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Analysis of Commercial Stevia Extracts Composition by HPLC and UHPLC-MS-MS-QTOF

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Samples of commercial Stevia Extracts from different countries (USA, China, Japan, France, Brazil and Paraguay) were analyzed quantitatively and qualitatively by HPLC and UHPLC-MS-MS-QTOF. Extract 03 (Japan) presented the lowest value of total glycosides (GTS = 74.7%) and extract 01 (USA) the highest value (GTS = 99.7%). Extracts 01 (USA) and 05 (Brazil) showed rebaudioside A as the major glycoside, while the other extracts presented stevioside as the main glycoside. In extract 6 (Paraguay), the presence of labdanic diterpenes (sterebins type B / C, D and I / J) was identified. In extract 05 (Brazil) the presence of phenolic compounds was observed. Extract 4 (France) with 87.4% of GTS, presented phenolic compounds and also sterebin I / J. Extracts 02 and 07 (China) were the only ones that exhibited the presence of oleamide and estereamide. These results show that commercially stevia extracts available may have a very varied composition, suggesting that many of the functional effects attributed to stevia sweeteners may be due to the presence of other substances, such as phenolic compounds and labdanic diterpenes.
Key words: Stevia rebaudiana, Stevia Extracts, UHPLC-MS-MS-QTOF, sterebin

1. Introduction

Many metabolic diseases, overweight and obesity are directly related to the abusive use of sucrose in foods and beverages. Recent studies have shown that in the United States and in many other countries, obesity continues to increase significantly despite awareness campaigns and efforts to develop non-caloric and safe sweeteners (Salazar et al., 2018). Stevia rebaudiana (Bert.) Bertoni is a shrub native to South America, belonging to the asteraceae family, used as a source of high intensity sweeteners. Steviol glycosides are found in stevia leaves, stems and roots. The major glycosides are stevioside, rebaudioside A, rebaudioside C and dulcoside A. Among the minority, the rebaudiosides D and E stand out. These glycosides differ in relation to sweetness and sweet taste quality. Steviosiddeo, rebaudiosideo C, rebaudiosideo B and dulcoside A present residual bitter taste. Rebaudiosides A, D and E have a sweet taste closer to that of sucrose. The total glycosides content in stevia leaves, depending on plant variety and growing conditions, may range from 10 to 24%. The stevia leaves also contain a number of other classes of substances such as phenolic compounds, lipids, amino acids, terpenoids, sesquiterpenoids, oligosaccharides, among others (Molina-Calle et al., 2017). The extracts commercially available around the world are obtained from different varieties of stevia by different techniques of extraction and purification (Pol et al., 2007; Chatsudthipong et al., 2009), being able to generate extracts with similar total steviol glycosides content, but with quite varied composition of steviol glycosides, which can negatively effect the organoleptic properties of final product. It should also be considered that even extracts with high purity, the residual presence of phenolic compounds, sterebins and lipids, for example, can

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to affect negatively the quality of the sweet taste and also confer functional properties such as ant diabetic, anti-oxidant, hypotensive, anti-inflammatory, among others (Chatsudthipong et al., 2009, Milani et al., 2017). The objective of this article was to determine the total steviol glycosides (TSG) by means of high performance liquid chromatography (HPLC) in stevia extracts produced in the USA, Japan, China, Brazil, Paraguay and France and also to investigate by means of **UHPLC-MS-MS-QTOF** the presence of steviol (aglycone), minor steviol glycosides, sterebins, lipids, phenolic compounds and other substances that can influence the functional properties of these commercially available extracts.

2. Materials and methods

2.1 Stevial extracts and reagents

Samples of commercial stevia extracts of different nationalities were provided by the Research Center on Natural Products - NEPRON, State University of Maringá.Stevioside, Rebaudioside A and C standards were obtained from Sigma-Aldrich. Acetonitrile, formic acid and deionized water (18 MΩ • cm) by Milli-Q plus system were supplied by Induslab. All solvents and standards were liquid chromatographic (**LC**) grade or higher.

2.2 UHPLC-MS/MS-QTOF Analysis

The commercial extracts of steviol glycosides were analyzed in **UHPLC-MS / MS-QTO**F for further identification of compounds following the methodology described by Formigoni et al. (2018). Chromatograms and ion spectra **(**MS2**)** in positive ionization mode were visualized using DataAnalysis 4.3 software, compared to literature and identified through databases such as METLIN (http://metlin.scripps.edu/), Food Database (http://foodb.ca/), MassBank (http://www.massbank.jp/), Respect for phytochimicals (http://spectra.psc.riken.jp/), and Human Metabolome Database (http http://www.hmdb.ca/). The precursor ion selected for identification was M+H, establishing a maximum error limit of 4 ppm for identification.

2.3 HPLC analysis

The steviol glycosides present in the commercial extracts were quantified through a high performance liquid chromatography (HPLC) coupled with the 5µm refractive index detector and 125x4.6 mm NH2 column using as the mobile phase acetonitrile and water (80:20) v/v by Dacome et al. (2005). The wavelength was set of 210nm. Further, an analysis was performed on the same equipment using a UV-Vis detector, C-18 column and mobile phase methanol and water (80:20) v/v. The reading was performed at a wavelength of 236nm.

3. Results and discussion

Outputs of the steviol glycosides content (stevioside, rebaudioside C and rebaudioside A) are reported in Table 01. Among the extracts, the total steviol glicosides (TSG) ranged from 74.7% to 99.7%, respectively, for EX3 (Japan) and EX1 (USA). Three extracts: EX2 (China), EX5 (Brazil) and EX6 (Paraguay) contained around total 80% of TSG. While extracts EX4 (France) and EX7 (China) presented TSG content of 87.4% and 86.7%, respectively. Among the extracts the stevioside content varies between Nd (not detected) for EX1 (USA) and 58.4% for EX2 (China) with respect to TSG. The highest content of rebaudioside C was 6.9% in EX4 (France). Content of rebaudioside A ranged from 19.4% (EX2-China) to 99.7% (EX1-USA). Therefore, the analysis shows that the extracts present homogeneous TSG values, but with great variation in relation to the relative composition of the three main major glycosides present in the stevia leaves. Only EX1 (USA) has a TSG content above 95% as recommended by 82nd Joint FAO/WHO Expert Committee on Food Additives (JECFA) meeting – Food additives.

Table 1. Quantification of major steviol glycosides in commercial extracts by HPLC-IR

Código	Origin	TSG* (%)	Steviol Glycosides(%)		
			Stevioside	Rebaudioside C	Rebaudioside A
EX1	USA	99.7	Nd	Nd	99.7
EX2	China	80.4	58.4	2.6	19.4
EX3	Japan	74.7	47.9	5.9	20.9
EX4	France	87.4	55.1	6.9	25.4
EX5	Brazil	81.8	9.9	3.5	67.7
EX6	Paraguay	80.6	53.8	5.1	21.7
EX7	China	86.7	53.6	5.4	27.7

* TSG -Total Steviol Glycosídes is represented as the sum of steviosíde, rebaudioside A and C; Nd: not detected

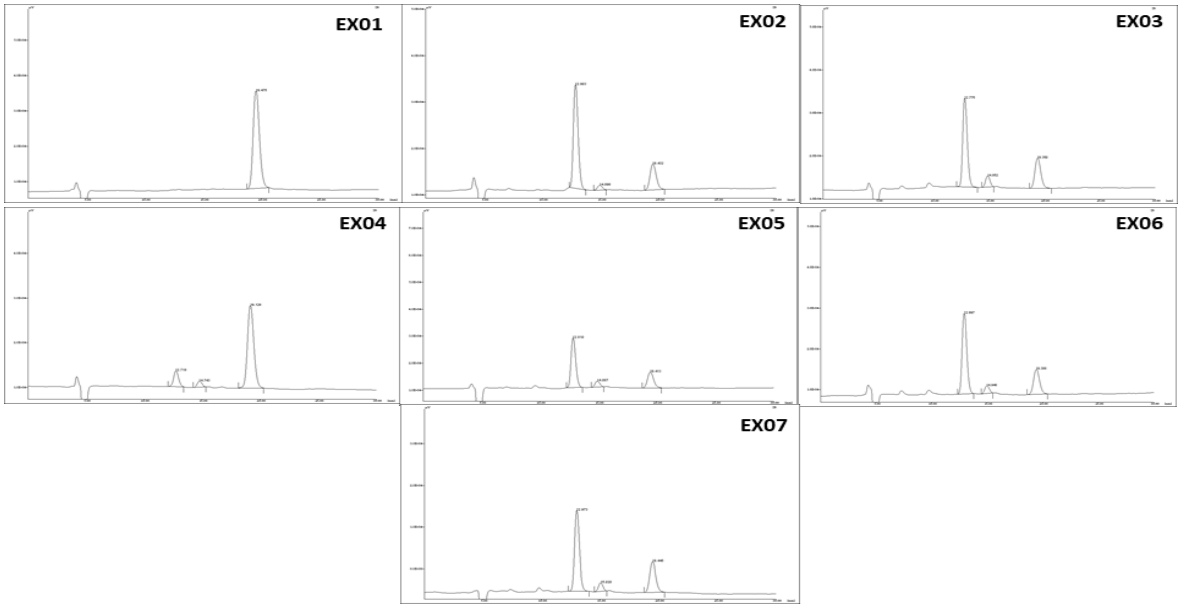

Figure 1. Chromatograms of commercial stevia extracts by HPLC-IR

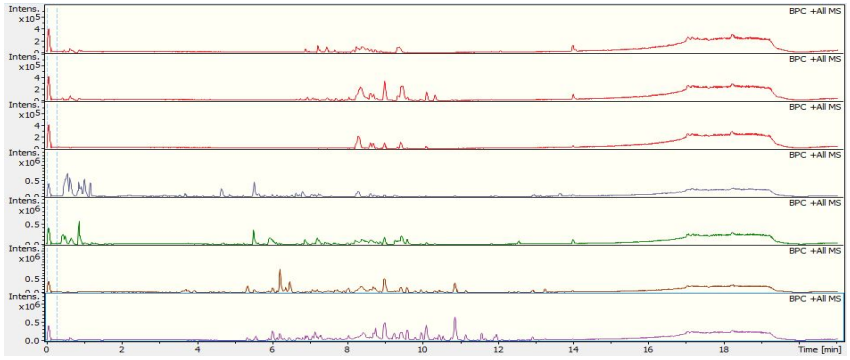

Figure 2. Total ion chromatograms MS/MS provided by the analysis of the extracts in the positive ionization mode in UHPLC-MS / MS-QTOF,

The content of major steviol glycosides were confirmed by HPLC-UV, however, additional peaks were observed in all extracts analyzed. The presence of steviol (aglycone), minor steviol glycosides and other classes of substances were investigated by means of **UHPLC-MS / MS-QTOF**. The Figure 2 shows the total ion chromatograms MS of all extracts in the positive ionization mode, in which it is observed that several groups of peaks, suggesting the presence of substances other than the major steviol glycosides determined by means of HPLC-IR. The identification was performed with the aid of a computer program associated with the database as cited in the literature, taking into account the degree of precision. The compounds identified, as well as their parameters, make up the Table 2.

Table 2. Identification of steviol glycosides in commercial extracts by UHPLC-MS / MS-QTOF (continue)

Compound	Molecular formula	Retention time	m/z	Fragment
EX01				
Rebaudioside N	$C_{56}H_{90}O_{32}$	7.29	1275.5477	633, 471, 309, 273, 147
Steviolbioside	$C_{32}H_{50}O_{13}$	7.38	643.3325	481, 463, 319, 301, 163
Rebaudioside M	$C_{56}H_{90}O_{33}$	7.44	1291.5425	649, 487, 325, 163
Rebaudioside K	$C_{50}H_{80}O_{27}$	7.47	1113.4943	627, 471, 309, 273, 147
Steviol	$C_{20}H_{30}O_3$	7.49	319.2268	319, 301, 273, 255, 165
Rebaudioside E	$C_{44}H_{70}O_{23}$	7.66	967.4365	481, 325, 319, 163

Table 2. Identification of steviol glycosides in commercial extracts by UHPLC-MS / MS-QTOF (continue)

Rebaudioside D	$C_{50}H_{80}O_{28}$	8.15	1129.4895	643, 479, 345, 163,
Rubusoside	$C_{32}H_{50}O_{13}$	8.25	643.3322	319, 301, 273, 163, 145, 127
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.38	805.3857	625, 481, 325, 271, 163
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.55	937.4272	457, 325, 295, 163
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.64	951.4428	627, 463, 309, 271, 147
Rebaudioside A	$C_{44}H_{70}O_{23}$	8.78	967.4371	626, 325, 319, 163
Dulcoside A	$C_{38}H_{60}O_{17}$	8.91	789.3897	481, 319, 309, 163, 147, 129
Stevioside	$C_{38}H_{60}O_{18}$	9.4	805.3851	625, 481, 319, 163, 145
EX02				
Steviol	$C_{20}H_{30}O_3$	7.46	319.2268	319, 301, 273, 255, 227, 165, 149
Steviolbioside	$C_{32}H_{50}O_{13}$	7.47	643.3323	481, 319, 301, 273, 163, 145
Rebaudioside D	$C_{50}H_{80}O_{28}$	8.16	1129.4900	643, 325, 319, 163, 145
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.28	805.3853	625, 481, 325, 319, 301 289, 163
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.62	951.4427	627, 463, 309, 271, 147
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.5	937.4272	325, 319, 163
Dulcoside A	$C_{38}H_{60}O_{17}$	8.64	789.3902	463, 465, 319, 309, 147, 87
Stevioside	$C_{38}H_{60}O_{18}$	9.37	805.3843	625, 319, 163, 145, 487, 481
Rebaudioside A	$C_{44}H_{70}O_{23}$	9.46	967.4372	643, 626, 325, 319,289, 163, 145
Retinal	$C_{20}H_{28}O$	9.99	285.2210	285, 267, 189, 137, 107
Palmitamide	$C_{16}H_{33}NO$	15.78	256.2630	256, 239, 102, 88
Oleamide	$C_{18}H_{35}NO$	15.99	282.2785	265, 111, 100
Estearamide	$C_{18}H_{37}NO$	16.91	284.2945	239, 133, 102, 88
13-Docosenamide	$C_{22}H_{34}NO$	18.06	338.3417	303, 135, 97, 83
N-stearoy valine	$C_{23}H_{45}O_3N$	16.73	384.3463	338, 321, 212, 97
EX03				
Stevioside	$C_{38}H_{60}O_{18}$	8.30	805.3748	325, 319, 289, 163, 127, 85
Rebaudioside A	$C_{44}H_{70}O_{23}$	8.27	867.4252	625, 481, 325, 319, 289, 163, 145
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.79	805.9741	625, 481, 325, 289, 163, 145
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.62	951.4297	465, 309, 273, 147,
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.52	937.4153	457, 325, 259, 163
Dulcoside A	$C_{38}H_{60}O_{17}$	8.65	789.3804	465, 309, 273, 255, 147
Steviol	$C_{20}H_{30}O_3$	10.12	319.2231	319, 273, 255, 121
Steviolbioside	$C_{32}H_{50}O_{13}$	8.31	643.3243	481, 319, 163, 145, 885
EX04				
Stevioside	$C_{38}H_{60}O_{18}$	8.28	805.3798	625, 481, 319, 163, 145, 127
Rebaudioside A	$C_{44}H_{70}O_{23}$	8.26	967.4311	649, 487, 325, 163, 145, 85
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.62	951.4355	627, 465, 309, 273, 147
Rebaudioside D	$C_{50}H_{80}O_{28}$	7.24	1129.482	812, 649, 487, 325, 163
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.8	805.9783	481, 319, 163, 145, 85
Dulcoside A	$C_{38}H_{60}O_{17}$	8.64	789.3849	609, 465, 309, 273, 147
Steviolbioside	$C_{32}H_{50}O_{13}$	9.01	643.3281	481, 417, 319, 163, 145
Steviol	$C_{20}H_{30}O_3$	8.98	319.2248	319, 301, 273, 255, 227, 165
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.53	937.4207	619, 457, 325, 319, 295, 163
Caffeic acid	$C_9H_8O_4$	4.04	181.0490	163, 145, 121
4,5-Dicaffeioylquinic Acid	$C_{25}H_{24}O_{12}$	6.84	517.1308	319, 163
1,5-Dicaffeioylquinic Acid	$C_{25}H_{24}O_{12}$	6.62	517.1300	319, 163
3,4-Dicaffeioylquinic Acid	$C_{25}H_{24}O_{12}$	7.13	517.1304	325, 319, 163
1-caffeoylquinic acid	$C_{16}H_{18}O_9$	4.67	355.1006	325, 163
Kempferol	$C_{15}H_{10}O_6$	7.24	287.0533	287, 247
Kempferol-3-glucoside	$C_{21}H_{20}O_{11}$	5.96	449.1049	287, 257
Quercetin	$C_{15}H_{10}O_7$	6.65	303.0481	303, 273
Quercetin-3-O-rhamnoside	$C_{21}H_{20}O_{11}$	6.66	449.1048	303, 129, 85
Luteolin	$C_{15}H_{10}O_6$	6.95	287.0535	287, 153
Luteolin-7-glucoside	$C_{21}H_{20}O_{11}$	6.58	449.1047	287, 145
Retinol	$C_{20}H_{30}O$	12.95	287.2351	287, 269

Table 2. Identification of steviol glycosides in commercial extracts by UHPLC-MS / MS-QTOF (continue)

Sterebin I/J	$C_{20}H_{32}O_4$	15.99	337.2332	337
Cinnamic acid	$C_9H_8O_2$	1.19	149.0594	131, 103
Palmitic amide	$C_{16}H_{33}NO$	15.76	256.2326	239, 102, 88
EX05				
Steviol	$C_{20}H_{30}O_3$	7.23	319.2252	319, 301, 273, 255, 227, 165
Stevioside	$C_{38}H_{60}O_{18}$	8.03	805.3800	625, 481, 319, 163, 145
Rebaudioside A	$C_{44}H_{70}O_{23}$	8.24	967.4324	649, 487, 325, 163, 145, 85
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.80	805.3806	625, 481, 325, 289, 163, 145
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.62	951.4377	627, 465, 309, 273, 147
Rebaudioside D	$C_{50}H_{80}O_{28}$	7.22	1129.4830	811, 649, 487, 325, 163
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.52	937.4227	619, 481, 457, 325, 319, 295, 163
Rebaudioside E	$C_{44}H_{70}O_{23}$	8.76	967.4320	889, 625, 325, 163
Steviolbioside	$C_{32}H_{50}O_{13}$	7.51	643.3286	481, 417, 319, 163, 145
Dulcoside A	$C_{38}H_{60}O_{17}$	9.62	789.3857	609, 465, 309, 273, 147
Chlorogenic acid	$C_{16}H_{18}O_9$	4.69	355.1007	163
Quinic acid	$C_7H_{12}O_6$	0.88	193.0695	157, 147, 129
Quercetin 3-O-rhamnoside	$C_{21}H_{20}O_{11}$	6.64	449.1052	303, 129
5uteolin-3',7-di-O-glucoside	$C_{27}H_{30}O_{16}$	4.39	611.1565	449,0287
Luteolin-4'-glucoside-7-rutinoside	$C_{33}H_{40}O_{20}$	4.43	757.2129	449, 287, 147
Kaempferol-3-O-galactose-rhamnose-7-O-rhamnose	$C_{33}H_{40}O_{19}$	5.71	741.2172	433, 147
Kaempferol-3-glucoside-3"-Rhamnoside	$C_{27}H_{30}O_{15}$	4.98	595.1618	448, 287
Apigenin-7-O-glucoside	$C_{21}H_{20}O_{10}$	6.53	433.1006	443, 271
1,5-Dicaffeoilquinic acid	$C_{25}H_{24}O_{12}$	6.83	517.1303	319, 163
3,4-Dicaffeoilquinic acid	$C_{25}H_{24}O_{12}$	7.06	517.1300	325, 319, 163
5-Caffeoilquinic acid	$C_{16}H_{18}O_9$	4.69	355.1003	325, 163
EX06				
Stevioside	$C_{38}H_{60}O_{18}$	8.03	805.3780	625, 481, 319, 163, 145
Rebaudioside A	$C_{44}H_{70}O_{23}$	7.39	967.4272	649, 487, 325, 163, 145
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.35	805.3785	649, 487, 325, 163, 145
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.61	951.4346	627, 465, 309, 273, 147
Rebaudioside D	$C_{50}H_{80}O_{28}$	8.14	1129.4805	649, 487, 325, 163
Rebaudioside E	$C_{44}H_{70}O_{23}$	7.85	967.4286	625, 325, 163
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.52	937.4193	619, 457, 325, 319, 295, 163
Dulcoside A	$C_{38}H_{60}O_{17}$	9.07	789.3842	465, 319, 273, 147, 129
Steviol	$C_{20}H_{30}O_3$	7.61	319.2240	319, 301, 273, 255, 227, 165
Steviolbioside	$C_{32}H_{50}O_{13}$	9.36	643.3274	481, 417, 319, 163, 145
Sterebin B/C	$C_{20}H_{32}O_5$	7.89	353.2292	335, 317, 293, 295
Sterebin D	$C_{18}H_{30}O_3$	9.20	295.2249	277, 259
Sterebin I/J	$C_{20}H_{32}O_4$	8.45	337.2351	337
EX07				
Stevioside	$C_{38}H_{60}O_{18}$	8.81	805.3763	625, 481, 319, 163, 145, 127
Rebaudioside A	$C_{44}H_{70}O_{23}$	8.12	967.4273	687, 325, 163, 145
Rebaudioside B	$C_{38}H_{60}O_{18}$	8.02	805.3753	643, 325, 163, 145
Rebaudioside C	$C_{44}H_{70}O_{22}$	8.63	951.4326	627, 465, 309, 273, 147, 85
Rebaudioside D	$C_{50}H_{80}O_{28}$	7.22	1129.4777	811, 649, 487, 325, 163
Rebaudioside E	$C_{44}H_{70}O_{23}$	8.26	967.4271	649, 487, 325, 163
Rebaudioside F	$C_{43}H_{68}O_{22}$	8.53	937.4175	457, 325, 319, 295, 163
Rebaudioside N	$C_{56}H_{90}O_{32}$	7.28	1275.5341	633, 471, 309, 273, 147
Dulcoside A	$C_{38}H_{60}O_{17}$	9.07	789.3815	465, 319, 273, 147, 129
Steviolbioside	$C_{32}H_{50}O_{13}$	7.79	643.3250	481, 319, 163, 145
Steviol	$C_{20}H_{30}O_3$	10.12	319.2233	319, 301, 273, 255, 165
Sterebin D	$C_{18}H_{30}O_3$	11.67	295.2239	277, 259, 201
Sterebin E/F/M	$C_{20}H_{34}O_4$	5.68	339.2493	321, 303, 267, 123
Sterebin I/J	$C_{20}H_{32}O_4$	8.46	337.2340	337

Table 2. Identification of steviol glycosides in commercial extracts by UHPLC-MS / MS-QTOF (continue)

Retinol	$C_{20}H_{30}O$	10.32	287.2336	287, 215, 133
Oleamide	$C_{18}H_{35}NO$	15.99	282.2768	282, 265, 247, 153
Estearamide	$C_{18}H_{37}NO$	16.91	284,2919	102, 88
Stevioside	$C_{38}H_{60}O_{18}$	8.81	805.3763	625, 481, 319, 163, 145, 127

The presence of steviol (aglycone) in all commercial extracts was identified by **UHPLC-MS / MS-QTOF.** In EX1 (USA) with 99.7% of rebaudioside A, another 13 steviol glycosides were identified, but no phenolic or sterebin compounds were identified. It was expected that EX3 (Japan) with lower TSG among the analyzed extracts, presented a great diversity of other substances, however it has only steviol glycosidesl. The low value of TSG for EX3 (Japan) is an indication that the extract can be diluted in maltodextrin. In EX4 (France) and EX5 (Brazil), in addition to minor steviol glycosides, a great number of phenolic compounds have been identified, which may confer antioxidant properties to such extracts, but on the other hand can compromise their organoleptic properties. In the EX06 (China) and EX07 (Paraguay) extracts the presence of sterebins (diterpenes labdanics) was identified. Even at low concentrations they can seriously to affect the organoleptic properties of stevia extract.

4. Conclusions

The analysis of commercial stevia extracts from several countries using HPLC showed that the composition of the major glycosides (stevioside, rebaudioside C and rebaudioside A) is quite variable among the samples, which may be a reflection of the variety of stevia or extraction and purification processes employed, and among the evaluated extracts only one presented a TSG above 95%. The presence of steviol in all extracts was identified by UHPLC-MS / MS-QTOF. In two extracts (EX4 e EX5) a series of phenolic compounds were identified and in the other two (EX6 e EX7) the presence of sterebins, compounds that may be responsible for many important biological effects attributed to the stevia extracts, but that on the other hand can affect negatively the organoleptic properties of such extracts.

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