechin	Out of Tolerance	03:37	289.0934	50515	39.3
-	Unknown 1	Unknown	03:45	431.2342	7701.3	-
2	Unidentified Peak A	Out of Tolerance	04:01	305.0932	102607	61.1
3	Procyanidin B2	Out of Tolerance	04:10	577.1857	75467	53.4
4	Epicatechin	Out of Tolerance	04:40	289.0943	138789	53.4
5	Procyanidin C1	Out of Tolerance	04:58	865.2709	27449	64.8
6	Procyanidin (Tetramer)	Match	05:09	1153.3114	4831	83.2
7	Clovamide	Out of Tolerance	05:19	358.1163	4785.6	51.3
-	Unknown 2	Unknown	05:22	275.1271	7312	-
8	Procyanidin II-G	Match	06:16	737.2098	7097.9	75.0
9	Procyanidin B5	Out of Tolerance	06:25	577.1767	14653	62.7
10	Procyanidin II-A	Match	6:30	707.2694	7540.8	73.8
11	Dideoxyclovamide	Match	07:02	326.1254	6902.8	96.1
12	Quercetin-Galactoside	Match	07:09	463.1278	13106	85.6
13	Quercetin-Arabinoside	Match	07:38	433.1087	13195	84.5
14	Unidentified Peak B	Out of Tolerance	08:11	497.3739	21993	38.7
15	Quercetin	Not Found	-	-	-	-
16	Unidentified Peak C	Out of Tolerance	09:46	723.5619	78164	48.0

Table 4. Comparison of Ivory Coast sample vs. reference chromatogram (Venezuela sample). Tolerance level of reference sample was set to 30%.

Peak #	Name	Type	R.T. (min:sec)	Unique Mass	Area	Concentration (%)
1	Catechin	Out of Tolerance	03:36	289.094	27479	22.3
2	Unidentified Peak A	Out of Tolerance	04:01	305.0955	86355	54.0
3	Procyanidin B2	Out of Tolerance	04:11	577.1906	61770	45.3
4	Epicatechin	Out of Tolerance	04:40	289.0965	115684	44.5
5	Procyanidin C1	Match	04:57	865.2826	29795	70.4
6	Procyanidin (Tetramer)	Match	05:09	1153.3545	4510	106.0
7	Unknown	Unknown	05:22	275.1206	6536	-
8	Procyanidin II-G	Out of Tolerance	06:16	737.2467	8946	152.3
9	Unknown	Unknown	06:21	393.2118	15175	-
10	Procyanidin B5	Match	06:25	577.1608	11220	71.7
9	Procyanidin II-A	Match	6:30	707.2426	5967	89.1
11	Dideoxyclovamide	Match	07:02	326.1277	3698	71.7
12	Quercetin-Galactoside	Match	07:09	463.1282	9164	75.1
13	Quercetin-Arabinoside	Match	07:38	433.1126	8864	70.9
14	Unidentified Peak B	Out of Tolerance	08:11	497.3755	21729	44.4
16	Unidentified Peak C	Out of Tolerance	09:46	723.5685	82774	54.9
7	Clovamide	Not Found	-	-	-	-
15	Quercetin	Not Found	-	-	-	-

The results of the automated comparison for (+)-catechin, (-)-epicatechin, and procyanidin B2 levels in the Bolivian, Madagascar, and Ivory Coast referenced to the Venezuelan sample are shown in Figure 2. The levels in the reference sample were normalized to 100%. The results indicate that the Venezuelan cocoa reference had the largest amount of (+)-catechin, (-)-epicatechin, and procyanidin B2 and the Ivory Coast sample had the least.

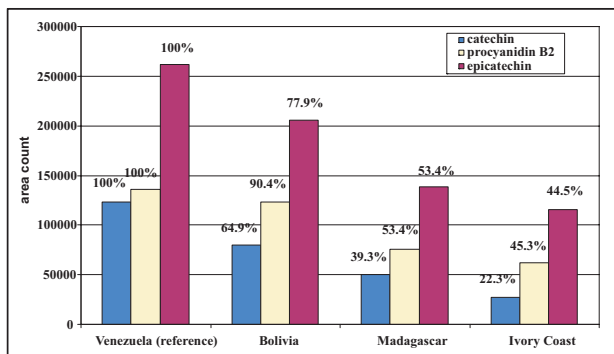


Figure 2. Comparison of catechin, epicatechin, and procyanidin levels in Bolivia, Madagascar, and Ivory Coast vs Venezuela reference sample.

4. Conclusion

A fast LC-TOFMS method for screening cocoa flavonoids from different countries has been developed. The capability of the Unique LC-TOFMS to provide full mass range data with good sensitivity combined with automated peak find allowed for rapid identification of all components within these samples. Using the automated ChromaTOF compare algorithm, variability of the flavonol content in cocoa from different countries was determined, with Venezuelan cocoa having the highest flavonol content and Ivory Coast cocoa having the least.

5. References

¹S. Collins et al., Relationship between procyanidin and flavonol contents of cocoa liquors from different origins, *J. Agric. Food Chem.* 2004, 52, 6243-6249.

²Andreas-Lacueva et al., An LC method for the analysis of cocoa phenolics, *LC-GC Eur.*, 2000, 902-905.

